Determining the Influence of Endocannabinoids in Skeletal Muscle Adiponectin sensitivity in Diet Induced Obesity and Diabetes

A thesis submitted by

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Contents

Abstract

Obesity kills more than 2.8 million people globally each year regardless of economic status or age. The endocannabinoid system is a widely distributed lipid signalling system that regulates appetite, fatty acid oxidation, glucose metabolism and inflammation. Adiponectin plays a protective role against metabolic disorders. Skeletal muscle plays a leading role in fatty acid oxidation and glucose metabolism. Activation of Cannabinoids in adipose tissue results in a decrease in adiponectin and an increase in inflammation causing ER stress. Blocking cannabinoids causes a decrease in inflammation and increase in adiponectin. This suggests a direct yet to be determined relationship between the Endocannabinoid system and adiponectin resistance, which was the focus of this candidature.

Sprague Dawley rats were fed a HFD (22%) for 12 weeks to elicit DIO, then injected daily (IP) with CB_1 Antagonist (AM251- 3mg/kg) for 6 wks. Animals were anesthetised, and skeletal muscles (Red /White fibre types) surgically removed. Skeletal muscle was immediately placed in organ bath (*37^C-95%$O_2$5%)* with Adiponectin for 30 minutes. Plasma analysis determined that chronic CB_1 Antagonism in these rats resulted in a significant reduction in food intake, weight reduction, a reduction in Peri-renal and brown adipose tissue weight, and a reduction in plasma leptin and Glucagon. There was an increase in inflammatory plasma cytokines (Il-1α, IL-2, Il-4, Il-5, Il-17α, Il-18, RANTES, IL12p70). Muscle analysis found that CB_1 Antagonism on whole muscle resulted in no changes in mitochondrial markers. Incubation of the soleus muscle with adiponectin showed a significant decrease in AdipoR1 expression. There was a decrease in markers of fatty acid oxidation in white skeletal muscle

Sprague Dawley rats were fed HFD (22%) for 12 weeks then injected daily (IP) with either CB_2 Agonist (AM1241-3mg/kg or CB_2 Antagonist (AM630- 0.3mg/kg) for 6 wks. Animals were anesthetised, and muscles (Red and White fibre types) were surgically removed. Muscle was placed in an organ bath (*37^C-95%O_2-5%)* with Adiponectin for 30 minutes. Plasma analysis determined that CB_2 modulation resulted in an initial decrease in food intake. CB_2 stimulation caused an increase in IL12p70 and a decrease in Leptin in plasma. CB_2 Antagonism caused a decrease in plasma Leptin, GLP-1, Ghrelin. Muscle analysis showed that blocking CB_2 caused an increase in mitochondrial activity in red fibres via elevated concentrations of citrate synthase. Adiponectin exposure resulted in CB_2 agonism causing a down regulation of the mRNA expression of both AMPK and PGC1α in the Extensor Digitorum Longus muscle.

Human skeletal muscle (rectus abdominus) were sourced from Obese and Diabetic individuals undergoing routine lap band surgery. Myotubes were treated for a 24-hour period with either CB_1 antagonist (AM251), CB_2 antagonist (AM630) in isolation or in combination with Adiponectin. Results showed significant increase of AdipoR2 in combination with Adiponectin in Diabetic tissue. Blocking CB_2 caused an increase in both AdipoR1 and AdipoR2 expression in diabetic tissue.

The results of this thesis are the first to support the hypothesis of synergistic mechanisms at play between Endocannabinoids and Adiponectin in the skeletal muscle of Obese and Diabetic skeletal muscle tissue.

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Declaration

I, Lannie O'Keefe, declare that the PhD thesis entitled 'Determining the Influence of Endocannabinoids in Skeletal Muscle Adiponectin sensitivity in Diet Induced Obesity and Diabetes' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Signature Date

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Joshua Kanaan

Isaac Daniel

Elijah Joseph

♥

Noah Aloysius

Mackay Anthony

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Published Manuscripts Arising From This Thesis

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Manuscripts in Preparation

1. **The effects of chronic CB_1 antagonsim AM251 on metabolic changes in a DIO model.** *Lannie O'Keefe, Anna C Simcocks, Kayte A Jenkin, Michael L Mathai, Deanne H Hryciw, Andrew J McAinch*

2. **Metabolic changes in a DIO model following chronic CB_2 agonism: Fat pad reduction increases in IL-12po.** *Lannie O'Keefe, Anna C Simcocks, Kayte A Jenkin, Michael L Mathai, Deanne H Hryciw, Andrew J McAinch*

3. **CB_2 antagonism in a DIO model reduces brown fat mass while increases citrate synthase in oxidative skeletal muscle.** *Lannie O'Keefe, Anna C Simcocks, Kayte A Jenkin, Michael L Mathai, Deanne H Hryciw, Andrew J McAinch*

Other Publications

Kayte A Jenkin, **Lannie O'Keefe,** Anna C Simcocks, Esther Grinfeld, Michael L Mathai, Andrew J McAinch, Deanne Helena Hryciw: *Chronic administration of AM251 improves albuminuria and renal tubular structure in obese rats*. Journal of Endocrinology 03/2015; 225(2). DOI:10.1530/JOE-15-0004

K A Jenkin**, L O'Keefe,** A C Simcocks, J F Briffa, M L Mathai, A J McAinch, D H Hryciw: *Renal Effects of Chronic Pharmacological Manipulation of CB2 in Rats with Diet Induced Obesity.* British Journal of Pharmacology 12/2014; 173(7). DOI:10.1111/bph.13056

Anna C. Simcocks, Kayte Jenkin, **Lannie O'Keefe**, Michael L. Mathai, Deanne H. Hryciw, Andrew J. McAinch: *O-1602 reduces plasma concentrations of leptin and ghrelin but increases AST, in diet-induced obese rats*. Obesity Research & Clinical Practice 12/2014; 8. DOI:10.1016/j.orcp.2014.10.117

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Anna Simcocks (nee Roy, **Lannie O'Keefe,** Kayte Jenkin, Michael Mathai, Deanne Hryciw, Andrew McAinch: *Chronic administration of O-1602 diminishes adiposity in diet induced obese rat model*. Obesity Research & Clinical Practice 12/2013; 7. DOI:10.1016/j.orcp.2013.12.586

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A. McAinch, **L. O'Keefe,** E. Grinfeld, D. Hryciw, P. O'Brien, J. Wentworth, M. Mathai: *CB2 antagonism upregulates ADIPOR2 mRNA expression in obese diabetic derived human skeletal muscle myotubes*. Obesity Research & Clinical Practice 10/2012; 6. DOI:10.1016/j.orcp.2012.08.137

L. O'Keefe, E. Grinfeld, P. O'Brien, J. Wentworth, D. Hryciw, M. Mathai, A. McAinch: *CB1 antagonism mediates adiponectin signalling in obese and obese diabetic derived human skeletal muscle myotubes*. Obesity Research & Clinical Practice 10/2012; 6. DOI:10.1016/j.orcp.2012.08.036

L. O'Keefe, E. Grinfeld, P. E. O'Brien, M. L. Mathai, A. J. McAinch: *Determining the effects of blocking cannabinoid receptors on adiponectin signalling in cultured human skeletal muscle myotubes*. Obesity Research & Clinical Practice 10/2011; 5. DOI:10.1016/j.orcp.2011.08.027

Role of Testosterone on Muscle Protein Syntheiss during Prostate Cancer Treatment

Erik D. Hanson, Andre Nelson, Dan W. West, John Violet, **Lannie O'Keefe[2],** Claudio L. Battaglini FACSM, Glenn K. McConell, Stuart M. Phillips FACSM, Alan Hayes .

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Oral Presentations

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Blocking Cannabinoid Receptor 2 increases skeletal muscle citrate synthase activity.

O'Keefe L, Simcocks, A1, Jenkin, K, Hryciw DH, Mathai ML , McAinch AJ1

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Australian Society for Medical Research (ASMR) Victorian Student Conference, May 2014, Melbourne, Australia Blocking Cannabinoid Receptor 2 increases skeletal muscle

citrate synthase activi**ty. Lannie O'Keefe[1],** Kayte A. Jenkin[1], Anna C. Simcocks[1], Michael L. Mathai[1,2], Deanne H. Hryciw3, Andrew J. McAinch.

Figures

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Abbreviations

2-AG	2-Arachidonoylglycerol
AEA	Anandamide
AEC	Animal Ethics Committee
BLAST	205Basic Local Alignment Search Tool
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CB_1	Cannabinoid Receptor 1
CB_2	Cannabinoid Receptor 2
cDNA	Complementary Deoxyribonucleic Acid
CTGF	Connective Tissue Growth Factor
DEPC	Diethylpyrocarbonate Treated Water
DNA	Deoxyribonucleic Acid
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
FBS	Foetal Bovine Serum
GAPDH	Glyceraldehydes-3-Phosphate Dehydrogenase
GPCRs	G-Protein Coupled Receptors
HFD	High Fat Diet
IL	Interleukins
IP	Intraperitoneal
kD	Kilo Daltons
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein
MJ/kg	Megajoules per kilogram
MOPS	3-(N-morpholino)Propanesulfonic Acid
mRNA	Messenger Ribonucleic Acid
NOS	Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPAR	Peroxisome Proliferator Activated Receptors
RNA	Ribonucleic Acid
T2DM	Type 2 Diabetes Mellitus
TBS	Tris Based Saline
TBST	Tris Based Saline Tween 20
THC	Δ^9 Tetrahydrocannabinol
TNF-α	Tumour Necrosis Factor-α
AdipoR1	Adiponectin Receptor 1

AdipoR2	Adiponectin Receptor 2
AEA	Anadamide
2-AG	2-Arachidonoylglycerol
a-MEM	Minimum Essestial Medium
AMPK	5’ Adenosine Monophosphate-activated protein kinase.
APPL	Adaptor protein containing PH domain, PTB domain and leucine zipper motif
BAR	Bin-Amphiphysin-RVS
CB_1	Cannabinoid Receptor-1
CB_2	Cannabinoid Receptor-2
cDNA	complementary Deoxyribonucleic acid
DEPC	Diethylpryrocarbonate
DMSO	Dimenthyl Sulfoxide
ECM	Extracellular Matrix Coating Medium
EtOH	Ethonal
FBS	Fetal Bovine Serum
gAd	Globular Adiponectin
HbA1c	Haemoglobin A1c
HSD	Honestly Significants Differences Test
JAK/STAT	Januse Kniase/Signal Transducers and activators pathway.
mRNA	messenger Ribonucleic Acid
NaCI	Sodium Chloride
P1	Passage 1
P2	Passage 2
P3	Passage 3
PBS	Phosphate Buffered Saline
PGC1-*alpha*	Peroxisome proliferator-activated receptor γ co activator – 1 *alpha*
PGM	Primary growth Medium
‘real time’ PCR	Reverse transcription polymerase chain reaction
SPSS	Statistical package for the social sciences

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Chapter 1 - The Cannabinoid Receptor 1 and its role in influencing peripheral metabolism

1.0 The Endocannabinoid System (ECS)

In the early 1960's the psychoactive compound in cannabis, Delta-9 tetrahydrocannabinol (ΔTHC), was first identified (Sugiura et al. 2006, Watkins et al. 2010). In 1992, the G protein coupled receptor (GPCR) cannabinoid receptor 1 (CB_1) was cloned (Devane et al. 1992). CB_1 is part of the endocannabinoid system which is a complex lipid signalling system, with CB_1 being widely distributed in a number of different tissues (Engeli et al. 2005). Human and animal trials have shown that this receptor plays a key role in the regulation of appetite and energy metabolism (ravine et al. 2004). CB_1 is activated by endocannabinoid ligands, which are small lipid molecules that are synthesized on demand (Alvheim et al. 2012), and precursors to these endocannabinoid ligands are circulating free fatty acids (Eckardt et al. 2009).

1.1 Endocannabinoid Receptors

There are two recognized cannabinoid receptors known as CB_1 and CB_2 which are both seven-transmembrane-domain GPCRs (Carayon et al. 1998). The endocannabinoid receptor, CB_1, was first thought to be expressed predominantly in the central nervous system and was initially termed a brain cannabinoid receptor being identified in the brain cortex, hippocampus, amygdala, pituitary and hypothalamus (Devane et al. 1992, Herkenham et al. 1991, Chorvat et al. 2012). However, it has subsequently also been identified in peripheral organs and tissues such as the gastrointestinal tract, adipose tissue, thyroid, adrenals, skeletal muscle, hepatocytes, kidneys, reproductive organs and endocrine cells of the pancreas (Chorvat et al. 2012; Pagotto

et al. 2006, Jenkin et al. 2010). CB_1 has subsequently been shown to play a significant role in energy metabolism, appetite control, endocrine and metabolic regulation (Pagotto et al. 2005).

1.2 CB_1 Ligands

The characterisation of the biologically active properties of tetrahydrocannabinol (ΔTHC) allowed the identification of endocannabinoid lipid ligands, which are compounds derived from long chain polyunsaturated fatty acids. Endocannabinoid ligands enter cells via the cannabinoid receptors (Ravient et al. 2004, Jenkin et al. 2010). The first endocannabinoid ligand identified in 1992 was anandamide (AEA) (Devane et al. 1992). It was later revealed that they are synthesised following an increase in intracellular calcium (Ca^{2+}) levels (Chorvat et al. 2012, Di Marzo et al. 2004). Over two decades later a variety of ligands, including oleoylethanolamide (OEA), 2-arachidonoylglycerol (2-AG) and palmitoylethanolamide (PEA) (Pagotto et al. 2006, Mechoulam et al. 1995, Capasso et al. 2001), have been identified with AEA and 2-AG the two most investigated ligands. Along with the discovery of these endogenous ligands, a number of synthetic analogues to the endocannabinoid system that both resemble (referred to as classical) and differ (referred to as alternative) from the chemical structure of ΔTHC have been synthesised (Gaoni et al. 1971, Howlett et al. 2002). Aminoalkylindoles which are novel cannabinoid mimetics have also been developed (Eissenstat et al. 1995). The discovery and development of these compounds have assisted in advancing our understanding of CB_1 mediated signalling.

1.3 CB_1 signalling

CB_1 is a GPCR that is located in the cell membrane where it is coupled to intracellular effector systems via G-proteins. CB_1 is a typical GPCR that contains seven transmembrane domains

connected by three extracellular and three intracellular loops with an extracellular N-terminal tail, and an intracellular C-terminal tail (Tura et al. 2010) [*Figure 1*]. CB_1 functions as a mediator of intracellular signalling as it is involved in a variety of regulatory roles including, cell growth and differentiation, neurotransmission, cardiovascular function, immune defence and cellular metabolism (Howlett et al. 2006). At a cellular level CB_1 has been found to participate in a variety of functions such as inhibiting voltage-gated Ca^{2+} channels, activating K^+ currents, inhibiting adenylyl cyclase, activating mitogen-activated protein kinases (MAPK), and increasing nitric oxide (NO) signalling (Mackie et al. 1992)

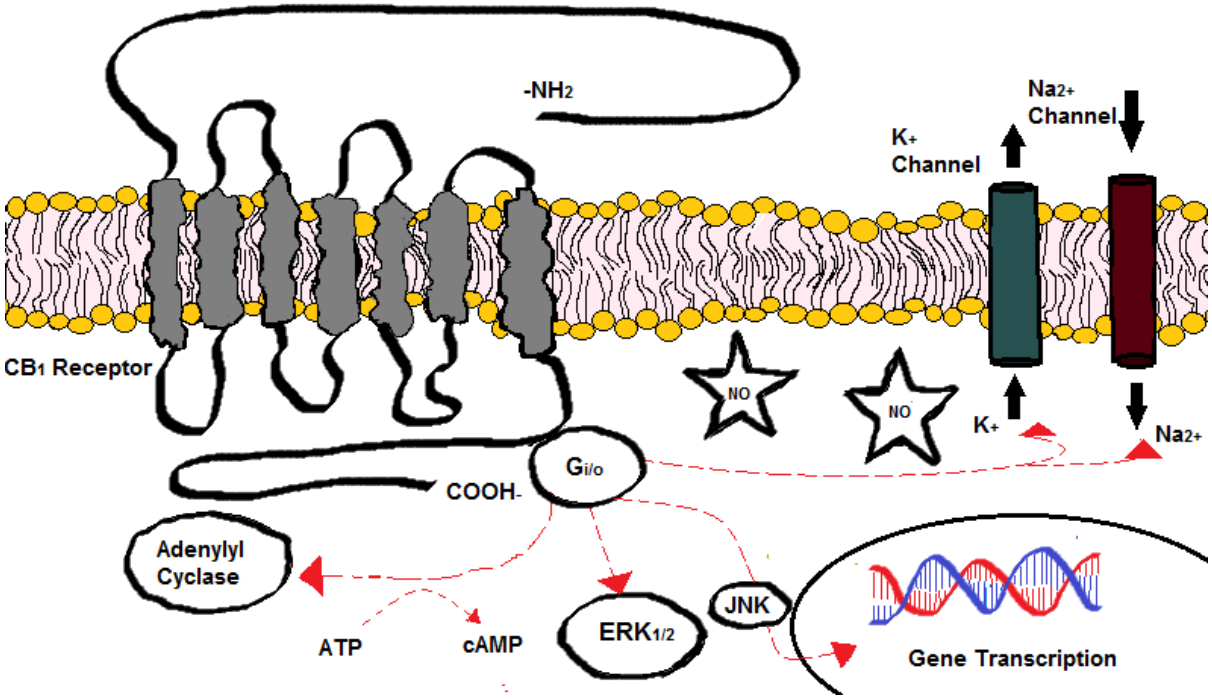


Figure 1 *A schematic view of the initial pathways that are activated via CB_1 signalling, including the inhibition of adenylyl cyclase, activation of mitogen-activated protein kinases (MAPK,) voltage-gated Ca^{2+} channels, activation of K^+ currents, and increases nitric oxide (NO) signalling (Adapted from Hohmann et al. 2006).*

In the brain, a primary function of the CB_1 signalling pathway is to participate in voltage-gated Ca^{2+} channels and activation of K^+ currents in the modulation of neurological responses to neural depolarisation (Herlitze et al. 1996). The calcium ions enter cells through gated Ca^{2+} channels stimulating rapid release of neurotransmitters influencing in turn intracellular K^+ channels (Adams et al. 2002). In the majority of other tissues, CB_1 activity is mediated through G coupled proteins (G_i, G_o, G_s, $G_{q/11}$). This coupling inhibits adenylyl cyclase, an enzyme that is responsible for cyclic adenosine monophosphate (cAMP) formation (Mackie et al. 1992). Adenylyl cyclase initiates the conversion of adenosine triphosphate (ATP) to cAMP. In peripheral tissues, cAMP functions as a cellular secondary messenger and operates with associated kinases to play a role in biochemical processes including the metabolism of glycogen, sugar, and lipids. For example, cAMP is required in the signalling of a series of pathways within skeletal muscle such as the biochemical responses to the hormones leptin and adiponectin, and initiating glucose transporter type 4 (GLUT4) translocation in response to insulin (Adams et al. 2002). Further, cAMP has been shown to increase CB_1 gene expression in neurons (Howlett et al. 2010).

Stimulation of CB_1 by endocannabinoid lipid ligands also leads to downstream phosphorylation and activation of MAPK (Bouaboula et al. 2005). MAPK are stimulated by G proteins at the tyrosine, serine and threonine residues that mediate the pathways of various growth factors and cytokines (Nishimoto et al. 2006, Brighton et al. 2009). One such pathway is the MAPK-modulated Extracellular Regulated Kinase 1 and 2 (ERK 1/2) phosphorylation cascade that plays an important role in cellular proliferation, differentiation, and survival (Sarnataro et al. 2006). CB_1 has been found to modulate ERK 1/2 signalling, with CB_1 specific agonists causing

a down regulation of this pathway (Yu et al. 2007). Conversely, when CB_1 activation is blocked using a CB_1 specific antagonist, ERK 1/2 levels are increased (Cowan et al. 2003).

CB_1 activation, which up regulates MAPK also initiates c-Jun N terminal kinases (JNK) which are kinases that bind and phosphorylate in response to stress stimuli, such as cytokines, they are also known to play a role in T cell differentiation and apoptosis. The JNK family consists of JNK1 and JNK2 which are widely expressed in peripheral tissues, while JNK3 is found exclusively in the nervous system and the heart (Davis et al. 2000; Tuncman et al. 2006, Bloomgarden et al. 2007). JNK are activated via G proteins following CB_1 receptor agonist stimulation (Tuncman et al. 2006, Bloomgarden et al. 2007). Levels of JNK are increased in conditions of excessive adipose storage affecting peripheral organs such as liver, skeletal muscle and adipose tissue (Togo et al. 2004). This over expression of JNK in obesity which results in inflammation and contributes to insulin resistance may at least be partially explained via CB_1 agonist activation in obesity (Eckardt et al. 2008).

The cellular activation of CB_1 is also found to affect pathways such as NO that are up regulated in pathological conditions. NO is synthesised in most biological tissues and it participates in a multitude of biological functions including neurotransmission, vasodilatation and macrophage function. Under normal homeostatic conditions NO plays a protective role assisting in blood pressure control through vasodilatation and inflammatory control (Nussler et al. 1993).

Increases in NO in the central nervous system (CNS) have also been suggested to be associated with pathologies of neurological decline such as dementia (Erdos et al. 2004, Steinert et al. 2010). In peripheral tissue NO has been implicated in the down regulation of mitochondrial biogenesis and inflammation in both adipose and skeletal muscle in conditions of excessive

lipid storage (Stenert et al. 2010, Valerio et al. 2006, Joost et al. 2007). In the liver, increased levels of NO have been found in conditions of liver dysfunction (El-Sherif et al. 2008).

1.4 CB_1 and Skeletal Muscle

The signalling pathway of CB_1 is an intricate cascade of reactions; its signalling role appears to have differing effects in both CNS and peripheral tissues (Cavuoto et al. 2007, Eckardt et al. 2009, Mackie et al. 1992). Extended stimulation of CB_1 via endocannabinoid ligands, in response to excess lipid accumulation results in a multitude of unfavourable physiological events (Miller et al. 2011). The CB_1 signalling pathways that are activated in the brain are well documented (Mackie et al. 1992, Herlitze et al. 1996). In the liver, less is known about the CB_1 pathway, with the direct activation of CB_1 receptors thought to stimulate lipogenesis through increased expression of sterol regulatory element binding protein 1-c (SREBP-1c), acetyl-CoA carboxylase–1 (ACC1) and fatty acid synthase (FAS) (Osei-Hyiaman et al. 2005). While CB_1 receptor expression has been identified in skeletal muscle, the downstream signalling pathways in this tissue are not well defined. Agonism of CB_1 receptors have been shown to influence intracellular signalling pathways associated with an increase in insulin resistance in skeletal muscle (Lipina et al. 2010). In agreement with this finding, blocking CB_1 in the skeletal muscle has been demonstrated to increase glucose uptake in oxidative muscle and the expression of genes involved in oxidative metabolism (Cavuoto et al. 2007, Pagotto et al. 2005, Liu et al. 2005, Esposito et al. 2008) .Blockade of CB_1 was shown to increase fatty acid oxidation in skeletal muscle myotubes by up-regulating genes in the energy metabolism pathways including 5′ adenosine monophosphate-activated protein kinase (AMPK) α1 and α2, pyruvate dehydrogenase kinase 4 (PDK4) and peroxisome proliferator-activated receptor co-activator-1α (PGC1-alpha) (Cavuoto et al. 2007). The fact that blocking CB_1 activity increases AMPK

gene expression in skeletal muscle is intriguing as AMPK responds to decreases in energy states (Kelley et al. 2002). AMPK activation restores ATP levels by initiating processes that generate ATP (Kemp et al. 2003). AMPK induces a cascade of events that effect fatty acid oxidation and skeletal muscle insulin resistance (Feldhoff et al. 1993). Decreases in AMPK have been implicated in the pathogenesis of metabolic syndrome (Davis et al. 2000, Feldhoff et al. 1993).

PDK4 phosphorylates and inactivates pyruvate dehydrogenase complex which converts pyruvate to acetyl CoA, in a rate-limiting step of glucose oxidation (Feldhoff et al. 1993, Stace et al. 1992). PDK4 gene expression has been found to be suppressed in the skeletal muscle of insulin resistance rats (Kim et al. 2006). Moreover, in Zucker diabetic fatty (ZDF) rats peroxisome proliferator-activated receptor (PPAR) agonists increased insulin sensitivity (Kanda et al. 2009). PPARs are a family of nuclear receptors that have been implicated in the control of metabolism (Kersten et al. 2000). It has been suggested that PPAR may play a role in inducing skeletal muscle fibre type composition to favour higher oxidative capacity (Luquet et al. 2003). Natural ligands for PPARs include fatty acids and eicosanoid derivatives (Sun et al. 2007). PPARγ has a high affinity for polyunsaturated fatty acids such as linoleic acid, arachidonic acid, and eicosapentaenoic acid, which are known precursors for ECS ligands (Herling et al. 2008). Interestingly, AEA has also been found to directly bind to and up regulate PPARγ in different tissues (Sun et al. 2007).

Antagonism of CB_1 increases energy expenditure via increased fatty acid oxidation, which is thought to contribute more to the body weight reduction that occurs when CB_1 is blocked than the decrease in food intake (Herling et al. 2008). The increase in fatty acid oxidation may occur via skeletal muscle (Tanner at el. 2002). Skeletal muscle is an essential tissue for whole body

energy metabolism, including fatty acid oxidation; fatty acid transporters and binding proteins (Andersson et al. 2002). The molecular changes that occur when the CB_1 signalling pathway is blocked may inhibit the influx of intracellular fatty acids, allowing increased expression of the genes involved in fatty acid oxidation and glucose control. These positive effects in metabolic peripheral tissues are independent of the CNS, making them a target for metabolic disorders (Tam et al. 2010).

1.5 CB_1 in Obesity

Obesity is a multifaceted disorder of energy balance that is a costly health problem that is increasing in western societies (Popkin et al. 2006). Obesity is a recognized risk factor contributing to metabolic disturbances (Kahn et al. 2006). The metabolic disturbances that occur in obesity and the association with CB_1 was demonstrated in Obese Zucker (*fa/fa*) rats via administration of a CB_1 specific antagonist for 14 days which resulted in reduced body weight and stimulated adiponectin release (Bensaid et al. 2003). Adiponectin has beneficial effects in skeletal muscle by improving glucose tolerance, increasing fatty acid oxidation and mitochondrial biogenesis (Lihn et al. 2005; Yang et al. 2005, Mao et al. 2006). In agreement with these findings CB_1 knockout mice ($CB_1^{-/-}$) are obese resistant and show decreased levels of plasma insulin and leptin and furthermore display no change in appetite regulation when compared to diet-induced obese wild type mice (Cavuoto et al. 2007). In humans, blocking CB_1 significantly decreases waist circumference which is indicative of a reduction in visceral fat deposits (Despres et al. 2005, Van Gaal et al. 2005, Pi-Sunyer et al. 2006, Scheen et al. 2006).

The effects of blocking CB_1 on the human hepatocyte- derived cell line (HEPG2) and mouse hepatocyte-derived cell line (AML-12) was researched by Wu (Wu et al. 2011), with CB_1

antagonism resulting in the activation of Protein Kinase A (PKA), a protein kinase involved in lipid metabolism, which subsequently suppressed SREBP-1c expression (Jourdan et al. 2010). Diet induced obesity results in an increase in the expression of CB_1 in both liver and adipose tissue (Jourdan et al. 2010). Following oral administration of a CB_1 specific antagonist, for a period of 6 weeks, these diet-induced obese mice normalised their CB_1 expression and had substantial reversal in hepatic steatosis (Cota et al. 2007).

Obesity associated hepatic steatosis and dyslipidaemia were also significantly reversed in Zucker *(fa/fa)* rats after 8 weeks of CB_1 specific antagonist treatment (Gary-BoBo et al. 2007)]. In supporting data, it has been demonstrated that blocking CB_1 in hepatocytes collected from lean and obese *(ob/ob)* mice increased the expression of genes involved in carbohydrate and lipid metabolism, and this result was reversed when CB_1 receptors are stimulated with a specific agonist, causing a decrease in key proteins that play a role in hepatic lipogenesis (Tam et al. 2010). This data suggests that blocking CB_1 in the liver has a direct peripheral effect on carbohydrate and lipid metabolism. The desirable results achieved when CB_1 is blocked in liver tissue have been replicated in the skeletal muscle (*Figure 2*) (Cavuoto et al. 2007).

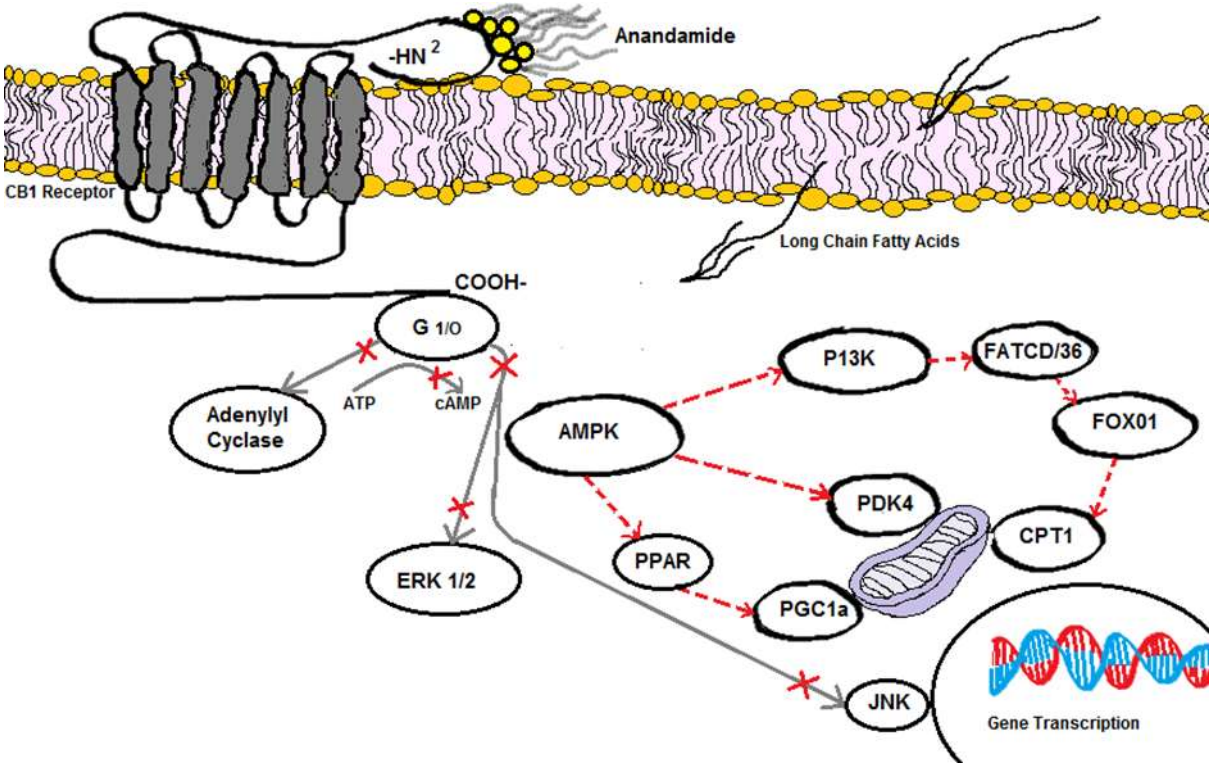


Figure 2: *A schematic view of the signalling cascade that may be activated to increase intracellular fatty acid metabolism in skeletal muscle. Blocking CB_1 signalling decreases the inhibition of adenylyl cyclase, blocks activation of mitogen-activated protein kinases (MAPK), and decreases NO signalling. This down regulation of the CB_1 signalling pathway causes AMPK to be up regulated resulting in a downstream signalling cascade involved in fatty acid oxidative metabolism and glucose control (Adapted from Cavuoto et al. 2007, Tam et al. 2010, Hohmann et al. 2006).*

Acute CB_1 manipulation on insulin sensitivity and glucose uptake was tested using wild-type mice (Song et al. 2011). The CB_1 receptor agonist HU210 significantly reduced glucose uptake in skeletal muscle and significantly decreased insulin stimulated signalling pathways. This effect was reversed when animals were administered a CB_1 antagonist (AM251) (Song et al. 2011).

The increase in circulating endocannabinoids and lipid storage in skeletal muscle that is observed in obesity was investigated by Blüher (2006). Muscle samples of both male and female participants were collected after open abdominal surgery. Results showed that mRNA

expression of enzymes that synthesise 2-AG had increased and the circulating levels showed a direct correlation with visceral obesity (Sarzani et al. 2009). In addition to this, CB_1 is also up-regulated in visceral fat deposits in obese states (Sarzani et al. 2009).

Liu (2005) used the pharmacological compound Rimonabant (a CB_1 antagonist) to investigate energy expenditure and glucose uptake in skeletal muscle of (*ob/ob*) genetically obese mice. All mice exhibited common risk factors that are associated with obesity (hyperglycaemia, hyperinsulinemia and insulin resistance) (Liu et al. 2005, Espositi et al. 2008). After a period of seven days, the mice receiving the antagonist displayed a reduction in food intake, reduction in body weight, a 68% increase in glucose disposal following an acute glucose load and interestingly, a 37% increase in basal metabolic rate (Liu et al. 2005). Crespillo (2010) investigated the impact of high fat feeding on the expression of lipid metabolism-related genes and endocannabinoid-signalling proteins. In male diet induced obese Wistar rats, 14 days of treatment with the CB_1 antagonist AM251 resulted in reduced caloric intake and a reduction in weight gain (Crespillo et al. 2010).

A supporting study by Guijarro (2008) looked at the down regulation of endocannabinoids and mitochondrial function of diet induced obese rodents after Roux-en-y gastric bypass surgery. CB_1 receptors were blocked using AM251 which resulted in a decrease in AEA and an up-regulation of mitochondrial respiration 28 days post-operative. Interestingly, circulating hormones such as adiponectin were increased at the end of the 28-day period (Guijarro et al. 2008).

1.6 Adipose Tissue and CB_1

Adipose tissue is an endocrine functional organ that plays a crucial role in obesity and its co-morbidities (Fain et al. 2004). Circulating endocannabinoid ligands bind to adipose tissue CB_1 receptors to be metabolised by the adipocytes (Spoto et al. 2006). Cultured primary adipose cells derived from C57BL/6N mice treated with a CB_1 agonist increased lipoprotein lipase in a dose dependent manner (Cota et al. 2003). Lipoprotein lipase is required by adipose tissue for fatty acid uptake and storage (Gonzales et al. 2007). In direct opposition, the up regulation was blocked after treatment with the CB_1 specific antagonist Rimonabant (Jbilo et al. 2005). Similarly, treatment with Rimonabant on cultured adipocyte cells initiated CB_1 stimulated expression of adiponectin (Lihn et al. 2005, Bensaid et al. 2003, Gary BoBo et al. 2006, Kim et al. 2012, Thornton-Jones, 2006). Rimonabant was also shown to have a direct influence on obese adipocytes changing gene expression in both white and brown adipose tissue (Jbilo et al. 2005). The cumulative effects of excess adipose on both the liver and skeletal muscle have been implicated in the deteriorating progression of normal tissue to insulin resistant tissue and finally diabetes (Wang et al. 2001, Jansen et al. 1992).

1.7 CB_1 and central mediated effects

The brainstem has been identified as having exiguously placed CB_1 expression (Jansen et al. 1992). Despite the low-level expression, when activated, CB_1 exhibit an influential effect on maintaining homeostasic conditions (Rademacher et al. 2003). Endocannabinoid ligands within the brain activate presynaptic CB_1 and again play a role in postsynaptic signalling (as reviewed by Di Marzo (2011). The exact role that the ECS plays in emotional psycho-behavioural responses is still unclear; CB_1 however, have been located in areas of the brain that are known to initiate responses in behavioural change (Christensen et al. 2007).

The anti-obesity effect of Rimonabant by Sanofi-Aventis was the first pharmacologically approved selective CB_1 inverse agonist/antagonist (Cota et al. 2003). The Rimonabant in Obesity (RIO) program consisted of large scale clinical trials spanning over one to two years. The initial findings from the RIO trials were promising, a large proportion of participants experienced significant weight loss, decrease in waist circumference and food intake, as well as an improvement in lipid profile and glucose clearance (Despres et al. 2005, Van Gaal et al. 2005, Pi-Sunyer et al. 2006, Scheen et al. 2006).

In the RIO- Lipids clinical trial (which involved 1,036 overweight and obese participants with untreated dyslipidemia), the average weight loss when compared to placebo on a dose of 20 mg of Rimonabant was 5.4 kg in one year (Despres et al. 2005). The lipid profile of participants taking Rimonabant improved, as did the risk of metabolic syndrome, which reduced by 41% in participants receiving the 20-mg dose daily (Despres et al. 2005).

RIO-Europe recruited 1,507 overweight and obese participants and was undertaken for 1 year. Participants received dietary advice and were assigned to a placebo, 5 mg Rimonabant or 20 mg Rimonabant group (Pi-Sunyer et al. 2006). Compared to control the 5 mg Rimonabant group experienced significant weight loss of 3.4 kg this loss was increased in the 20-mg group with a weight loss of 6.6 kg this result shows the impact that blocking CB_1 signalling has on obesity (Pi-Sunyer et al. 2006).

RIO-North America was a two-year trail involving 3,045 overweight and obese participants. This study found that when compared to the control group, participants on 20 mg Rimonabant showed a decrease in waist circumference and an overall improvement in lipid profile (Scheen et al. 2006). The average weight loss was 7.4 kg. Interestingly, at the completion of the first-

year participants receiving 20 mg of Rimonabant were split into two groups with half continuing to receive Rimonabant and the other half received a placebo. Whilst the Rimonabant group maintained weight loss, the placebo group proceeded to regain weight demonstrating the long-term effectiveness of the drug (Pi-Sunyer et al. 2006). Finally, the RIO-Diabetes study used diet/exercise and Rimonabant in overweight and obese participants with controlled type 2 diabetes. These participants received lifestyle modification techniques and 20 mg Rimonabant daily. At the completion of the study, these participants showed an overall weight loss of 3.4 kg coinciding with improvements in both waist circumference and glycated haemoglobin (HbA1c) levels (Scheen et al. 2006). Despite Rimonabant having such positive effects on the pathologies of obesity it was withdrawn from market shortly after its release due to continual concerns over a small percentage of participants in these studies experiencing acute psychiatric syndrome (depression; anxiety or became suicidal) (Christensen et al. 2007).

1.8 Peripheral targeted CB_1 Antagonism – *The road forward*

The potency of an unregulated ECS on appetite, lipid distribution and storage in obesity is powerful. Controlling its effects has potent health implications on obesity and its associated co-morbidities (Tam et al. 2010, Gary-Bobo et al. 2007). This thesis has highlighted evidence to support that the positive effects in metabolic peripheral tissues when CB_1 signalling is blocked and has provided evidence to support that these positive effects are independent to any CNS effects.

On the basis of emerging data, the evidence clearly demonstrates that targeting peripheral CB_1 is a promising therapeutic target to induce weight loss and improve metabolic disorders. The

adverse psychoactive effects of CB_1 blockage in the brain would be greatly attenuated if a CB_1 selective antagonist was to target peripheral tissues and not cross the blood brain barrier (BBB).

Initial investigations into the development of such compounds met with disappointing results, showing that the positive effects were short lived or did not share the same effects as the brain penetrating parent compound, Rimonabant (Wu et al. 2011). Further research however has seen the development of a range of newer, more effective peripheral CB_1 targeted compounds. Some of the most recent compounds being investigated and their effects on obesity are discussed below.

AM4113 [*N*-piperidin-1-yl-2,4-dichlorophenyl-1h-pyrazole-3-carboxamide analog]

Research has suggested that the compound AM4113, a CB_1 antagonist, may provide some therapeutic benefit. Sink (2007), tested AM4113 in Sprague-Dawley rats fed either a normal diet or a diet high in carbohydrates or fat. Analyses of these animals determined that AM4113 crossed the BBB however, displayed properties of being CNS neutral. There were no apparent psychoactive effects as determined by contextual fear retention tests, this selective antagonist was shown to reinforce appetite control (Sink et al. 2007). Later testing showed that this change in appetite was due to peripheral control of the ECS. While AM4113 injected into the brain was shown to react with neural CB_1, the positive appetite control did not alter between the CNS activated CB_1 or the peripherally targeted CB_1. This finding gives additional evidence that supports the therapeutic benefit of peripherally targeted CB_1 antagonism (Son et al. 2010).

AM6545 [5-[4-[4-cyanobut-1-ynyl]phenyl]-1-[2,4-dichlorophenyl]-4-methyl-*N*-[1,1-dioxo-thiomorpholino]-1*H*-pyrazole-3-carboxamide]

Studies have investigated the use of AM6545 in obesity management. AM6545 is a high affinity and selective CB_1 peripheral antagonist that showed a low level of BBB permeability (Tam et al. 2010). In diet induced obese mice, AM6545 displayed the same effects as Rimonabant, by reducing the effects of fatty liver and improving lipid profile. The AM6545 treatment group and the Rimonabant treatment group both showed an increase in glucose plasma clearance after treatment (Tam et al. 2010). This is further evidence to support targeting peripheral receptors in the treatment of obesity and diabetes can be as effective in improving obesity metabolic parameters as the CNS permeable Rimonabant (Tam et al. 2010).

Compound 1

Compound 1 is a CB_1 specific antagonist that is likely to be peripherally selective; the exact mechanisms behind Compound 1 selectivity (either structural or modulating mechanisms) are unclear (Son et al. 2010). Son et al (2010) compared Rimonabant with Compound 1 in a diet induced obesity model in mice (Son et al. 2010). Despite having decreased levels of endocannabinoids in the brain and increased circulating levels in plasma, both treatment groups showed a reduction in food intake and weight loss compared to the control group (Son et al. 2010).

LH-21 [5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-3-hexyl-1*H*-1,2,4-triazole]

The CB_1 antagonist, LH-21, has also been investigated as a therapeutic. LH-21 displays poor brain penetration and it has been shown to have positive effects in peripheral CB_1 targeting *in vitro* studies (Alonso et al. 2012). In an animal study, following 10 weeks of high fat feeding,

rats were treated with LH-21 for 10 days; the rats displayed reduced food intake, reduction in weight gain and a decrease in gene expression of leptin and lipogenic enzymes (Alonso et al. 2012). These positive markers were independent of plasma changes between the LH-21 groups when compared to control. When the liver was analysed for changes, there was an increase in fatty acid accumulation in the diet-induced obese rats. In this study, LH-21 had no effect on altering the fatty acid composition of the liver. This finding was confirmed by Pavon (2008) who found that LH-21 did not influence the same positive physiological effects that the previous centrally active antagonists displayed (Pavon et al. 2008). To better determine if LH-21 is a valid target for peripheral antagonism of CB_1 receptors research investigating its effects in skeletal muscle is fundamental.

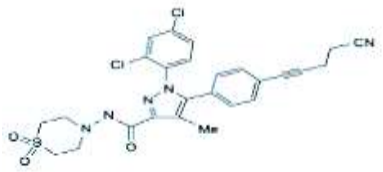
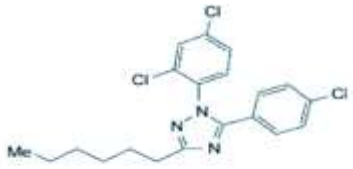
JD5037

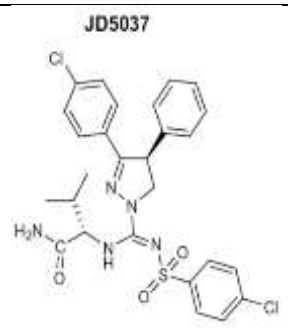
Finally, a new compound JD5037 has shown propitious results in the treatment of obesity (Tam et al. 2012). The CB_1 antagonist/inverse agonist was shown to block peripheral CB_1 receptors following both acute and chronic administration in diet-induced obese mice (Tam et al. 2012). The treated animals displayed reduced body weight and food intake when compared to the control group. This compound reversed the effects of hepatic steatosis which is similar to the effects of Rimonabant. Interestingly the JD5037 treatment group showed improved insulin sensitivity (Tam et al. 2012). Determining if this glucose clearance was due to an improvement in fatty acid oxidation and glucose uptake in skeletal muscle will provide further evidence to support this compound as the peripheral CB_1 target compound that has the same positive effects in obesity and diabetes as those seen in the pharmacological treatment with Rimonabant.

The quest for a successful peripheral CB_1 antagonist should not ignore the translation of the possible adverse psycho-behavioural effects when central CB_1 receptors are blocked. Studies

investigating the behavioural effects of Rimonabant have been limited. In rats, acute administration of Rimonabant increases anxiety like behaviours (Tallett et al. 2007), while in mice however, Rimonabant has been shown to have an anti-depressive effect in a mild stress model (Shearman et al. 2003). Interestingly only LH-21 of the peripherally targeted CB_1 antagonists has been investigated in regard to psycho-behavioural effects (Griebel et al. 2005). Despite the new peripheral compounds showing that they either fail to cross the BBB at all or they are present in trace amounts it must be remembered as previously discussed that any stimulation of CB_1 receptors in the CNS can exhibit potentially deleterious responses.

Table 1 Compound Structure

Compound	Compound Structure	Reference
AM6545	 **AM6545**[a] [5-(4-[4-cyanobut-1-ynyl]phenyl)-1-(2,4-dichloro phenyl)-4-methyl-N-(1,1-dioxo-thio-mopholino)-1H-pyrazole-3-carboxamide]	Tam et al. 2012
LH-21	 **LH-21**[a] [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-3-hexyl-1H-1,2,4-triazole]	Alfonso et al. 2012,

JD5037		Tam et al. 2012

Obesity results in dysfunction in many different human systems. The implications of this stress are evident in all tissues. There is sufficient evidence in both *in vitro* and *in vivo* studies implicating an up regulated ECS in a series of pathological conditions. The cellular CB_1 signalling pathway is a complex serious of pathways that when blocked results in whole body weight-loss and improvements in obesity and its associated co-morbidities. In the CNS however, CB_1 antagonism results in adverse psychological side effects. In adipose and liver tissue, blocking CB_1 signalling increases lipolysis and reverses steatosis, while in skeletal muscle blocking CB_1 signalling increases glucose uptake and fatty acid oxidation. Appetite, lipid distribution and storage in obesity are all up regulated by an increased CB_1 activity resulting in serious health implications. Controlling the CB_1 pathway in metabolic peripheral tissues has positive effects and does not require CB_1 antagonism on the CNS. The new generation of peripheral targeted pharmacological CB_1 antagonists are showing promising evidence to support CB_1 peripheral antagonism as a viable target for the treatment of obesity and its associated co- morbidities.

1.9 The cannabinoid receptor 2 (CB_2) and its enigmatic role in obesity and skeletal muscle metabolic signalling

CB_2 is a member of the G- protein coupled receptor (GPCR) protein family and was first cloned in mice spleen three years after its predecessor CB_1 (Munro et al. 1993). The CB_2 receptor however shares only 44% homology with the CB_1 receptor (Munro et al. 1993), despite distinct but overlapping distribution. The shared ancestry between these two receptors is such that although they may be biologically related and although they share similar signalling pathways, they have varying individual roles (Munro et al. 1993). As a receptor, CB_2 is a single chained polypeptide that contains seven transmembrane α-helices, with an extracellular glycosylated N-terminus and an intercellular C-terminus (Cabral et al. 2009). The role of CB_2 in metabolism is unclear as there is very limited information available. Therefore, its potential as a novel target in metabolic diseases including obesity is yet to be determined.

1.10 Inflammation and CB_2

Cytokines are a large group of low-molecular weight proteins secreted by various tissues which are directly involved in cell communication, inflammation and immune response (Ziring et al. 2006, Caër, 2016). They assist in the regulation of cell differentiation, proliferation, and apoptosis (Valerio et al. 2006, Miceli, 2016). They are essential for the maintenance and regulation of biological functions at the cell, tissue and organ level (Valerio et al. 2006). Obesity causes a metabolic disturbance which results in the production of increased levels of inflammatory cytokines (Ziring et al. 2006). This increase in inflammatory cytokines may result in skeletal muscle damage (Haiyan et al. 2013). It has been demonstrated in CB_2 knockout mice are deficient in a range of immune cells. This alteration in levels of immune

cells contributes to the initiation of inflammation. (Ziring et al. 2006; Ostanin et al. 2005; Elmes et al. 2004). CB_2 may also play a role as a modulator in immune responses; however, its exact role is not clear. Reviewing all the possible signalling pathways that may be involved in skeletal muscle and possible CB_2 inflammatory pathway is beyond the scope of this literature review. It will however present recent evidence in tissues other than skeletal muscle that have a relationship between CB_2 and well-known cytokines, Interlukin-6 and tumour necrosis factor - alpha and NF-κB.

1.11 NF-κB Pathway and CB_2

NF-κB is situated in the cytosol bound to inhibitors IķB. The phosphorylation of IķB occurs when activated by a variety of inflammatory markers or a GPCR agonist resulting in the migration of NF-κB to the nucleus. In microglial cells of the central nervous system (CNS) activation of CB_2 via an antagonist through the activation of ERK1/2 and JNK MAPKs activity inhibited NF-κB (Correa et al. 2009). In skeletal muscle NF-κB is of particular interest in its role in insulin resistance (Wang et al. 1999). As a consequence of altered intracellular signalling via PKB/Akt, IKKβ is able to activate NF-κB, which in turn regulates the production of proinflammatory cytokines such as TNFα and IL-6 (Puckett et al. 2013). CB_2 signalling activation in human coronary artery endothelia cells resulted in the down regulation of TNF-α signalling via NF-κB (Rajesh et al. 2007). In the skeletal muscle of diet induced obese male Wistar rats that were fed a high carbohydrate, highfat diet for 16 weeks, the content of skeletal muscle NF-κB was significantly increased compared to a lean control group (Sishi et al. 2010). Given that CB_2 modulation influences NF-κB in tissue of the CNS, the understanding of the role of CB_2 in skeletal muscle will assist in our knowledge of its role in insulin resistance.

1.12 CB_2 and Interlukin-6 (IL-6)

IL-6, an anti-viral protein, is a cytokine predominately derived from fibroblasts, macrophages and tumour cells and locally in skeletal muscle (Feng et al. 2014, Stensberg et al. 2002). It has been shown that CB_2 modulation in a human B lymphocyte continuous cell line SKW 6.4, significantly inhibited IL-6 secretion (Feng et al. 2014). IL-6 is secreted as a reaction to trauma, especially tissue damage leading to inflammation (Steensberg et al. 2002; Febbraio et al. 2004). IL-6 has been shown to be released in skeletal muscle upon muscle contractions during intense, prolonged exercise in healthy skeletal muscle (Steensberg et al. 2002; Febbraio et al. 2004; Haddad et al. 2005). However, in obesity there is chronic production of IL-6 (Steensberg et al. 2002). The implications of the continuous production of IL-6 and its relationship to CB_2 in obesity and diabetes, is not yet known.

1.13 Tumour necrosis factor alpha (TNFα) and CB_2

TNF-α contributes to the regulation of immune cells, which can induce inflammation (Cornelli et al. 2007). TNF-α and other pro-inflammatory cytokines such as IL-6 has been found to significantly increase the expression of CB_2 in the immune cells of peripheral blood taken from individuals of whom suffered an auto-immune inflammatory disorder. A range of concentrations were tested including low expression to mimic that of usual inflammatory circumstances (Jean-Gilles et al. 2015). Those with continuous inflammation showed a six-fold increase of TNF-α mRNA expression compared to the control group. This increase was further correlated with a fivefold increase of CB_2 mRNA expression. It was also confirmed in this study that the increase of CB_2 expression by TNF-α involved NF-κB signalling pathways (Jean-Gilles et al. 2015). In skeletal muscle, it has been demonstrated that TNF-α down regulates the

expression of electron transport chain genes, thus inhibiting fatty acid oxidation (Dahlman et al. 2006). This down regulation may cause a modulation to intracellular signalling resulting in an increased risk of the incidence of insulin resistance (Dahlman et al. 2006). Valerio et al. (2006) concluded that TNF-α impairs mitochondrial metabolic function in obese animal models (Valerio et al. 2006). TNF-α mRNA expression has also been demonstrated to be 2.5-fold higher in adipose tissue extracted from obese individuals (Pradhan, 2007; Holamisligil et al. 1995). The exact mechanisms for this up-regulation are still unknown; however recent studies have indicated that an inflammatory response is triggered by an accumulation of adipocytes causing increased TNF-α mRNA expression (Pradhan, 2007). Obese mice which were TNF-α deficient are protected from the cellular signalling modulation that exists in insulin resistance and have decreased levels of circulating fatty acids (Uysal et al. 1997).

TNF-α has also been shown to induce ceramide generation (Samad et a. 2006; Modur et al. 1996). Ceramides are sphingolipids, lipid molecules which are found in the membrane walls of cells (Samad et al, 2006; Helge et al. 2004). Ceramide is important in the structure of cells and acts as a signalling molecule involved in multiple signalling pathways which regulate a variety of biological events (Sawai et al, 2005). Abnormalities in ceramide metabolism have been implicated in muscle tissue in the pathogenesis of obesity (Samad et al, 2006).

1.14 CB_2 and Obesity

Obesity results in the altered regulation of a number of the body systems. Diet type and the compilation of nutrients influence inflammation in humans (Esposito et al. 2004). Obesity is negatively correlated with whole body insulin sensitivity (Arkan et al. 2005). Obesity has been found to be associated with chronic inflammation, resulting in abnormal cytokine production

and over activation of a network of inflammatory signalling pathways (Valerio et al. 2006). The first study to investigate the role of CB_2 and its possible mechanisms in obesity was researched by Agudo et al. (2010). Mice deficient in CB_2 (-/-) were compared against wild type control mice CB_2 (+/+). In addition, C57BI/6J wild type mice were treated with a highly selective CB_2 inverse agonist SR144528 *abcam*®. (Agudo et al. 2010). They were treated daily by intraperitoneal injection for 28 days (3mg/kg/bw) and saline was used for the control group. Results showed that obese CB_2 (-/-) knockout mice fed a high fat diet had improved insulin sensitivity (Agudo et al. 2010). Moreover, the CB_2 antagonist group, despite showing no change in body weight, had an improvement in insulin sensitivity (Agudo et al. 2010). The links between obesity, a high fat diet, inflammation and reduced insulin sensitivity in skeletal muscle are well documented (Valerio et al. 2006, Arkan et al. 2005, Esposito et al. 2004). Skeletal muscle due to its high physiological mass and capacity to switch between glucose and fatty acids as different fuel sources is a major consumer of fatty acids and glucose (Furler at al 2000; Shumen et al. 1990).

1.15 CB_2 and Diabetes

Diabetes is characterised as an increase in glucose resistance and potential modulation of intramyocellular signalling. Glucose uptake occurs via the translocation of the glucose transporters. Insulin binds to membrane receptors and triggers a cascade of phosphorylation of target proteins to initiate the relocation of glucose transporters on the Golgi apparatus to the cell membrane, where they take up glucose into the cell (Kim et al. 2000). This interruption of the glucose transporters causes a decrease in the amount of glucose that can enter the cell, thus resulting in insulin resistance (Kim et al. 2000; Turcott & Fisher, 2008). The exact mechanisms of this interruption to insulin signalling have not been determined (Bonen et al. 2004). Clinical

studies have shown a direct link between obesity and the associated development of diabetes (Kelley et al. 2002). This link is accompanied by an alteration in the endocannabinoid system and its effects on a variety of metabolic organs and systems. The activation of tissue specific CB_1 has been shown to result in a reduction in insulin sensitivity (Bermudez-siva et al. 2006).

The effects of chronic endocannabinoid tissue activation in a diet-induced obese mouse model was investigated by Guerts et al. (2013) Following a three-week control diet mice were separated into either a control, HF - high fat diet (60% - kcal/100g) or LPS – (Lipopolysaccharide) group that consumed an additional amount of carbohydrates (20% - kcal/100g). They were fed the diets for a further three weeks. The mice were implanted with a subcutaneous mini-osmotic pump at the beginning of the six week dietary treatment that delivered either HU210 (50µg/kg/day) a potent agonist of both CB_1 and CB_2 receptors, LPS from escherichia coli (0.55:B5 – (300µg/kg/day) and a saline control group (Guerts et al. 2014). Interestingly, the results showed that CB receptor agonist treatment caused a significant increase in CB_1 and CB_2 mRNA expression in skeletal muscle however this increase did not occur in the adipose tissue. The mice receiving the LPS treatment showed no alteration in cannabinoid receptor mRNA. The treatment groups showed no differences in body weight, body fat percentage or food intake. The CB agonism group combined with a HF diet resulted in diet induced glucose intolerance, HF diet induced inflammation in the skeletal muscle and an increase in the content of lipid in muscle (Guerts et al. 2014).

The intrinsic role that CB_2 plays in immunity regulation was investigated Deveaux et al. (2009). Obesity induced inflammation and insulin resistance was the focus of this study; which looked at CB_2 knockout mice that were fed a high fat diet for a total of 15 weeks (Deveaux et al. 2009). In findings parallel with previous studies, the knockout mice showed no obesity-induced

inflammation compared to the wild type mice (Deveaux et al. 2009). Moreover, the control mice developed both hyperglycaemia and hyperinsulinemia in contrast to the knockout mice, which showed no insulin resistance (Deveaux et al. 2009). This novel finding highlights CB_2 as a potential target into obesity-induced insulin resistance.

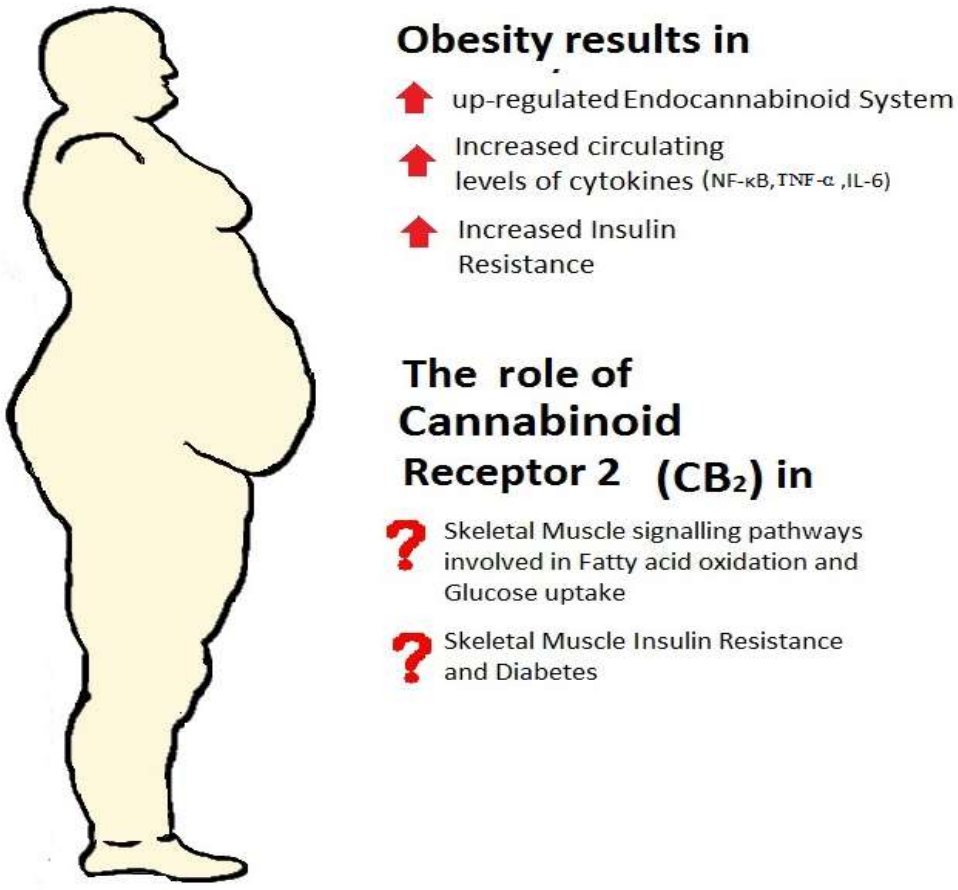


Figure 3: A schematic diagram detailing what we know about the effects of obesity on humans. Obesity results in an up-regulation of the Endocannabinoid system; increased levels of circulating inflammatory cytokines and an increase in insulin resistance. It also displays that the role of Cannabinoid Receptor 2 in skeletal muscle signalling pathways that are involved in fatty acid oxidation and glucose uptake; is not known. Equally its role in insulin resistance and diabetes has yet to be determined.

1.16 CB_2 and skeletal Muscle

The cannabinoid receptor 2 was first detected via *in-vitro* studies in rodent myogenic cell line (L6) and in lean human skeletal muscle myotubes, developed from muscle collected from the rectus abdominus muscle during gastric surgery (Cavuoto et al. 2007). CB_2 was identified in the human samples in cell culture and whole muscle (Cavuoto et al. 2007). It was later confirmed that the CB_1 and CB_2 were involved in the regulation of fatty acid oxidative pathways in skeletal muscle (Zhao et al. 2010). The research performed to initially investigate CB_2 and its role in pain and immune responses discovered that localized skeletal muscle damage increases the activity of CB_2 within hours. This was considered a possible tool for forensic pathologists to determine exact age of tissue wounds (Tian-Shiuet et al. 2010). Fifty Sprague Dawley rats were divided into control and contusion groups ranging from 3 hours to 14 days post injury time points. Contusion was administered by a mechanical drop device and measured at instant impact velocity (*V*) and elastic deformation (DF). Using macrophage markers via confocal laser microscopy, protein and mRNA expression it was shown that CB_2 expression increased almost two-fold an hour and remained increased for up to 14 days post injury (Tian-Shiuet et al. 2010). The same research group more investigated CB_2 modulation and the effects on skeletal muscle (soleus and gastrocnemius) and the repair process. Tianshiu et al. (2015) replicated the same model of contusion as the previous study., Fifteen healthy adult Sprague-Dawley rats were injected (1 ml/kg) at day 0, 1 and every second day for 14 days in the same localised site with either JWH-133 (a potent selective CB_2 receptor agonist), AM630 (a potent selective antagonist for CB_2 that also has weak partial agonist binding to CB_1) or saline treatment. Compared to the control group the fibrogenic markers (Tgfb1, Fn1 and Acta2) where up-regulated in the CB_2 antagonist group, it was also found that the number

of myofibroblasts increased following CB_2 antagonism, in contrast the CB_2 agonist group who showed a significant decrease in myofibroblasts. These acute effects emphasise the synergistic role that CB_2 plays with the localized immune response. However, a whole-body response to the effects of chronic CB_2 modulation on skeletal muscle signalling pathways is still not known.

Zheng et al. (2013) investigated CB_2 signalling pathways in C2C12 myotubes in a continuous mouse derived cell line which closely resembles metabolically normal skeletal muscle (Iwabu et al. 2010). Following molecular cloning and plasmid construction the cultured cells were investigated to determine the effects of CB_2 on the SIRT1/PGC-1α pathway (Zheng et al. 2013). The results showed that blockade of CB_2 receptors inhibited the SIRT1/PGC-1α pathway. This pathway at a hypothalamic level has been demonstrated to have a direct effect on whole body metabolism (Su, 2011). The implications of this are evident in skeletal muscle. Increased levels of circulating fatty acids infiltrate skeletal muscle; leading to an accumulation of intramyocellular triglycerides which in turn causes a disruption to the signalling pathways that initiate fatty acid oxidation (Malenfant et al. 2001; Hilton et al. 2008). This disruption places pressure on cells causing an increase in ceramide production (Samad et al. 2006). Because of an accumulation of ceramide within cells, inflammatory cytokines are manufactured and released from both skeletal muscle and adipose tissue (Bruun et al. 2003, Samad et al. 2006).

1.17 CB_2 and cellular signalling pathways

CB_2 shares an array of signalling pathways with CB_1 resulting in homogenous intracellular signalling (O'Keefe et al. 2013). CB_2 signalling pathways are not well researched and despite their potential application as a therapeutic target, little is understood about the mechanisms

behind CB_2 signalling and its intracellular responses. In existing literature, CB_2 has been found to couple with pertussis toxin sensitive $G_{i/o\alpha}$ proteins resulting in inhibition of adenylyl cyclase resulting in a reduction in cyclic adenosine monophosphate (cAMP) (Baywitch et al. 1995). This response is well known as a target of G-coupled protein receptors (GPCR) including CB_1 and CB_2.

1.18 Cyclic AMP regulates a plethora of cellular functions including activation of enzymes responsible for glycogen and fat metabolism in skeletal muscle. The regulation of cAMP is responsible for increased lipolysis, reduced glycogen synthesis and increased glycogen breakdown. The mechanisms of cAMP are controlled by its activation of protein kinases which in turn regulates phosphorylation. CB_2 agonism in thymoma-derived T-cells decreased the adenlyl cyclase/cAMP pathway by decreasing IL-2 expression. This decrease caused a down regulation of intracellular cAMP levels and inhibited the protein kinase A (PKA) mediated activation of transcription factors cAMP (Condie et al. 1996).

1.19 Nitric Oxide (NO) is commonly found in tissues of humans and mammals it is synthesised from the amino acid arginine. It has been shown that NO regulates mitochondrial respiration in skeletal muscle (Kobzick, et al. 1995). CB_2 agonism in cardiac cells resulted in CB_2 activation of NO signalling (Shmist, et al. 2006). Interestingly cytokines and inflammation have been found to induce NO in a C2C12 skeletal muscle cell culture model (Williams et al. 1994). An increase in NO isoform activity has been found to exist in the state of metabolic syndrome and obesity (Zahedi et al. 2008, Ghaseui et al. 2010, Kraus et al. 2012). There appears to be enough evidence to suggest mechanistic roles between NO signalling and ECS modulation in skeletal muscle. If these roles can influence mitochondrial gene regulation or initiate cytokine activity in skeletal muscle is yet to be determined.

1.20 5' adenosine monophosphate-activated protein kinase (AMPK) is a heterotrimic kinase that consists of α, β and γ subunits. AMPK plays a role within the metabolic regulation pathways of a cell. The homeostasis of the cells energy sources is geared towards the use of available and stored energy. AMPK is known as a chief regulator in skeletal muscle energy metabolism having been demonstrated to activate genes (such as PGC1α) in both fatty acid oxidation and glucose uptake (Lee et al. 2006, Iglesias et al. 2004). AMPK have been found to have an established role in signalling pathways in the central nervous system (Choi et al. 2013). The relationship between CB_2 and AMPK in skeletal muscle has not yet been established. In the obese state AMPK is down regulated and AMPK signalling is altered (Martin et al. 2006). A reduction in AMPK activity and an increase in inflammation have been found in white adipose tissue and whole-body insulin resistance in obese humans (Gauthier et al. 2011). It has also been found that AMPK activity is lower in adipose tissue of obese patients who are insulin resistant compared to insulin sensitive obese patients. Obesity in this cohort correlated with increased expression of multiple inflammatory markers (Gauthier, et al. 2011). This increase in inflammatory markers has been shown to cause an increase in skeletal muscle ceramide production (Adams et al. 2004).

These pathways present as being possible targets of investigation in CB_2 skeletal muscle cellular signalling. However, the endocannabinoid system has been shown to have differing effects on different tissues. For example, CB_2 agonism has been shown to have favourable effects on many tissues including immune cells, brain, cardiomyocytes, epithelial cells and pancreatic cells (Di Filippo et al, 2004, Mukhopadhyay et al. 2007, Zhang et al. 2007, Marquez et al. 2009, Michalski, et al. 2008). This thesis has researched the benefits of CB_2 antagonism.

This is further complicated by the varying effects displayed in the modulation of the ECS and its effects on both the CNS and the Peripheral Nervous system.

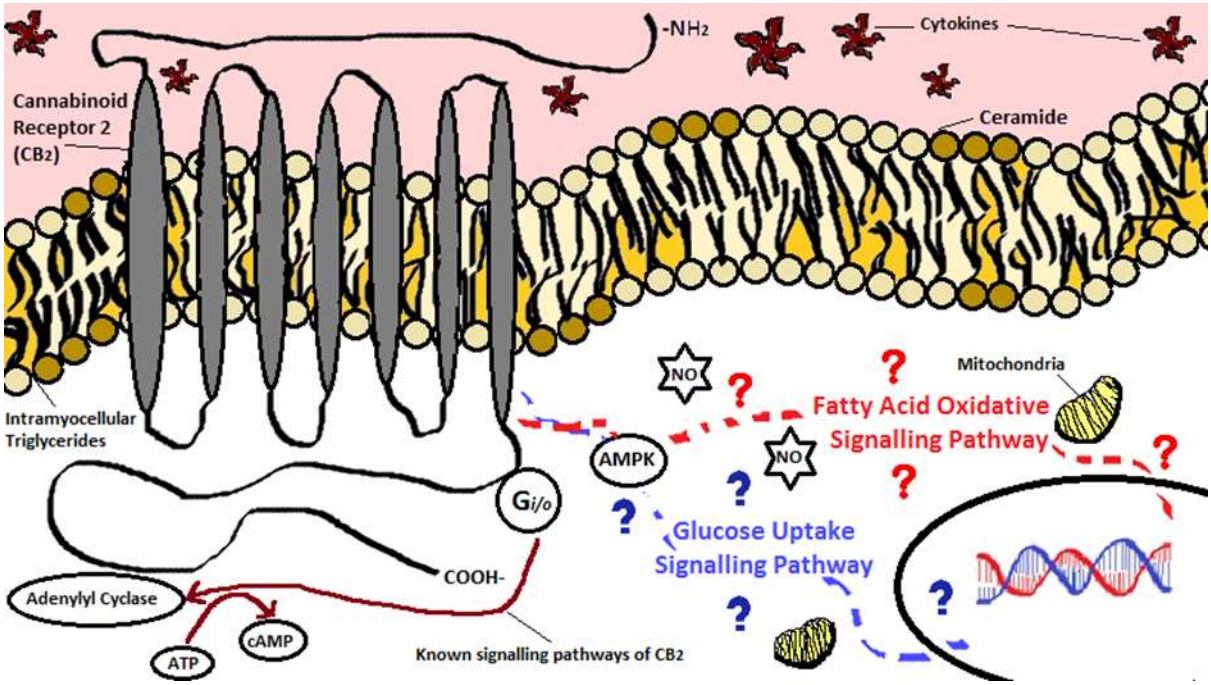


Figure 4: An intramyocellular schematic view of a proposed obesity effected skeletal muscle cell; showing what pathways we know CB_2 plays a role in. Also highlighted is the unknown role in both the fatty acid oxidative signalling pathway and the glucose uptake signalling pathway.

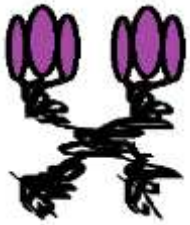
As a novel target CB_2 has been researched by numerous pharmaceutical and academic laboratories (Kumar et al. 2014). The therapeutic potential of both CB_2 agonism and antagonism is well recognised. It is important to remember however that the expression and mechanisms of CB_2 can be up regulated and mechanisms may alter in disease states (Smit et al. 2007). CB_2 has been shown to play many roles in a variety of biological systems, what is puzzling however is how it is activation and down regulation can affect different metabolic tissues. This in turn can result in either favourable or unfavourable results depending on the homeostatic state of the samples being studied.

This literature review has looked at the evidence that exists in skeletal muscle and CB_2 modulation. The endocannabinoid system is a major system involved in whole body metabolism. The functional mechanisms of CB_2 and skeletal muscle are not well elucidated, research suggests a possible link that may affect metabolic profiles further research into to CB_2 in the presence of obesity and diabetes is required to determine the possible role of CB_2.

1.21 Adiponectin resistance in Obesity and Skeletal Muscle Metabolic Signalling

Adiponectin (Acrp30, GBP23, AdipoQ) plays an innate biological role in exerting anti-inflammatory, anti-diabetic and strong insulin sensitizing mechanisms (Waki et al. 2004, Goto et al. 2014). This highly abundant circulating hormone in healthy lean individuals (Tsatsanis 2005) has a structure consisting of a globular C-terminal domain and a collagenous N-terminal domain and was originally thought to be secreted exclusively from adipocytes (Scherer et al. 1995). It has since been discovered that it is also released by skeletal muscle, salivary glands, liver, pituitary glands, cardiomyocytes, the colon and placenta (*as reviewed by Vaiopoulos et al. 2012*). It is known to play a protective role against metabolic disorders such as obesity (Lihn et al. 2005; Bruce et al. 2005).

Table 2: Biologically adiponectin presents in three different multimer forms:

Adiponectin Complex	Schematic View	Biology
Low Molecular Weight (LMW)		• Trimer • ~90kDa • 3 Adiponectin Molecules
Middle Molecular Weight (MMW)		• Hexamer (Isoform) • ~180kDa • 6 Adiponectin Molecules
High Molecular Weight (HMW)		• Higher Order Multimers (Isoform) • ~360-400kDa • 12-18 Adiponectin Molecules

(Table 2 adapted from Goldstein et al. 2009).

Adiponectin associates into multimeric higher-order of structures via disulphide bonds (Castro et al. 2006). In human's adiponectin presents as three multimer forms (*see Table 1*). There appears to be evidence implicating that the type of monomer of adiponectin that is either abundant or sparse may be directly associated with metabolic modulation, such as diet induced diabetes (Nakashima et al. 2006, Zhu et al. 2010, Gato et al. 2014). One example is the discovery that elderly individuals with type 2 diabetes have significantly lower levels of circulating LMW adiponectin (Graessler et al. 2011). Moreover, LMW adiponectin has been associated with anti-inflammatory influence (Schober et al. 2007). The ratio of the different types of monomers has also been implicated as a contribution to

metabolic syndrome (Bobbert et al. 2005, Waki et al. 2005). The exploration and determination of the specific benefits of adiponectin monomers is beyond the scope of this thesis therefore total adiponectin will be focused upon.

1.22 Adiponectin and Obesity

In the obese state, circulating adiponectin concentrations have been found to be reduced, which correlates negatively with the level of adipose tissue in the body (Yang et al. 2006; Kopp et al. 2005; Bruce et al. 2005). It has been identified that not only total fat intake but the composition of fatty acids within the diet may have an influence on stored and membrane structural lipids and plays an important role in obesity (Andersson et al. 2002). A diet high in saturated fatty acids has a strong correlation with obesity and other metabolic disorders; whereas in comparison, a diet high in monounsaturated and polyunsaturated fatty acids has been shown to have the opposite effect (Flachs et al. 2006). This is of particular interest; as dietary polyunsaturated fatty acids are precursors of endocannabinoid ligands AEA and 2-AG (Ueda et al. 2000; Flachs et al. 2006). The composition of dietary fat intake may have an influence on adiponectin levels (Yang et al. 2012). However, this is yet to be determined and further research in this area is warranted. In humans, there appears to be little evidence to support the hypothesis that postprandial effects on adiponectin are influenced by either the intake or the metabolic state of the individual. A study that investigated this hypothesis looked at the effects of a high fat food (51.5%) intake compared to water intake in three groups of middle aged men (~43-56 years). Individuals were fasted and either placed in a lean, obese or obesity induced diabetic group. Following intake, the participants were tested hourly for six hours (Phillips et al. 2013). Their blood plasma showed that there was no overall difference in inflammation, glucose, insulin or triglyceride levels. Furthermore, there were no changes in either total or HMW

adiponectin levels (Phillips et al. 2013). The chronic effects of obesity however have an undisputed effect biologically. It is strongly associated with an altered metabolic state, inflammation, and diet induced diabetes (Maahs et al. 2005, Yang et al. 2006).

1.23 Adiponectin and Diabetes

Type 2 diabetes comprises 90% of people with diabetes around the world and is largely the result of Obesity and physical inactivity (Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus. Geneva, World Health Organization, 1999 (WHO/NCD/NCS/99.2). Diabetes increases the risk of heart disease and stroke. In a multinational study, 50% of people with diabetes die of cardiovascular disease (primarily heart disease and stroke) combined with reduced blood flow, neuropathy (nerve damage) in the feet increases the chance of foot ulcers, infection and eventual need for limb amputation (Morrish NJ, Wang SL, Stevens LK, Fuller JH, Keen H Mortality and causes of death in the WHO Multinational Study of Vascular Disease in Diabetes. Diabetologia 2001, 44 Suppl 2: S14–S21).

Differing roles of adiponectin receptors and circulating adiponectin concentrations in revascularization were investigated in a knockout model by Parker-Duffen et al. (2014). AdipoR1–KO and AdipoR2-KO mice were fed a high fat, high sucrose diet (36.0%) for 12 weeks resulting in metabolic dysfunction. The study mimicked ischemia with restricted blood flow via the femoral artery to test the role of adiponectin in vasculature responses, gastrocnemius muscle was removed and tested glucose tolerance testing insulin tolerance testing (Parker-Duffen et al. 2014). AdipoR1-KO and the wild-type control mice showed similar recovery time, however AdipoR2-KO mice displayed a notably longer recovery time and displayed skeletal muscle atrophy (Parker-Duffen et al. 2014). The AdipoR2-KO mice

were also susceptible to necrosis and in some of the mice in this group displayed total foot necrosis leading to auto-amputation (Parker-Duffen et al. 2014). The circulating levels of adiponectin were unchanged amongst the groups however once treated with an adiponectin plasmid. The HMW isoform plasma levels increased and accelerated the vascularization and tissue repair of the control mice. However, in the AdipoR2-KO mice the circulating levels of adiponectin increased but without the AdipoR2 the necrosis continued: indicating that the AdipoR2 is required for vascularisation (Parker-Duffen et al. 2014). In contrast when looking at the knockout mice in relation to metabolic syndrome. The AdipoR1-KO knockout mice displayed increased weight gain, elevated levels of insulin, leptin and circulating TNF-α (Parker-Duffen et al. 2014). The AdipoR2-KO mice did not display any diet induced weight gain and showed an increased ability to clear glucose and an accelerated tissue sensitivity to insulin (Parker-Duffen et al. 2014).

1.24 Adiponectin and Inflammation

In humans, there appears to be a contradictory association between inflammatory markers and circulating adiponectin concentrations (Krikitos et al. 2004). Similarly, when macrophages derived from porcine plasma were tested in-vitro following exposure to lipopolysaccharide (known to induce a strong immune response in mammalian cells) (Rietschel et al. 1994). The cells displayed increased production of TNF-α and IL6 (Pro-inflammatory cytokines) which in turn was suppressed when exposed to adiponectin (10 µg/mL for 24 + 8 hours). Furthermore, adiponectin increased the production of IL-10 (anti-inflammatory cytokine) (Wulster-Radcliffe et al. 2004). This anti-inflammatory effect by adiponectin was found to have been achieved via the inhibition of NFκB activation and the suppression of ERK1/2 signalling (Wulster-Radcliffe et al. 2004).

1.25 Adiponectin signalling

There are two structurally related cell receptors that interact with adiponectin, AdipoR1 and AdipoR2. These receptors contain seven transmembrane domains not unlike the G protein-coupled receptor family; their structural topology is opposite however, with their NH2-terminus located inside of the cell and their COOH-terminus outside of the cell, there signalling occurs via the Januse kinase/ signal transducers and activators of transcription (JAK/STAT) pathway (Rawlings et al. 2004, Ogawa et al. 2010). To induce cellular change adiponectin must attach to an adiponectin specific receptor to enable a cascade of signalling events involving multiple cell responses, for example intracellular adiponectin binds directly with Adaptor protein containing PH domain, PTB domain and leucine zipper motif (APPL) to initiate a downstream cascade of intramyocellular signalling (Mao et al, 2006; Wang et al. 2009). There are two known APPL proteins APPL1 and APPL2; APPL1 functions as a positive mediator in the adiponectin pathway (Mao et al. 2006). Studies have shown that APPL1 plays a crucial role in insulin stimulated glucose transporter signalling (Michael et al. 2001). Over expression of APPL1 increases, and suppression of APPL1 level reduces, adiponectin signalling (Wang et al. 2009). APPL2 is an isoform of APPL1, and shares up to 54% identity in protein sequences (Mao et al. 2006; Wang et al. 2009). APPL2 has been found to play a vital role in cell proliferation and embryonic development (Wang et al. 2009). APPL2 is thought to play an opposing role to APPL1 since it has been shown to bind to AdipoR1 and AdipoR2 thereby inhibiting the ability of APPL1 to initiate adiponectin's positive effects on insulin sensitivity and fatty acid oxidation (Wang et al. 2009).

1.26 Adiponectin and Skeletal Muscle

Investigating the relationship between adiponectin levels, in skeletal muscle and its role in obesity and insulin resistance Yang et al. (2006) found that degrees of obesity coincided with increased levels of insulin resistance and a decrease of tissue adiponectin levels (Yang et al. 2006). Although these study results were recorded in rodent muscle samples; Buettner et al. (2007) found that after a period of 8 weeks a high fat diet fed to rodents can induce models that closely resemble the metabolic disorders seen in human obesity. Many studies have shown that in the obese state adiponectin levels are decreased in humans (Fantuzzi, 2007; Lihn et al. 2005; Mao et al. 2006; Bruce et al. 2005). Skeletal muscle is an essential tissue for whole body energy metabolism, including fatty acid oxidation; fatty acid transporters and binding proteins (Andersson et al. 2002). Clinical studies have shown that skeletal muscle in the obese or obese diabetic state have a significant increase in intramyocellular triglycerides (Malenfont et al. 2001). Intramyocellular fatty acid accumulation places stress on mitochondrial ability to oxidise fatty acids (Malenfont et al. 2001), this can result in chronic low-grade inflammation (Kopp et al. 2005). In a homeostatic environment inflammation is regulated by a variety of functions (Helge et al. 2004). Adiponectin regulation of cytokines derived from adipose tissue was investigated in a study by Bruun et al. (2003). The study tested three groups of people who were obese, measuring cytokine levels and adiponectin levels (Bruun et al. 2003). They discovered that cytokines inhibited adiponectin production (Bruun et al. 2003). This pattern would suggest that intramyocellular triglyceride levels that are seen in obesity cause cellular stress and thus low-grade inflammation, resulting in the down regulation of adiponectin. These results conflict with the results of Mullen et al. (2008) and colleagues, whose study suggested a decrease in adiponectin levels precede the accumulation of skeletal muscle lipids and insulin

resistance in high fat fed rats (Mullen et al. 2008). Spraugue-Dawley rats were put on a feeding regime of a high fat diet (Mullen et al. 2008). They were tested for adiponectin resistance; plasma insulin and fasting blood glucose at the time points of 3 days; 2 weeks and 4 weeks (Mullen et al. 2008). This was to determine if a down regulation of adiponectin caused an increase in insulin resistance and intra cellular triglyceride levels (Mullen et al. 2008). This result would suggest that lipid accumulation causes a decrease in adiponectin production, which resulted in increased levels of intramyocellular triglycerides and thus inflammation.

1.27 Adiponectin Resistance

The decrease in circulating adiponectin levels that occur in obesity reduces the efficiency of macrophages rendering them more tolerant to other pro-inflammatory stimulation (Tsatsanis et al. 2005). It has been proposed that internal cellular damage such as obesity induced endoplasmic reticulum (ER) stress may be play a role in adiponectin down regulation (Liu et al. 2010). Moreover *C57BL/6J* male mice fed a high fat diet (60%) for 16 weeks showed distinct markers of obesity for example insulin resistance, increased plasma free fatty acids, triglycerides and impaired glucose intolerance (Yuzefovych et al. 2013). Following the sixteen weeks of treatment the mixed gastroc was tested for changes in mitochondrial efficiency. The study showed that the skeletal muscle of the mice on the high fat diet had elevated dysfunctional mitochondria compared to the control group indicating ER stress which was further supported by a decline in the ATP levels (Yuzefovych et al. 2013). Moreover, adiponectin has a yet to be determined role in cellular ceramide levels (Holland et al. 2011). AdipoR1-KO and AdipoR2-KO mice develop severe metabolic dysfunction. Upon *in-vitro* exposure to adiponectin, ceramidase activity increased resulting in amelioration of cellular ceramide levels (Holland et al. 2011). Overall there is substantial evidence to advocate adiponectin's role in

obesity and diabetes at both circulating and molecular levels. However, the role of adiponectin in skeletal muscle and its possible link to the ECS has not yet been determined.

1.28 Summary and Aims

Rimonabant in Obesity (RIO) large scale clinical trials *(As discussed in the introduction (Section 1.1.7)* concluded that in the RIO-Lipids study, 1 year of Rimonabant 20mg (CB_1 Antagonist) treatment altered increased circulating levels of adiponectin in participants by 57 percent. Intriguingly, this significant increase could not be attributed to weight loss alone (Despres et al. 2006). To investigate the contributing mechanisms behind this increase in adiponectin, an *in-vitro* study was conducted looking at abdominal adipose tissue collected from 30 obese individuals undergoing bariatric surgery (Ge et al. 2013). Adipocytes were cultured and a CB_1 Antagonist (Rimonabant) or CB_2 Agonist (ACEA – arachidonyl-2-chloroethlamide, a CB_1 agonist which is a low affinity agonist to CB_2) was added to groups of cells for 24 hours. In addition, three additional groups were treated with protein kinase inhibitors. JNK (c-jun NH2-terminal protein kinase), p38MAPK (p38 mitogen-activated protein kinase and ERK1/2 (Extracellular Signal-Regulated kinases)). Adiponectin concentration levels were measured in the medium of the cultured adipocytes. Gene expression was then analysed using *real time* – PCR (RT-PCR). The results showed that by blocking $CB_{1,}$ adiponectin levels increased by 40%. Moreover, inflammatory markers were down regulated, (MIP)-1β by 26% and IL-7 by 32%. In order to test adiponectin signalling ACEA was used to stimulate cannabinoid activity (Ge et al. 2013). CB_1 stimulation increased phosphorylation of p38MAPK which is responsive to stress stimuli such as cytokines. Similarly, CB_1 activation down regulated adiponectin and this effect was reversed by CB_1 blockage (Ge et al. 2013). In summary, activation of cannabinoid signalling caused a decrease in adiponectin and an increase

in inflammation, resulting in ER stress. Deactivation of cannabinoids caused a decrease in inflammation and an increase in adiponectin. Interestingly this suggests a direct, yet to be determined relationship between the Endocannabinoid system and adiponectin resistance in adipose tissue.

It is yet to be determined whether the same effects occur in the skeletal muscle in the presence of obesity and diabetes. Skeletal muscle is responsible for more than half of the body's glucose clearance, fatty acid oxidation and energy production and is a major player in metabolic disorders. The exploration into the role that the cannabinoid system plays in adiponectin resistance in the presence of metabolic disorders in skeletal muscle will provide possible pharmacological targets for the treatment of obesity and diabetes. Therefore, the aims of studies describe in this thesis are:

Aim 1 Investigate the effect of chronic CB_1 antagonism on obesity plasma hormone and cytokine markers.

Aim 2 Investigate the effects of chronic CB_1 antagonism and adiponectin resistance on fatty acid oxidative pathways in skeletal muscle.

Aim 3 Investigate the role of chronic CB_2 agonism and antagonism on obesity plasma hormone and cytokine markers.

Aim 4 Investigate the effects of chronic CB_2 agonism and CB_2 antagonism on adiponectin resistance on fatty acid oxidative pathways in skeletal muscle.

Aim 5 Investigate the effects of cannabinoid receptor antagonism on adiponectin receptor mRNA expression and adiponectin signalling in obese and obese diabetic derived human skeletal muscle myotubes.

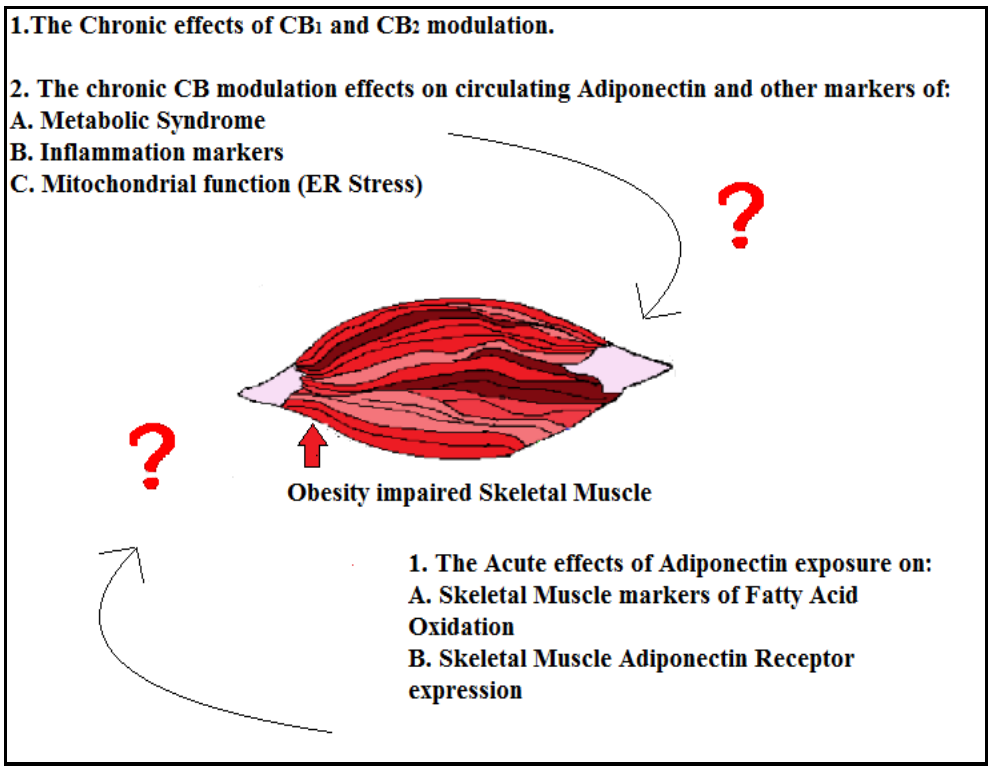


Figure 5 Displays a schematic view of the novel pathways in the skeletal muscle of both animals and humans that will be investigated in thesis to determine - *The Influence of Endocannabinoids in Skeletal Muscle Adiponectin sensitivity in Diet Induced Obesity and Diabetes*

Chapter 2 - Materials and Methods

2.0 Animals – DIO model

Seven-week-old male Sprague Dawley rats (weight range 200-250g) were ordered from The Animal Resource Centre (ARC, Perth). Following a seven-day acclimatisation period, the rats were randomly designated into one of five groups. Sprague Dawley rats were selected for use in this thesis because of their ability to gain weight on a high fat diet (Farley & Cook et al. 2003). This strain of rodent displays a variable response to weight gain which is similar to humans (Hager et al. 1998). While consuming a high fat diet some of the rats display traits resembling obese resistance (lean phenotype) and some display traits that resemble the adipose storage that are exhibited in some human of who are predisposed to obesity (obese phenotype) when they consume a chronic high fat diet (Levin, Dunn-Meynell et al. 1997; Farley, Cook et al. 2003).

To ensure that all treatment groups have evenly matched characteristics, a ranking system was used to evaluate the status of each rat in groups. This outcome was achieved via the following protocol: at the end of the 9 week feeding regime, individual rats were ranked and given a score which was calculated as an average of scores across 9 measurements (Body Weight, Body Fat %, Lean Body Weight %, Blood Pressure, Insulin Sensitivity Test, and Glucose Tolerance Test). Ranking scores ranged from 1 (physiologically worst) to 5 (physiologically best).

Table 2.1 Treatment groups

Treatment Groups
Standard Chow with vehicle
High Fat Diet (HFD) with vehicle
HFD with CB_1 Antagonist (AM251)
HFD with CB_2 Agonist (AM1241)
HFD with CB_2 Antagonist (AM630)

The animals were maintained for nine weeks on either a high fat diet (22% fat 0.15% cholesterol, Specialty Feeds SF00-219) or standard chow (5% fat, Rat & Mouse Feed, Barastoc); Speciality Feeds is in Perth, WA and Barastoc is in Pakenham, VIC (Manufactured by Ridley Agriproducts). Following the nine-week feeding regime, a six-week treatment period of daily IP injection occurred. Treatment consisted of commercially available CB_1 antagonist AM251, CB_2 agonist AM1241 or CB_2 antagonist AM630. Listed above (Table 2.1) are the treatment groups, see Table 2.2 below for dosage amounts. Vehicle control groups were treated with 7.5 µl Tween 80 per 1 ml of 0.9% saline solution (Tween 80 was sourced from Science Supply, Labchem 2510 – Castle Hill, NSW, saline solution McFarlene Medical).

Table 2.2 Treatment groups and concentrations

Treatment Group	Compound	Concentration (mg/kg)	References for concentration of compounds
Control	7.5 µl Tween 80 per 1 ml of 0.9% saline solution		
CB1 Antagonist	AM251	3	(Janiak et al., 2007)
CB_2 Agonist	AM1241	3	
CB_2 Antagonist	AM630	0.3	

The compound concentration within this thesis was chosen from the articles presented in the above table: Rationale was supported by the following evidence based research. AM251 administered to the obese rats at a concentration of 2.5 g/kg was found to be the lowest dosage

at which the anorectic effects of CB_1 antagonism started to have an effect on food intake (Chambers et al. 2004). Similarly, the selective CB_1 receptor antagonist AM251 was administered via IP injection at 3 mg/kg dose to male Sprague Dawley rats weighing 240 – 310g. The IP injection resulted in suppression of cumulative food intake in rats deprived of food for 16 h (Ching-Heng et al. 2015).

There are few studies that have investigated the obesity related effects of CB_2 agonist – AM1241 and CB_2 antagonist – AM630. The dosages selected were based on research that showed that the minimal effect of AM1241 occurred at 3 mg/kg in mice (Curto-Reyes et al. 2010). Similarly, a dosage of 3 mg/kg of AM1241 was seen to have a biological effect in rats (Beltramo et al. 2006). AM630 selective CB_2 receptor antagonist has previously been provided via IP injection in rats at different doses; at 0.3 mg/kg AM630 decreased the cumulative food intake for the 12 hours following in freely fed rats (Ting et al. 2015). The adiponectin concentration was chosen to be the same as the concentration added used in the cell culture methods of Chapter 7, which mimic the concentration utilised in McAinch, A, Steinberg, G et al. (2016) and Chen, M, McAinch, A et al. (2005).

Power calculations that determine the n required to produce adequate sample size to reach significance was done by using the following equation: to achieve a power of 90% and observe a 1.25 fold change between treatments, sample size required: n = ((2 x 0.82)/ 1.252) x 10.51 = 8.6 rats per group, where: 0.8 is the standard deviation expected, and α is 0.05. The number reached by the power calculation was similar to that published by other researchers using similar research techniques, animal studies (Alonso et al. 2012, Erdös et al, 2004, Herling et al. 2008, Eckardt et al. 2009, Kelley et al. 2002).

2.1 High Fat Diet

A semi-pure high fat diet formulation for laboratory rats and mice (22% Fat, 0.15% Cholesterol - Specialty Feeds SF00-219) was utilised to mimic a "Western fast food diet". Semi-pure diets are made from more refined ingredients, such as sucrose, starch, casein, and refined oils. The vitamins and minerals are still added as premixes, but because levels of these nutrients in the raw materials are lower, they can be more closely controlled (Speciality Feeds, Great Western Highway, Glen Forrest, WA, 6071). This high fat diet is widely accepted as equivalent to the Western diet (Posey and Clegg, D 2009; Buettner, R, Scholmerich, J, Bollheimer, L 2007) although this may not replicate fully the types of dietary fats consumed within the population (Naughton, SS et al. 2015).

1 Table 2.3 High Fat diet – Nutritional parameters

Calculated Nutritional Parameters - High Fat Diet (SF00-219) – (% = total amount measured within the completed diet).					
Protein	19.0%	**Calculated Amino Acids**		Vitamin K (Menadione)	12.5 mg/Kg
Total Fat	21.0%	Valine	1.20%	Vitamin C	(Ascorbic acid) 700 mg/Kg
Crude Fibre	4.7%	Leucine	1.80%	Vitamin B1 (Thiamine)	11 mg/Kg
AD Fibre	4.7%	Isoleucine	0.80%	Vitamin (Riboflavin)	11 mg/Kg
Digestible Energy	19.4MJ/Kg	Threonine	0.80%	Niacin (Nicotinic acid)	50 mg/Kg
% Total calculated digestible energy from lipids	40.0%	Methionine	0.80%	Vitamin B6 (Pryridoxine)	11 mg/Kg
% Total calculated digestible energy from Protein	17.0%	Cystine	0.06%	Pantothenic Acid	34 mg/Kg
Ingredients		Lysine	1.50%	Biotin	200 µg/Kg
Casein (Acid)	195 g/Kg	Phenylanine	1.00%	Folic Acid	1 mg/Kg
Sucrose	341 g/Kg	Tyrosine	1.00%	Inositol	55 mg/Kg
Clarified Butter (Ghee)	210 g/Kg	Tryptophan	0.30%	Vitamin B12 (Cyancobalamin)	18 µg/Kg
Oxicap E2	0.04 g/Kg	**Calculated Total Minerals**		Choline	3 860 mg/Kg
Cellulose	50 g/Kg	Calcium	0.60%	**Calculated Fatty Acid Composition**	
Wheat Starch	154 g/Kg	Phosphorous	0.30%	Saturated Fats C12:0 or less	1.80%
DL Methionine	3.0 g/Kg	Magnesium	0.10%	Myristic Acid14:0	2.6
Calcium Carbonate	17.1 g/Kg	Sodium	0.12%	Palmitric Acid16:0	7.00%
Sodium Chloride	2.6 g/Kg	Chloride	0.16%	Stearic Acid 18:0	2.40%
AIN93 Trace Minerals	1.4 g/Kg	Potassium	0.40%	Palmitoleic Acid 16:1	0.40%
Potassium Citrate	2.6 g/Kg	Sulphur	0.23%	Oleic Acid 18:1	5.50%

Potassium Dihydrogen Phosphate	6.9 g/Kg	0Iron	80 mg/Kg	Linolenic Acid 18:2 n6	0.40%
Potassium Sulphate	1.6 g/Kg	**Calculated Total Vitamins**		alpha Linolenic Acid 18:3 n3	0.55%
Choline Chloride (75%)	2.5 g/Kg	Vitamin A(Retinol)	11650 IU/Kg	Arachidonic Acid 20:4 n6	Trace
SF00-219 Vitamins	10 g/Kg	Vitamin D (Cholecalciferol)	1 100 IU/Kg	Total n3	0.35%
Cholesterol	1.5 g/Kg	Vitamin E (a Tocopherol acetate)	64 mg/Kg	Total n6	0.41%
Cholesterol	0.15%	Total Polyunsaturated Fats	0.77%	Total Saturated Fats	13.99%
Total Mono Unsaturated Fats	6.23%				

Barastoc Rat & Mouse Standard Chow
Ingredients: Cereal grains and cereal by-products, legumes and legume by-products, vegetable protein meals, fats and oil, vitamins, minerals and Yucca Schidigera extracts. Product manufactured in Victoria and South Australia, may contain traces of animal protein meals.
Estimated Nutritional Composition:

Crude Protein (minimum)	20.0%	Copper (added)	7.5mg/kg
Crude Fat (minimum)	5.0%	Selenium (added)	0.1mg/kg
Crude Fibre	5.0%	Calcium (minimum)	0.8%
Salt (maximum added)	0.5%	Phosphorus (minimum)	0.45%

2.2 Weight and Food Consumption

Rat weight and food consumption was monitored and recorded daily, typically during the morning hours between 7:00 -11:00 am, in the beginning phase of the light cycle.

2.3 Body Composition

Body compositions of rats were analysed using EchoMRI ™ Whole Body Composition Analyser (EchoMRI, Houston TX). The machine was calibrated according to manufacturer's instructions using canola oil as a calibration medium. Live, conscious rats were restrained within a clear plastic cylinder which limited their ability to turn around, but did not restrict breathing. The restrained rats were then placed inside the EchoMRI™ Analyser for a two-minute scan, which determined fat and lean tissue as well as free and total body water content of the animals. Each animal was scanned in duplicate, to ensure accuracy of measurements.Animals were restrained for no longer than 10 minutes and were returned to cages following the scans

Table 2.4 Tissue collection and storage and use

Tissue	Storage	Use
Extensor digitorumlongus muscle (EDL)x2	LN^2 Fixed and stored 80^0C	Organ bath (~200mg each) prior to storage
Soleus muscle x2	LN^2 Fixed and stored 80^0C	Organ bath (~260mg each) prior to storage
Gastrocnemius muscle x2	LN^2 Fixed and stored 80^0C	Immunohistochemistry and separation into red and white Gastrocnemius whole muscle.
Liver	LN^2 Fixed and stored 80^0C	Stored for subsequent analyses
Heart	LN^2 Fixed and stored 80^0C	Stored for subsequent analyses
Epididymal fat pad	LN^2	Stored for subsequent analyses
Peri-renal fat pad	LN^2	Stored for subsequent analyses
Brown fat	LN^2	Stored for subsequent analyses
Cardiac blood/plasma	-20^0C +-80^0C	Bioplex

2.4 Skeletal muscle collection

To establish any biological changes in the rats after chronic administration of CB compounds it is important to consider the relationship between the ECS and inflammation. To properly understand how CB modulation affects skeletal muscle cellular signalling and adiponectin resistance in obesity. It was deemed vital that the rats not be deprived of food prior to death to avoid the risk of any induced inflammation or biological changes prior to death.

The rats were deeply anesthetised following isoflurene administration each animal underwent identical opposing surgical removal. The left hind limb extensor digitorum longus (EDL) muscle was removed first, followed by the soleus muscle and gastrocnemius. Each muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. Immediately after their removal longitudinal strips were incubated in an oxygenated organ bath. Each longitudinal strip was placed in an individual chamber of Krebs-Heseleit buffer with either an Adiponectin treatment for the EDL and soleus from the left leg and no Adiponectin for the muscles from the right leg, as detailed below.

After incubation, the soleus and EDL were surgically sliced into ¾ and ¼ pieces, then the slices were snap frozen in LN_2 for future analysis. The gastrocnemius muscle was removed from the left and right limbs and sectioned into red and white portions and snap frozen in LN_2 for skeletal muscle mitochondrial enzyme activity, mRNA analysis and future analysis.

2.5 Rat Skeletal Muscle Preparation

Immediately after their removal, longitudinal strips of the soleus and EDL were incubated in an oxygenated organ bath (37ºC pre-gassed at 95% O_2 -5% CO_2). Each longitudinal strip was placed in an individual chamber of Krebs-Henseleit buffer with either 0.1 µg/ml of adiponectin (AD+) or the contralateral hind limb muscle exposed to control (AD-) for a period of 30 minutes. The muscles were taken from opposing limbs from each animal to ensure that alternate muscles where exposed to AD treatment for example: rat one- right then left hind limb muscle then rat 2 left and then right limb muscle was treated with the adiponectin or control this was then rotated for each subsequent rat. Following the treatment muscle samples were snap frozen and stored in liquid nitrogen for subsequent analysis.

2.6 mRNA

Total RNA was isolated from the soleus and EDL muscle by using TRIzol Reagent (Invitrogen, Carlsbad, CA) as previously described (McAinch et al., 2006). In short, rodent muscle tissue extracts (approximately 15 mg) were dissociated using 1000 mg of ceramic/silica beads and RNA was extracted in TRIzol and treated with the RQ1 RNase–free DNase kit (Promega Corporations, Madison, WI) RNA concentration of 1µg/9µg total volume. First strand cDNA was then generated from 0.3 µg of template RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) using random hexamers and oligo dTs. cDNA was stored at -20 ºC for subsequent analysis. 'Real-time' PCR was conducted using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and the MyiQ™ single colour 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA). Forward and reverse oligonucleotide primers are shown in Table 2.5. 'Real-time' PCR reactions were run for 50

cycles of 95 ºC for 15 s and 60 ºC for 60 s. The PCR normalized to housekeeping genes, GAPDH and Cyclophilin. Relative changes in mRNA abundance was quantified using the 2(-Delta Delta Ct) method as previously detailed (Livak and Schmittgen, 2001) and data is reported in arbitrary units and expressed as Mean ± SEM.

Table 2.5 Rat oligonucleotide primers for 'real-time' PCR primers

Genes	Accession number	Forward primer	Reverse primer
Cyclophilin	NM_017101.1	CTGATGGCGAGCCCTTG	TCTGCTGTCTTTGGAACTTTGTC
GAPDH	NM_017008.3	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA
AdipoR1	NM_207587.1	TGAGGTACCAGCCAGATGTC	CGTGTCCGCTTCTCTGTTAC
AdipoR2	NM_001037979.1	TCCATGGAGTCTCAACCTG	GGAGAGTATCACAGCGCATC
APPL1	XR_007603	TCACTCCTTCCCCATCTTTC	TAGAGAGAGGGCAGCCAAAT
APPL2	NM_001108741	TGCTCGGGCTATTCACAA	AAACAGGCCCGTGACACT
FAT/CD36	NM_031561.2	GACCATCGGCGATGAGAAA	CCAGGCCCAGGAGCTTTATT
AMPK	NM_023991.1	ACTCTGCTGATGCACATGCT	AGGGGTCTTCAGGAAAGAGG
PDK4	NM_053551.1	GGGATCTCGCCTGGCACTTT	CACACATTCACGAAGCAGCA
PGC-1α	NM_013261.3	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC

GAPDH: *glyceraldehyde-3-phosphate dehydrogenase;* ***AdipoR1:*** *Adiponectin receptor 1;* ***AdipoR2:*** *Adiponectin receptor 2;* ***APPL1*** *adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1;* ***APPL2****: adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2;* ***FAT/CD36:*** *fatty acid translocase/CD36;* ***AMPK:*** *5'adenosine monophosphate-activated protein kinase a-2;* ***PDK4:*** *pyruvate dehydrogenase kinase 4;* ***PGC-1a:*** *peroxisome proliferator-activated receptor gamma co-activator 1 alpha.*

2.7 Skeletal muscle Mitochondrial Enzyme Activity

To determine if chronic cannabinoid receptor modulation influenced the oxidative capacity of red and white gastrocnemius, citrate synthase (CS) activity was measured. Citrate synthase is a rate sensitive enzyme that catalyses the first step of the tricarboxylic acid cycle (TCA). To assay the activities of CS, whole frozen muscles were pulverised using a mortar and pestle (pre-submerged in liquid nitrogen). Next 4-12 mg of powered muscle was weighed out and homogenised for 30-40 seconds, using pellet pestels, in eppendorf tubes containing ice cold buffer [0.175 M KCL (Sigma P-9333), 2 mM EDTA (Sigma E-6511); pH 7.4] at a 50 µl buffer/mg of muscle, according to a previously established protocol (Wadley et al. 2008). Preceding this, muscle homogenates underwent 3 freeze-thaw cycles in liquid nitrogen before being centrifuged (Allegra™ 25R Centrifuge, Beckman Coulter™, Lane Cove west, NSW) at 13,000 rpm for 1 minute to separate particular matter (Wadley et al. 2000). CS was assayed according to the protocol of Srere (1969). Briefly, after the addition of 5 µl of muscle homogenate, 230 µl of reagent cocktail [3 mM Acetyl CoA (Sigma A-2056, Castle Hill, NSW), 100 nM Tris buffer (BioRad, Oakleigh East, VIC) 161-0719; pH 8.3), 1 mM DTNB (Sigma D-8130)] and 15 µl of 10 mM Oxalacetate (Sigma 0-4126, Castle Hill, NSW). CS activity was measured at room temperature by recording the increases in the colorimetric compound (5,5-dithiobis-2-nitrobenzoate), which is linked to citrate production, at 412nm (xMark™ Microplate Spectrophotometer, BioRad, Oakleigh East, VIC), readings of CS activity were recorded every 15 seconds for 3 minutes. All enzyme activity was expressed relative to wet weight (Betick et al. 2008). The change in absorbance/min was calculated from the equation below to measure CS activity. All muscle samples were measured in duplicates, and the

average activity used to characterise the oxidative capacity of both the red and white gastrocnemius.

$$\text{CS activity} = \frac{(\text{absorbance/min (sample)} - \text{absorbance/min (blank)}) \times \text{total volume}}{6.22 \times \text{sample volume}}$$

2.8 Plasma Collection

At the end of the experimental period, rats were deeply anesthetised (as detailed earlier) and killed via cardiac puncture using a 10-ml syringe with 18-gauge needle. Blood was transferred into 10 ml Ethylenediaminetetraacetic acid (EDTA) BD Vacutainer® tubes (McFarlene Medical, Surry Hills, VIC). Tubes containing blood were gently mixed to ensure even distribution of EDTA within samples to prevent clotting and samples were stored on ice until samples were centrifuged at 4000 x *g* for 10 minutes at 4°C. The plasma layer was carefully aspirated off and aliquoted into 2 ml Eppendorf tubes and stored at -80°C until further analysis.

2.9 Intra-Peritoneal Glucose Tolerance Test (IPGTT)

The IPGTT was performed on the animals at weeks 9 and 14. Rats were fasted overnight for approximately 16 hours (still having access to water). The weight of the rat was recorded to determine the correct insulin dosage. The tail was swabbed with alcohol to sterilise. The tail end was snipped removing just enough tissue to create a small bleed (no more than 1mm of the very tip of the rat tail). Droplets of blood were collected being gently eased out via light pressure of the tail external to the vain. Glucose was administered via IP injection of 2 g per kg of glucose/ 0.9% saline solution using a 3-mL syringe 25-gauge needle (a larger gauge needle is needed because of the increased volume of glucose needed for injection). Blood glucose levels are monitored using glucose monitor (Optimum Xceed, Abbott, USA) at 15, 30, 60, 90, 120 minutes.

2.10 Intra-Peritoneal Insulin Sensitivity Test (IPIST)

The IPIST was performed on the animals at weeks 9 and 14. Rats were fasted overnight for approximately 16 hours (still having access to water). The weight of the rat was recorded to determine the correct insulin dosage. The tail was swabbed with alcohol to sterilise. The tail end was snipped removing just enough tissue to create a small bleed (no more than 1 mm off the very tip of the rat tail). Droplets of blood were collected being gently eased out via light pressure of the tail external to the vein. Insulin was administered via intraperitoneal injection 0.75U/kg Humalog fast acting insulin (Eli Lilly® Melrose Park, NSW) in a 0.9% saline solution using a 1 mL syringe 27-gauge needle via IP injection.

2.11 Multiplex Protein Analysis

Cardiac puncture plasma concentrations of each rat were analysed for their diabetes and cytokine profile. This was determined using multiplex protein arrays (Biorad, Biorad Laboratories GmgH, Munich, Germany), enabling the quantification of all parameters across a single sample.

The Diabetes Hormone Panel – The diabetes 4-Plex Panel Rat kit (Biorad, Biorad Laboratories GmgH, Munich, Germany).

Table 2.6 Diabetes Hormone Panel

Hormone	Function
Ghrelin	A hormone, produced and released by the stomach and duodenum, elevated levels may influence food intake (Castañedaa et al. 2010).
Leptin	A peptide associated with obesity and thought to be an appetite suppressant (Myers et al. 2010).
GLP-1	Glucagon-like peptide-1 **(GLP-1),** an appetite suppressing substance found in the brain (satiety signalling) and intestine (gastric emptying and insulin signalling) (Nauck et al, 2010).
PAI-1	Plasminogen activator inhibitor-1 **(PAI-1)** produced by the endothelium (cells lining blood vessels), but is also secreted by other tissue types eg. Adipose tissue. An inhibitor of fibrinolysis, the physiological process that degrades blood clots (Czekay et al. 2011).

Table 2.7 The Cytokine Panel

The Cytokine Panel – The Rat Cytokine 24-Plex panel kit (Biorad, Biorad Laboratories GmgH, Munich, Germany).

Cytokine	Function
EPO	Erythropoietin (EPO) is a glycoprotein responsible for the regulation of growth and differentiation of erythroid precursor cells and of the maintenance of physiological levels of erythrocytes in the blood stream (Rankin et al. 2005).
G-CSF	Granulocyte Colony Stimulating Factor (G-CSF) is a glycoprotein released from activated monocytes, macrophages and neutrophils. Primarily involved in the proliferation and differentiation of haematopoietic progenitor cells of the neutrophil/granulocyte lineage (Ordelheide et al. 2016).
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) is a glycoprotein released in many cells in response to antigen or mitogen activation. It has a predominant role in the survival, proliferation and differentiation of granulocyte-macrophage cell population (Yamaguchi et al. 2015).
GRO/KC	Growth-related oncogene (GRO/KC) is a CXC-chemokine which attracts neutrophils and activated T lymphocytes (Dong et al. 2012).
IFN-γ	Interferon Gamma (IFN-γ) is a glycoprotein produced by T-cells and NK cells in response to antigen and mitogen activation. It exhibits potent anti-viral activity and acts synergistically with other cytokines. It induces antigens,

	macrophage activation and increases immunoglobulin production (Fruchta et al. 2001).
IL-α and IL-β	Interleukin 1-*alpha* (IL-α) & Interleukin 1- *beta* (IL-β) are proteins that are involved in many immune responses including activation of macrophages and resting T cells, modulation of the inflammation response and can also induce adrenocorticotrophic release (Kamari et al. 2012).
IL-2	Interleukin 2 (IL-2) is a polypeptide that is up regulated by activated T cells. It induces proliferation and stimulation of T cells to produce other cytokines (Boyman et al. 2015).
IL-4	Interleukin 4 (IL-4) is a cytokine involved in the proliferation and differentiation of activated B cells (Bao et al. 2015)
IL-5	Interleukin 5 (IL-5) is produced predominately by T Cells and is responsible for the production, activation and localisation of eosinophils. This cytokine has been implicated in various allergic reactions (Molfino et al. 2015).
IL-6	Interleukin 6 (IL-6) is a glycoprotein produced predominately by activated monocytes and macrophages it is also activated by many tissue types. It influences antigen-specific immune and inflammatory reactions. Is known to induce B Cell differentiation, immunoglobulin secretion, T cell growth and differentiation (Lacroix et al. 2015).
IL-7	Interleukin 7 (IL-7) is a cytokine exhibiting multiple biological activities. These include activation, stimulation and inhibition of other cytokines (Nguyen et al, 2015).
IL-10	Interleukin 10 (IL-10) is a homodimeric protein involved in inhibition of pro-inflammatory cytokines from activated monocytes and macrophages. Promotes B cell proliferation and immunoglobulin secretion (Boks et al. 2015).
IL-12p70	Interleukin 12 (IL-12p70)-*P70* describes the active heterodimer that are formed after protein synthesis. A glycoprotein heterodimer that is activated by peripheral lymphocytes involved in activation, stimulation and inhibition of other cytokines (Jana et al. 2014).
IL-13	Interleukin 13 (IL-13) is a cytokine produced from different T cell subsets. Induces B cell differentiation, decreases macrophage derived pro-inflammatory cytokines and chemokines. It also plays a role in the induction of monocyte differentiation and an increase in the activity of JAK cells (Sheikh et al. 2015).
IL-17α	Interleukin 17 (IL-17α) is a cytokine produced by activated T Cells and plays a role in the stimulation of other cytokines and prostaglandin synthesis from a range of cells.
IL-18	Interleukin 18 (IL-18) is a cytokine that induces IFN-γ inducing factor (Tsutsumi et al. 2014).
M-CSF	Macrophage colony-stimulating factor (M-CSF) is a cytokine which influences hematopoietic stem cells to differentiate into macrophages or other related cell types (Rosenfeld et al. 2013).
MCP-1	Monocyte Chemotactic Protein 1 (MCP-1) is a member of the C-C chemokine family, and a potent chemotactic factor for monocytes (Deshmane et al. 2009).

MIP-1α and MIP-3a	Macrophage Inflammatory Protein 1α (MIP-1α) and Macrophage Inflammatory Protein 3α (MIP-3α). MIP-1α and MIP-3α are chemokines and play a pivotal role in regulating the trafficking of leukocytes, local recruiting and activating of lymphocytes, and modulate the reaction of Th1 or Th2 in secondary lymphoid organs (Song et al. 2007).
RANTES	Regulated on activation, Normal cell Expressed and secreted (*RANTES*) are chemokines it is known to induce the chemotaxis of multiple immune cells (Adada et al. 2013).
TNF-α	Tumor Necrosis Factor Alpha (TNF-α) TNF plays a role in the recruitment of circulating immune cells to sites of inflammation by up-regulating adhesion molecules (intracellular adhesion molecule and vascular cell adhesion molecule) (Adada et al. 2013).
VEGF	Vascular Endothelial Growth Factor (VEGF) is a family of cytokines that mediate angiogenesis which typically occurs as a response to hypoxia (Vempati et al. 2014)

Multiplexing was performed in accordance with manufacturer's instructions (Bio-rad Laboratories, United States). Briefly, beads coated with anti-rat antibodies against the examined biomarker antigen were mixed with 200 µl each diluted sample (50 µl supernatant and 150 µl dilution buffer) and then incubated for 30 minutes. Following a wash cycle, a biotinylated detection antibody specific to another epitope of the examined biomarker-antigen was added and the samples were incubated for an additional 30 min. A second wash cycle was then performed, after which streptavidin-phycoerythrin was added to the beads and a third wash cycle was conducted, followed by incubation for 10 mins. Following the removal of excess conjugate, the bead mixture was analysed using a Bioplex 200 system (Bio-Rad Laboratories, Inc. Oakleigh East, VIC).

Raw data was initially measured as the relative fluorescence intensity and then converted to a fluorescence ratio using pre-dyed internal standard beads (Bio-Rad Laboratories, Inc. Oakleigh East, VIC). A series of calibrators was analysed with the samples to convert the fluorescence ratio to international units per millilitre. All samples were measured in duplicate. Standard

curves and concentrations were calculated using Bioplex Manager 6.1 software (Bio-Rad Laboratories, Inc. Oakleigh East, VIC). A step by step guide on the multiplex analysis is detailed below.

2.12 Bio-Plex Assay – Detailed Methods

Materials

- Vortex
- Bioplex Multi-plex Rat Diabetes/Cytokine Kit
- Sterile 200 µl pipettes
- Sterile 1000 µl pipettes
- Single 200 µl capacity pipette
- Single 1000 µl capacity pipette
- Multichannel pipette <200 µl
- Aluminum foil
- Orbital Shaker
- 10 sterile 1.5 eppendorf containers
- X2 sterile15ml Falcon tube
- Centrifuge
- Rack
- Reagent reservoir (x2)

The Bio-Plex Pro diabetes assays were developed on the **high PMT** (25,000) setting using the Bio-Plex 200 system. Protocols using alternative standard dilution series or low PMT settings should be validated by the end user, for example when mixing diabetes assays with cytokine assays.

#All components must be at room temperature before running the kit.

#Pipette to side of well – **AVOID BUBBLES**

#Assay incubations are carried out in the dark on a shaker at **850 ± 50rpm.** Cover the plate with provided sealing tape and protect from light with aluminium foil.

Preparation

A. Prepare Plasma Samples

To completely remove platelets and precipitates centrifuge samples at **10,000 x g for 10 minutes at 4^0C**

B1.Reconstitute a Single Vial of Diabetes Standards

This procedure prepares enough material to run each dilution in duplicate.

1. Gently tap the vial containing the lyophilized standard.
2. Add **500 µl** of the appropriate standard diluent. Do not use assay buffer or sample diluent to reconstitute the standards.

B2. Prepare diabetes standard Dilution

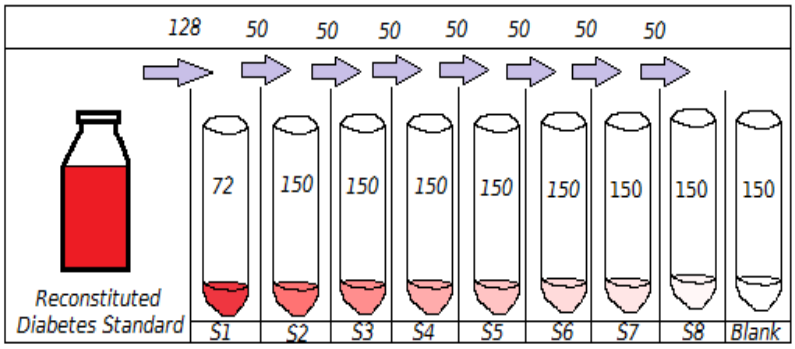
The following procedure produces an eight-point standard curve with a fourfold dilution between each point. Pipet carefully using calibrated pipets and use new pipet tips for every volume transfer.

1. Label nine 1.5 ml Eppendorf tubes labelled S1 through S8 and Blank.

2. Add the specified volume of standard diluent to each tube.

3. Vortex the reconstituted standards gently for **5 seconds** before removing any volume. Add **128 µl** into the S1 tube containing **72 µl** of standard diluent. Vortex at medium speed for **5 sec**, then use a new pipet tip to transfer 50 **µl** from S1 tube to S2 tube.

4. Continue with 1:4 (fourfold) serial dilutions from tube S2 to S8 as shown in the figure below. Use reconstituted and diluted standards immediately. **Do not freeze** for future use.



C. Prepare Coupled Beads

1. Add the required volume of Bio-Plex assay buffer **(4,896µl)** to a **15 ml** falcon tube.

# of Wells	20x beads µl	Assay Buffer, µl	Total Volume, µl
96	288	5,472	5,760
# of 20 x beads	3 x 288		
3	864	4,896	5,760

2. Vortex the stock coupled beads at medium speed for **30 sec**. carefully open the cap and pipet any liquid trapped in the cap back into the tube. This is important to ensure maximum bead recovery. ***Do not centrifuge the vial; doing so will cause the beads to pellet***

3. Dilute coupled beads to 1x by pipetting the required volume into a 15-ml tube. Vortex. Each well of the assay plate requires **2.5 µl (20x stock)** diluted to a final volume of **50 µl** in assay buffer.

4. Protect the beads from light with aluminium foil. Equilibrate to room temperature prior to use.

D. Add coupled Beads, Standards, Blanks, Samples and Controls.

1. Cover unused wells with sealing tape.

2. Vortex the diluted (1x) coupled beads for 30 sec at medium speed. Pour the diluted coupled beads into a reagent reservoir and transfer 50 µl to each well of the assay plate.

Tip: A multichannel pipet is highly recommended for ease of use and efficiency.

3. Wash the plate two times (Mag x2) with 100 µl Wash Buffer

4. Gently Vortex the diluted standards, blanks, samples and controls for 5 secs. Transfer 50 µl to each well of the assay plate. Changing the pipette tip after each sample.

5. Cover plate with a new sheet of sealing tape and protect from light with aluminium foil. Incubate on shaker at 850 ± 50 rpm for 1 hour at room temp (RT).

E. Prepare and Add Detection Antibodies

# of Wells	20x beads	Detection Antibody Diluent, µl	Total Volume, µl
96	150	2,850	3,000
# of Coupled Beads Included in Kit	**150 x 3**	**3000 - 450**	
3	450	2550	3,000

1. Work out required volume of antibodies required.

2. Detection Antibodies should be prepared 10 minutes before use.

3. Add the required volume of Bio-Plex detection antibody diluent (2,850 µl) to a 15 ml Falcon tube.

4. Vortex the stock detection antibodies for 15–20 sec at medium speed, then perform a 30 sec spin to collect the entire volume at the bottom of the tube.

5. Dilute detection antibodies to 1x by pipetting the required volume into the 15-ml tube. Each well of the assay requires either 1.25 µl (20x stock) adjusted to final volume of 25 µl in detection antibody diluents.

6. After Incubating the beads, samples, standards and blank slowly/carefully remove and discard the sealing tape.

7. Wash the plate three times (MAG x3) with 100 µl wash buffer.

8. Vortex the diluted (1x) detection antibodies gently for 5 sec. Pour into a reagent reservoir and transfer 25 µl to each well of the assay plate using a multichannel pipet.

9. Cover plate with sealing tape and protect from light with aluminium foil. Incubate on shaker at 850 ± 50 rpm for 30 min at room temperature (RT).

F. Prepare and Add Streptavidin-PE (SA-PE)

1. Streptavidin-PE should be prepared 10 min before use.

2. Add the required volume of assay buffer to a 15 ml Falcon tube.

3. Vortex the 100x SA-PE for 5 sec at medium speed. Perform a 30 sec spin to collect the entire volume at the bottom of the vial.

4. Dilute SA-PE to 1x by pipetting the required volume into the 15 ml Falcon tube. Vortex and protect from light until ready to use. Each well of the assay requires 0.5 µl (100 x stocks) adjusted to a final volume of 50 µl in assay buffer.

# of Wells	100x SA-PE, µl	Assay Buffer, µl	Total Volume, µl
96	60	5,940	6,000

5. After the detection antibody incubation, slowly remove and discard the sealing tape.

6. Wash the plate three times (MAG 3) with 100 µl wash buffer.

7. Vortex the diluted (1x) SA-PE at medium speed for 5 sec. Pour into a reagent reservoir and transfer 50 µl to each well using a multichannel pipette.

8. Cover plate with sealing tape and protect from light with aluminium foil. Incubate on shaker at 850 ± 50 rpm for 10 min at room temperature.

9. After the streptavidin-PE incubation step, slowly remove and discard the sealing tape.

10. Wash the plate three times (MAG 3) with 100 µl wash buffer.

11. Each well requires 125 µl of assay buffer to allow the Bioplex to read the plate.

# of Wells	125 µl, per well	20% excess, µl	Total Volume of Assay Buffer, µl
96	1200	3000	15000

12. To resuspend beads for plate reading, add 15000 µl into a clean reagent reservoir. After this using, Multichannel pipettes add 125 µl of assay buffer to each well. Cover the plate with a new sheet of sealing tape. Shake the plate at room temperature at 850 ± 50 rpm for 30 sec, and slowly/carefully remove the sealing tape. Ensure that the plate cover has been removed.

13. Read Plate – Allow 15 minutes to put bead Region into computer (specific with each kit – see Yellow sticker in kit). Plate will take 40 minutes to run.

2.13 Adiponectin Assay

Adiponectin levels in plasma were measured using the adiponectin enzyme-linked immunosorbent assay (Elisa, SanDiego, USA) kit, AdipoGen - Adiponectin Rat ELISA assay (Cat. No. AG-45A-0005TP-KI01). Samples were diluted 1:1000 (1µl adiponectin: 900µl manufactures diluent). All measurements were completed according to the manufacturer's instructions. The kit can measure total adiponectin amount including all native isoforms (high, medium and low-molecular-weight-adiponectin).

2.14 Quantification of Results and Statistical Analysis

All statistical analysis was conducted using PASW statistics version 18 (SPSS Inc., Chicago, USA). All data is reported as mean ± SEM. Independent one or two tailed t-tests or analysis of variance (ANOVA) with Tukey's post-hoc test Statistical significance was accepted at $p < 0.05$.

2.15 Human Primary Skeletal Muscle Cell Culture

2.16 Cell Culture Establishment

Human primary skeletal muscle myotubes were cultured according to the methods of McAinch et al. (2006). Following surgical collection, the dissected muscle samples were cleaned of visible adipose tissue and blood vessels. The samples were then placed in a 15-ml cell culture

tube containing 10 ml ice-cold α-MEM (minimum essential medium). Muscle samples were approximately 50 mg. A 25cm^2 flask was coated with extracellular matrix (ECM) and left in the cell culture Lamina Flow Cytotoxic safety cabinet to dry. Sample tissue was then washed twice briefly with α-MEM prior to removal. The samples were then washed three times in ice cold PBS (approx 10 ml per wash, with care taken so not to lose tissue). Samples were then transferred to a Petri dish and any remaining adipose tissue or connective tissue was removed. Following which 3 ml 0.05% Trypsin-EDTA (Invitrogen® Castle Hill, NSW # 25300) was added and the tissue was minced with a sterile scalpel blade (Swann-Morton®, Sheffield, UK - Carbon Steel Surgical Blades-large) and forceps. After mincing the samples were transferred along with the medium to a sterile conical flask. 12 ml of 0.05 % Trypsin-EDTA (Invitrogen® Castle Hill, NSW # 25300) was added to the flask. The flask (covered with sterile paper to ensure no contamination while shaking) was then placed on a shaker at low speed (setting 60-70) for 20 minutes at room temperature.

The supernatant was collected and placed in a 50 ml Falcon tube containing 5 ml of FBS on ice to enable the inactivation of trypsin. Another 15 ml of 0.05% Trypsin-EDTA (Invitrogen® Castle Hill, NSW # 25300) was then added and the procedure was repeated three times and the supernatants pooled. The supernatant was then filtered through a 100 µm cell filter (Dismic-25cs®, Toyo Roshi Kaisha, Ltd, Japan) to remove any connective tissue and clumps. It was then centrifuged for 7 min at 1600 rpm at room temperature. The supernatant was then removed and the pellet of cells was re-suspended in 5 ml of primary growth medium (PGM). The cells were then seeded onto an uncoated 25 cm^2 flask, and placed in the incubator at 37 ^{o}C and 5% CO_2 for a period of 20 minutes. This allows for the majority of fibroblasts to attach to the surface of the flask. The medium containing the re-suspended cells was then transferred to an

ECM coated 25 cm^2 flask. Cells were then cultured at 37 ^oC and 5% CO_2. The media was changed following the first 24 hours and every second day thereafter, myotube cultured cells became visible under a microscope within ~8 days. Cells were now considered at Passage 1 (P1).

2.17 Cell Culture Passaging and Freezing

Once cells had reached a confluency of ~80%, 3 ml of trypsin was added to the flask for 3 minutes and incubated allowing the cells to dissociate from the flask. The trypsin was then inactivated by adding 7 ml of PGM. Cells were then spun down at 1500 rpm for 5 minutes. The supernatant was then discarded and the pellet of cells was resuspended in 40 ml of PGM. This was then split onto five 75 cm^2 flasks (8ml in each flask) that had been pre-coated with ECM. The media was changed following the first 24 hours and every second day thereafter. The cells were now considered at Passage 2 (P2).

When cells reached 70-80% confluency one of the flasks had 3 ml of trypsin added for 3 minutes and incubated allowing the cells to dissociate from the flask. The trypsin was then inactivated by adding 7 ml of PGM. Cells were then spun down at 1500 rpm for 5 mins. The supernatant was then discarded and the pellet of cells was resuspended in 40 ml of PGM. This was then split onto ten 75 cm^2 flasks (8 ml on each) pre-coated with ECM. The media was changed following the first 24 hours and every second day thereafter. The cells were now considered at Passage 3 (P3).

The cells from the other 4 75 cm^2 flasks at P2 were frozen, into 8 sterile cryotubes containers. This was done by washing out the flask of confluent cells with sterile PBS twice. Then 3 ml of trypsin was added to each flask for 3 minutes and incubated allowing the cells to dissociate

from the flask. The trypsin was then inactivated by adding 7ml of PGM. The cells were then spun down at 1500 rpm for 5 minutes. The combined supernatant from the four flasks was then discarded and the pellet of cells was resuspended in 2 ml PGM, 2 ml of FBS (Invitrogen® Castle Hill, NSW #10099-141) and 4 ml of freezing medium. 1 ml aliquots were then transferred to labelled cryotubes and to decrease cell death they were frozen down slowly at –20ºC for 30 mins. Cells were then left overnight in –80ºC, the following day cells in the cryotubes were then stored in liquid nitrogen. Similarly, when the cells in the ten 75 cm^2 flasks at passage 3 (P3) were >70% confluent they were frozen down into 20 cryotubes and placed in liquid nitrogen for storage.

The selected cells were then thawed quickly at 37 ºC and were resuspended in 9 ml of PGM. The supernatant was then added to an uncoated flask for 20 minutes to further remove any traces of fibroblasts. The supernatant was then removed and added to an ECM coated 75 cm^2 flask. Cells were then incubated at 37 ºC and 5% CO_2. The media was changed following the first 24 hours and every second day thereafter. When cells reached 70-80% confluency they were washed with sterile PBS twice. Then 3 ml of trypsin was added to the flask for 3 minutes and incubated allowing the cells to dissociate from the flask. The trypsin was then inactivated by adding 7 ml of PGM. The cells were then spun down at 1500 rpm for 5 minutes. The PGM containing the trypsin was then discarded and the pellet of cells was resuspended in 36 ml PGM. The cells were then divided into three 6 well pre-coated ECM plates (Greiner, Georgia, United States); with 2 ml added to each well (this enabled the cells to be placed at Passage 5 (P5). The media was changed following the first 24 hours and every second day thereafter; the cells were routinely incubated at 37 ºC and 5% CO_2.

2.18 Treatment

Upon reaching 70-80% confluency the cells were washed twice with sterile PBS. 3 ml of Differentiation Medium (500ml of α-MEM (Invitrogen® Castle Hill, NSW #12 571,); 0.5% of fungizone; 0.5% of pen-strep and 2% v/v of Horse Serum (Invitrogen® Castle Hill, NSW # 16050).was added to each well and the cells were left for a period of four days to enable the development of mature myotubes. After four days, the cells were washed twice with sterile PBS and serum starved with serum free medium (α-MEM and 0.1 BSA (bovine serum albumin) for a period of two hours. Cells were then treated in triplicate with 1 ml of each treatment being added to a single well. Treatments for CB1 antagonist 5 µM - AM251 (Sigma® Castle Hill, NSW # 71670) and CB_2 antagonist 50 µM - AM630 (Enzo® Redfern, NSW # CR-107) were dissolved in Ethanol (EtOH). Globular Adiponectin was used at a concentration of 0.1 µg/ml and was dissolved in EtOH. Thus, the total EtOH in each dish is 0.2 % (vehicle treated control).

2.19 Extraction of total RNA for PCR

Once the treatments had been on the cells for a total of exactly 24 hours the media was discarded and the cells were washed twice with 1 ml of PBS. 300 µl of TRIzol® reagent (Invitrogen® Castle Hill, NSW #15596-018) was added to each well. The TRIzol® was then gently swirled within the well to completely cover the well surface to properly lyse the cells. The TRIzol® Castle Hill, NSW, homogenate was then removed and placed in a sterile 1.5ml tube. All triplicate treatments were placed in the one tube for example each tube now had~ 900µl TRIzol® Castle Hill, NSW, of homogenate of each treatment. The homogenate was then stored at -80˚C until they were ready for extraction. Upon thawing on ice 250 µl of chloroform (Sigma® # C2432, Castle Hill, NSW) was added to each homogenate and were vortexed briefly. Homogenates were then left on ice for 5 minutes and then centrifuged at 13,000 rpm for 15

minutes at 4˚C. This created a separation of the chloroform. The clear upper phase was carefully removed (making sure neither interface nor any chloroform was collected) and transferred to a clear sterile eppendorf tube, containing 600 µl of isopropanol (Sigma® Castle Hill, NSW #191516) and 10 µl of 5 M NaCI. Treatments were then vortexed briefly and placed overnight in -20˚C. The following day, they were centrifuged at 13,000 rpm for 20 mins at 4˚C which enabled the RNA to form a white pellet. All liquid was aspirated off and the RNA pellets were placed in 400 µl of ethanol (75%, made with Diethylpryrocarbonate (DEPC Invitrogen® Castle Hill, NSW) and H_2O. They were then centrifuged for 8 minutes at 9000 rpm. The ethanol was aspirated off and the pellets were left to air dry at room temperature for~5 minutes (care was taken not to let the pellets dry out completely). Pellets were then dissolved in 10 µl of DEPC H_2O pre-heated to 60 ˚C.

2.20 Reverse Transcription of RNA

0.5 µg of RNA was added to the required amount of DEPC H_2O (Invitrogen® Castle Hill, NSW # 46-2224) to make a total solution volume of 5 µl. Using an iScript TM cDNA Synthesis Kit (Bio-Rad® Oakleigh, VIC, # 170-8890) containing a combination of oligos and random hexomers, 2 µl of iScript reaction mix and 0.5 µl of iScript reverse transcriptase was added to each sample. This solution of RNA was subsequently incubated for a total of three reaction times being 5 minutes at 25 ˚C; 30 minutes at 42 ˚C; 5 minutes at 85 ˚C using the PCR Express Thermal Cycler (Bio-Rad® Oakleigh, VIC). The 10 µl of cDNA was then diluted to a volume of 200 µl using nuclease free water and stored at -20 °C for subsequent analysis.

2.21 Real Time PCR

Real-Time PCR was used to assess mRNA expression. Real Time PCR was performed using the MY iQ® single colour real-time PCR detection system (BIO-RAD Oakleigh, VIC, model no – My iQ® - optics model; serial number – 569BR/0611) and BIO-RAD iQ® Oakleigh, VIC SYBR® Green Supermix. A master mix solution of 8 µl of 2 X SYBR Green; 2 µl of forward primers; 2 µl of reverse primers; 6 µl of sterile milli-Q water and 2 µl of sample cDNA was heated for 3 minute at 95 °C followed by 40 cycles of 10 seconds at 95 °C followed by 45 seconds at 60 °C, followed by 1 minute at 95 °C , followed by 1 minute at 55 °C, followed by 81 cycles of 10 seconds starting at 55 °C, and for the heat dissociation step utilising the BIO-RAD My iQ® Oakleigh, VIC sequence detection system. The heat dissociation step involved heating samples over 55-95 °C following the final cycle of the PCR. The increase in temperature causes PCR products to undergo denaturation that is accompanied by a decrease in fluorescence for solutions containing SYBR green chemistry. Denaturation enables the molecules to be separated to form single strands. This step allows the specific annealing between the primers and the single strands of the target DNA.Analysis of PCR results were calculated using the 2(-Delta Delta Ct) method normalized to the house keeping genes cyclophilin and GAPDH.

Table 2.8 Human primer design

Genes	Accession number	Forward primer	Reverse primer
Cyclophillin	NM_017101.1	CTGATGGCGAGCCCTTG	TCTGCTGTCTTTGGAACTTTGTC
GAPDH	NM_017008.3	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA
AdipoR1	NM_207587.1	TGAGGTACCAGCCAGATGTC	CGTGTCCGCTTCTCTGTTAC
AdipoR2	NM_001037979.1	TCCATGGAGTCTCAACCTG	GGAGAGTATCACAGCGCATC
APPL1	XR_007603	TCACTCCTTCCCCATCTTTC	TAGAGAGAGGGCAGCCAAAT
APPL2	NM_001108741	TGCTCGGGCTATTCACAA	AAACAGGCCCGTGACACT
FAT/CD36	NM_031561.2	GACCATCGGCGATGAGAAA	CCAGGCCCAGGAGCTTTATT
AMPK	NM_023991.1	ACTCTGCTGATGCACATGCT	AGGGGTCTTCAGGAAAGAGG
PDK4	NM_053551.1	GGGATCTCGCCTGGCACTTT	CACACATTCACGAAGCAGCA
PGC-1α	NM_013261.3	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC

GAPDH: *glyceraldehyde-3-phosphate dehydrogenase;* ***AdipoR1:*** *Adiponectin receptor 1;* ***AdipoR2:*** *Adiponectin receptor 2;* ***APPL1*** *adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1;* ***APPL2:*** *adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2;* ***FAT/CD36:*** *fatty acid translocase/CD36;* ***AMPK:*** *5'adenosine monophosphate-activated protein kinase a-2; 1;* ***PDK4:*** *pyruvate dehydrogenase kinase 4;* ***PGC-1a:*** *peroxisome proliferator-activated receptor gamma co-activator 1 alpha.*

Chapter 3 – The effects of chronic CB_1 antagonism on plasma hormone and cytokine markers in obesity

3.0 Abstract

The endocannabinoid system is well established as a viable anti-obesity target. The knowledge of the effects of chronic CB_1 antagonist treatment of AM251 in a DIO rodent model on systematic metabolic markers is not yet fully elucidated. The aim of this study was to characterise the effects on metabolic and cytokine markers following chronic treatment with the CB_1 antagonist AM251 in a model of DIO. Male Sprague–Dawley rats were fed a high-fat diet (HFD: 40% energy from lipids) for 9 weeks to elicit DIO. After 9 weeks consuming a HFD, rats were injected daily for 6 weeks with 3 mg/kg AM251 (n=9) or saline (n=9) via IP injection, while continuing to consume the HFD. Chronic blocking of CB_1 with AM251 resulted in a reduction in epididydmal and brown fat pad mass and a transient decrease in food intake. Chronic CB_1 antagonist treatment resulted in a decrease in circulating plasma leptin levels and decrease in plasma glucagon levels. Increases in many plasma inflammatory cytokines were also observed following 6 weeks of treatment with AM251.

3.1 Introduction

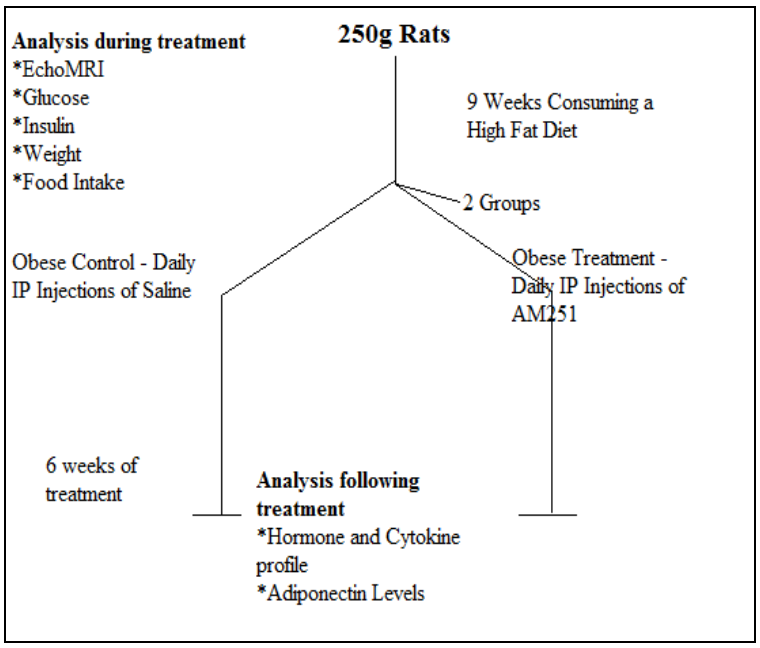
Global obesity contributes to the death of more than 2.8 million people each year regardless of economic status or age (World Health Organisation – Global status report on non-communicable diseases, 2010). In 2011 there were more than 40 million children under the age of five that were overweight. Children who are overweight have an increased risk of obesity and diabetes as adults (The GBD 2013 Obesity Collaboration). The overconsumption of energy dense nutrients plays a considerable role in the accumulation of intramyocellular triglycerides.

This increase in fatty acids has a deleterious effect on whole body insulin action resulting in impaired glucose tolerance. This increase in free fatty acids has been shown to correlate with a systematic up-regulation of the endocannabinoid system (Samocha-Bonet et al. 2010; Turner et al. 2013).

The endocannabinoid system is a widely distributed lipid signalling system that regulates appetite, fatty acid oxidation, glucose metabolism and inflammation. It is now widely accepted that CB_1 is expressed in numerous tissues and in varying concentrations (Cavuoto et al. 2007; Facci, 1995; Staton et al. 2008; Ziring et al. 2006; Ostanin et al. 2005; Elmes et al. 2004; Jenkin et al. 2013). It is also known that the endocannabinoid system plays a significant role in metabolism (Bluher et al. 2006). There is sufficient evidence to demonstrate that blocking CB_1 has an effect in the obese state. There are limitations on this knowledge however due to the centrally mediated psychotropic effects of CB_1 antagonism (Bermudez-Silva et al. 2007).

Obesity is associated with a continuous low-grade flow of inflammatory signaling pathways. This chronic abnormal cytokine production has been found to have a negative impact on insulin sensitivity (Arkan et al. 2005, Valerio et al. 2006). There are numerous classes of cytokines that have both autocrine and paracrine effects, and perform a variety of roles which are a part of an extensive signaling network. Pro-inflammatory cytokines are involved in the induction of an immune response. Anti-inflammatory cytokines are involved in inhibiting the actions of inflammation. The influences of obesity on metabolic hormones, is well established (Pasquali et al. 1991). Hormones such as leptin, ghrelin, and glucagon play significant roles in hunger, fat storage and food intake (Lustig et al. 2004, Tschöp_et al. 2001, Raben et al. 2001). Therefore, the aim of this study is to determine the effect of chronic CB_1 antagonism in a DIO rodent model on hormone and plasma cytokines markers. It is hypothesised that chronic

treatment with a CB_1 antagonist will reduce plasma hormone and cytokine markers in the DIO rodent model.



3.2 Materials and Methods

3.3 Animals and Experimental Protocol

Seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24 °C) with a 12-hour light/ dark cycle (7:00 - 19:00). Rats were fed ad libitum a HFD (containing 21% fat by weight, sourced from Specialty Feeds, Glen Forrest, Australia) for nine weeks to induce obesity. Obesity was determined when the treatment animals were compared to chow fed only animals at week 9; there was significantly higher weight gain in the high fat compared to the chow treatment groups (data not shown).Rats were then matched using many physiological parameters and put into either obese control or CB_2 agonist or antagonist treatment groups (n = 9 per group). For six weeks, rats were maintained on the HFD and treated daily, via IP injection, with either vehicle control (0.9% isotonic saline solution containing 0.75% Tween 80) or 3 mg/kg of AM251 dissolved in

vehicle solution. Following the conclusion of the experimental protocol, rats were deeply anesthetized and killed via cardiac puncture. Organs were removed, weighed and stored for further analysis, as outlined in Section 2.6. Experimental procedures were approved by Howard Florey Animal Ethics Committee (AEC 11-036).

3.4 Biological Measurements

Rat weight and food was recorded daily throughout the experimental period. An IPGTT (intraperitoneal glucose tolerance test) and IPIST (intraperitoneal insulin sensitivity test) were performed to determine the effects on whole body glucose homeostasis and was measured a week prior to drug treatment (week nine) and a week prior to euthanizing of the animal (week 15). The IPGTT was performed at the start of week nine and week fifteen the IPIST was performed at the end of week nine such that there was less impact of stress on the animal.

3.5 Hormone and Cytokine assay

Cardiac blood was extracted at the time of death using an E.D.T.A vacutainer system. Samples were immediately spun at 4000 RCF at 4 °C for 10 minutes. Plasma was then aspirated and checked for turbidity. The plasma was then stored at –80 °C. Prior to analysis samples were thawed on ice and spun at 1792 RCF at 4 °C for 10 minutes and 25 µl of plasma removed for analysis on the rat diabetes 4-plex panel and the rat cytokine 23-Plex panel multiplex protein arrays (Biorad, Biorad Laboratories GmbH, Munich, Germany), enabling the quantification of all parameters across a single sample. The diabetes 4-plex panel consisted of the following measurements: 1. Ghrelin; 2. Leptin; 3. GLP1 (Glucagon-like peptide-1); 4. PAI-1 (Plasminogen activator inhibitor-1). The rat cytokine 23-Plex panel kit consisted of the following measurements: 1. EPO (*Erythropoietin*), 2. G-CSF (*Granulocyte Colony Stimulating*

Factor), 3. GM-CSF (*Granulocyte Macrophage Colony Stimulating Factor*), 4.GRO/KC (*Growth-related oncogene*), 5. IFN-γ (*Interferon Gamma*), 6. IL-α (*Interleukin 1-alpha*), 7. IL-β (*Interleukin 1- beta*), 8. IL-2 (*Interleukin 2*), 9. IL-4 (*Interleukin 4*), 10. IL-5 (*Interleukin 5*), 11. IL-6 (*Interleukin 6*), 11. IL-7 (Interleukin 7), 12. IL-10 (*Interleukin 10*), 13. IL-12p70 (*Interleukin 12*), 14. IL-13 (Interleukin 13), 15. IL-17α (*Interleukin 17*), 16. IL-18 (*Interleukin 18*), 17. M-CSF (*Macrophage colony-stimulating factor*), 18. MCP-1 (*Monocyte Chemotactic Protein 1*), 19. MIP-1α (*Macrophage Inflammatory Protein 1α*), 20. MIP-3 (*Macrophage Inflammatory Protein 3α*), 21. RANTES, 22. TNF-α (*Tumor Necrosis Factor Alpha*), 23. VEGF (*Vascular Endothelial Growth Factor*)

3.6 Adiponectin

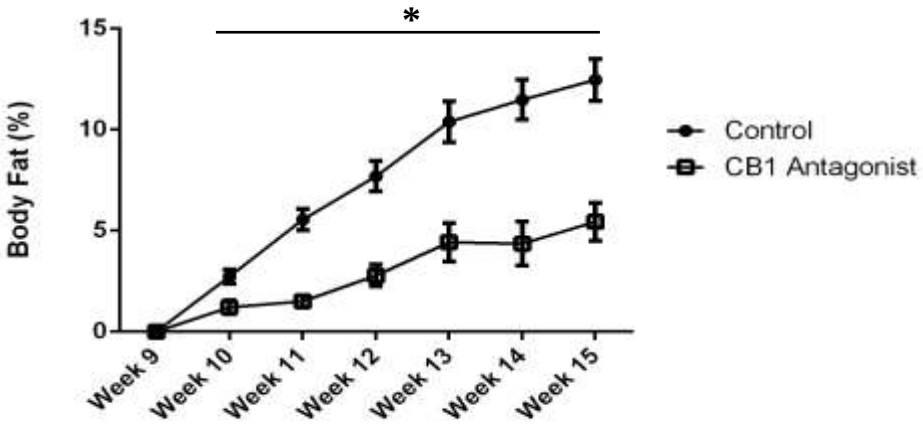
Cardiac blood was extracted and stored as detailed above in section 3.2.3. Plasma samples were thawed on ice and plasma adiponectin was analysed using an enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical, Tokushima, Japan). The kit measures the total adiponectin amount including all native isoforms (high, medium and low-molecular-weight-adiponectin). The test was carried out following manufactures instructions. Absorbance was read using a microtiter plate reader at 450 nm.

3.7 Statistical Analysis

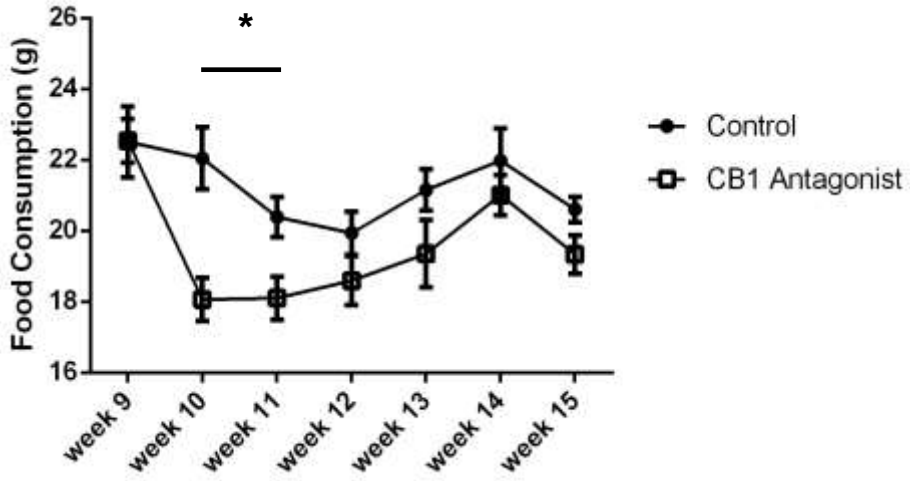
SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group direct analysis or mixed model ANOVA with Tukey's post-hoc tests for analysis of pre- and post- treatment measurements between obese control and AM251 treated groups. Significance was accepted when $p \leq 0.05$.

3.8 Results

Obesity Markers

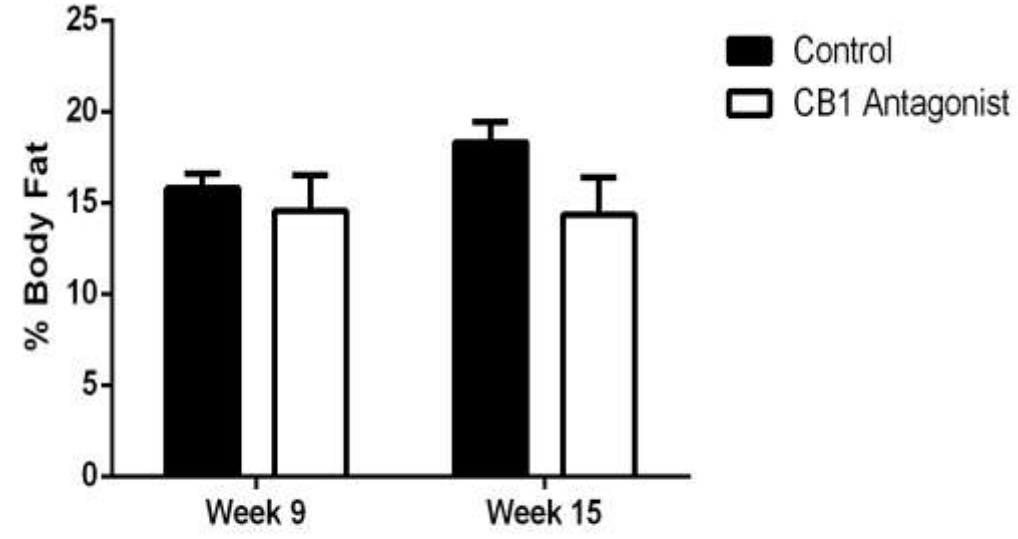


A

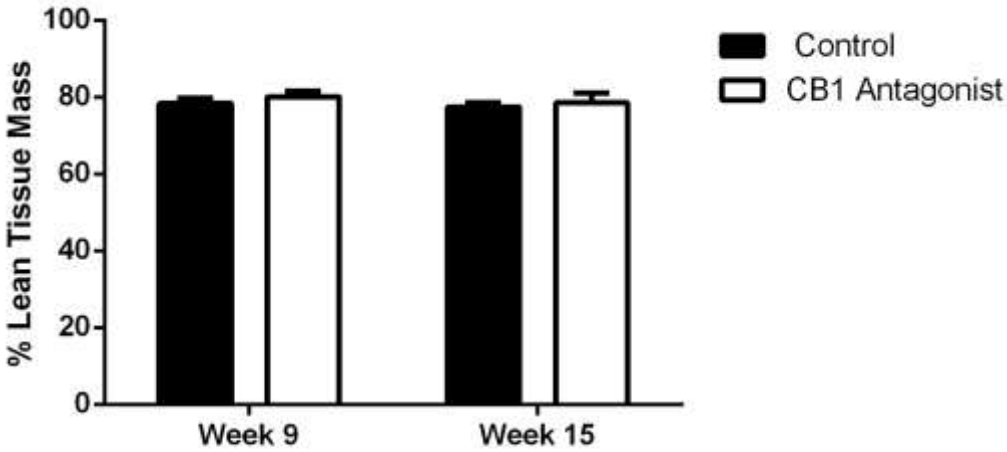


B

Figure 3.1.A *Percentage weight gain during 6 weeks of treatment with CB_1 Antagonist AM251 following 9 weeks of HF diet.* ***B*** *Food consumption following 9 weeks on a HF Diet and treated with CB_1 Antagonist AM251 for six weeks compared to DIO obese controls. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*



A

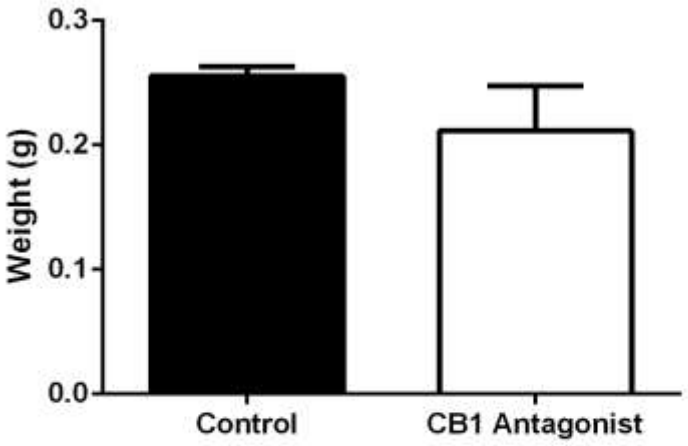


B

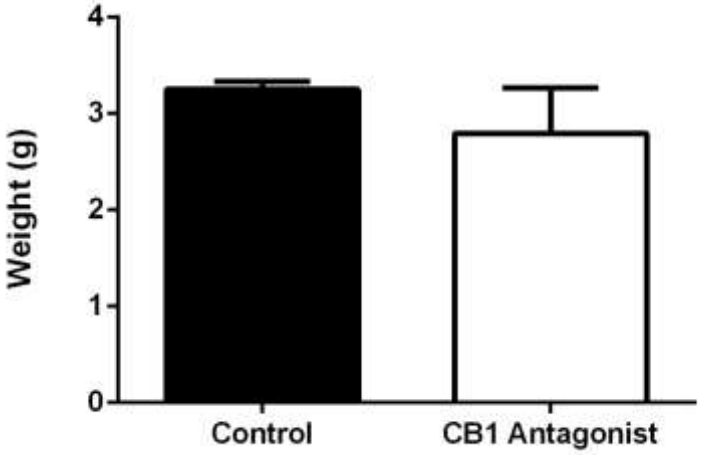
Figure 3.2.A *Percentage body fat in obese rats fed a HF diet for 9 weeks and treated with CB_1 Antagonist AM251 for six weeks compared to obese controls.* ***B*** *Percentage body fat in obese rats fed a HF diet for 9 weeks and treated with CB_1 Antagonist AM251 for six weeks.* ***B*** *Displays the change in lean tissue mass that occurred during six week treatment of CB_1 Antagonist AM251 (n=9).*

Despite a difference in weight gain throughout the six-week treatment period (Figure 3.1.A $P<0.05$), there was no difference in percentage of body fat (Figure 3.2.A) or lean tissue (Figure 3.2.B) ($p=0.39$) detected following Echo MRI at week 9 and week 15 in the AM251 group compared to the obese control animals receiving saline (Figure 3.3.2b). Food consumption (g/day) was however significantly decreased at weeks 11 and 12 (Week 11 =$p=0.007$, Week 12 = $p=0.024$) between the obese AM251-treated group and the obese control group (Figure 3.1.B).

Organ weight



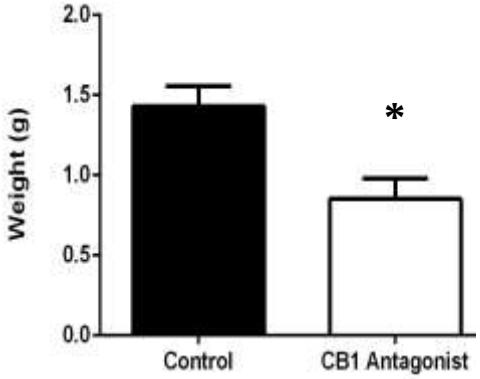
A; Heart



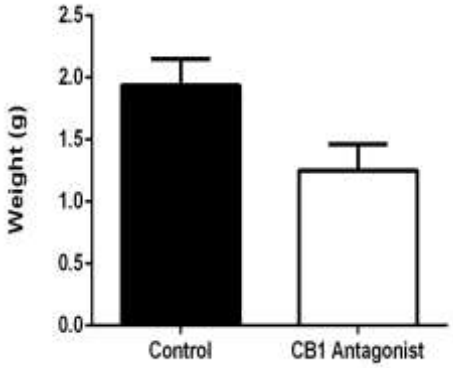
B; Liver

Figure 3.3.A *Heart Weight (g) following treatment of obese rats fed a HF diet for 9 weeks and treated with either the CB_1 Antagonist AM251 for six weeks..* ***B*** *Indicates the Liver weight (g) at death of obese rats fed a HF diet for 9 weeks and treated with either AM251 CB_1 Antagonist for six weeks compared to control.*

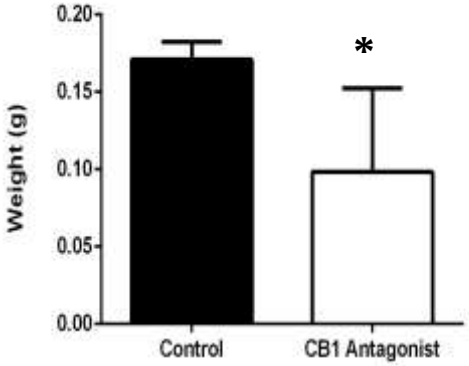
Fat Pad Weights



A; Epididymal Fat Pad



B; Peri-Renal Fat Pad

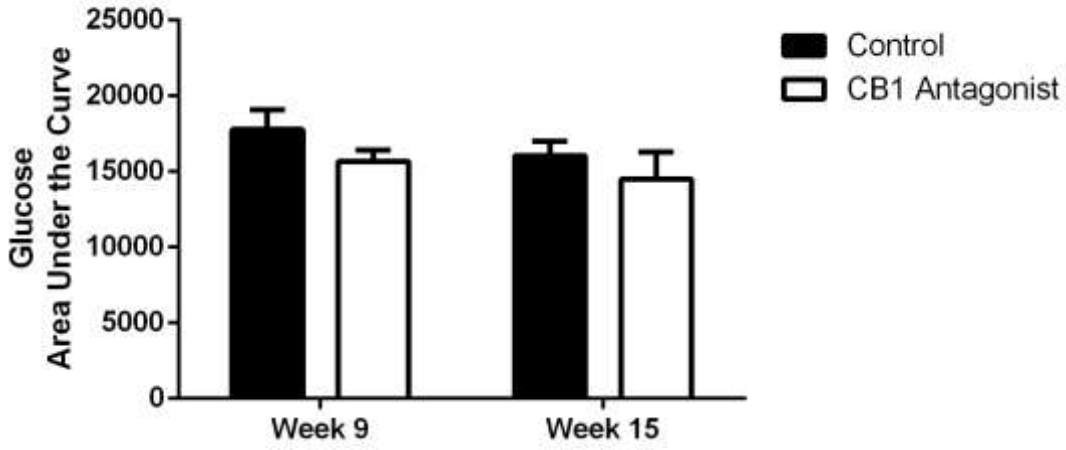


C; Brown Fat Pad

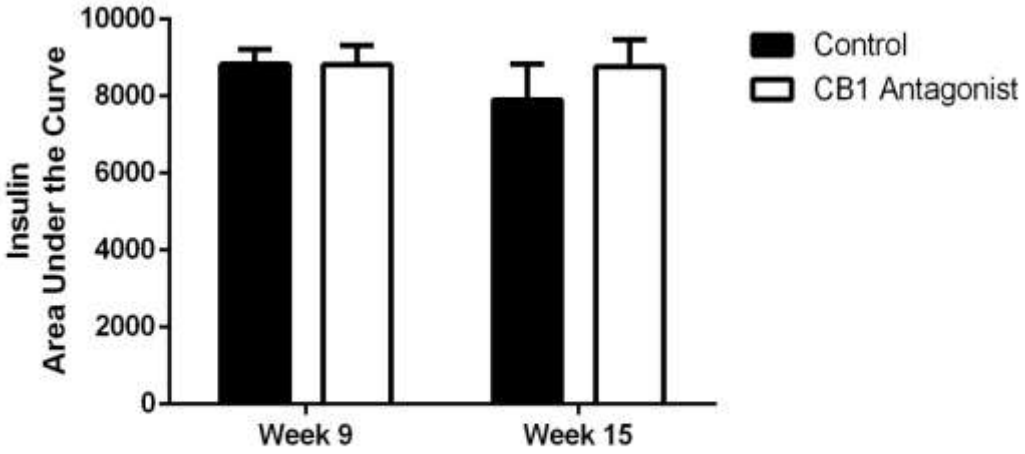
Figure 3.4.A *Epididymal Fat Pad (g) at death of obese rats fed a HF diet for 9 weeks and treated with CB_1 Antagonist AM251 for six weeks, compared to the obese control.* ***B*** *Peri-Renal Fat Pad (g) at death of obese rats fed a HF diet for 9 weeks and treated with CB_1 Antagonist AM251 for six weeks compared to control.* ***C*** *Brown Fat Pad from the high fat of either the AM251 CB_1 Antagonist group compared to control. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

There was no difference in the size of the heart between the obese AM251-treated group compared to the obese control animals receiving saline (Figure 3.3.A, p=0.31). Liver size between the AM251 group and the obese control group showed no significant difference in weight (Figure 3.3.B, p=0.36). Compared to the high fat control group, the AM251 group showed a significant decrease in the size of the epididymal fat pad (Figure 3.4.A, p=0.01), no difference in the size of the peri-renal fat pad (Figure 3.4.B, p=0.08), however brown fat was found to be significantly reduced in the AM251 group (Figure 3.4.C, p=0.01).

Glucose and Insulin



A; Glucose

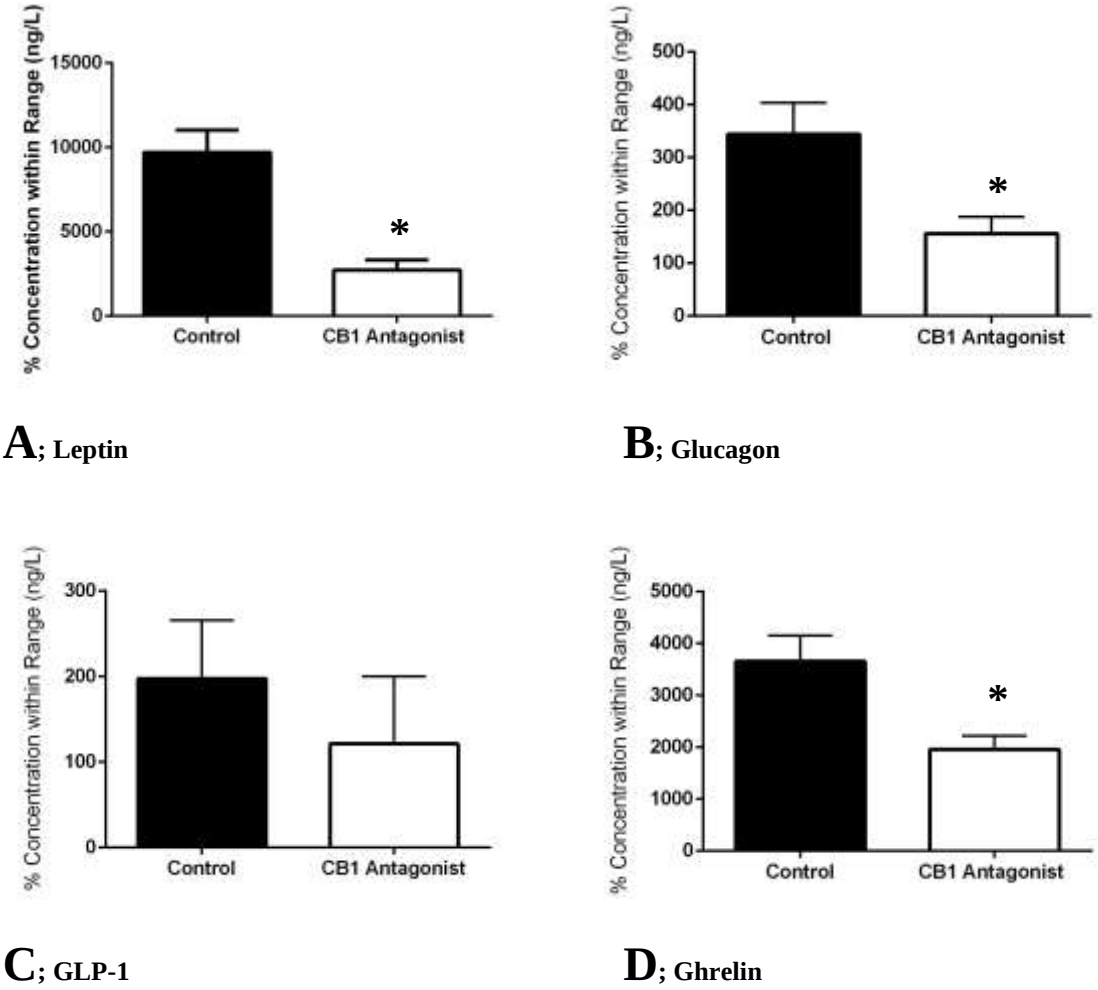


B; Insulin

Figure 3.5.A *Glucose tolerance test area under the curve prior to treatment (Week 9) and following six weeks of treatment with either* CB_1 antagonist AM251 or *obese control.* ***B*** *Insulin tolerance test area under the curve prior to treatment (Week 9) and following six weeks of treatment with either the* CB_1 antagonist AM251 or *compared to obese control Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

There was no significant difference between the two groups (Figure 3.5.A, p=0.17) following 9 weeks of HFD or following 6 weeks of treatment with the AM251 CB_1 antagonist or saline. Insulin levels were also not different (Figure 3.5.B, p=0.46) prior to or following treatment.

Hormone detection



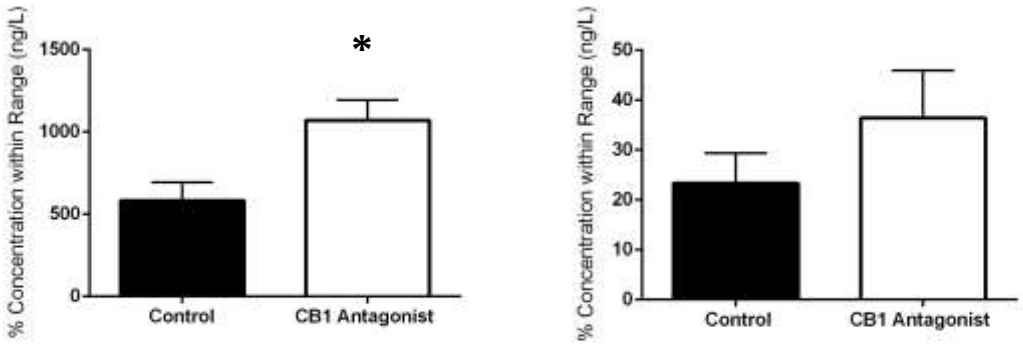
A; Leptin **B; Glucagon**

C; GLP-1 **D; Ghrelin**

Figure 3.6.A *Leptin levels in plasma in obese rats fed a HF diet for 9 weeks and treated with* CB_1 antagonist AM251 or control ***B*** *Glucagon levels in plasma in obese rats fed a HF diet for 9 weeks and treated with either CB_1 antagonist AM251 or control.* ***C*** *GLP-1 levels in the plasma of obese rats fed a HF diet for 9 weeks and treated with either CB_1 antagonist AM251 when compared to controls.* ***D*** *Ghrelin levels following treatment with AM251. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

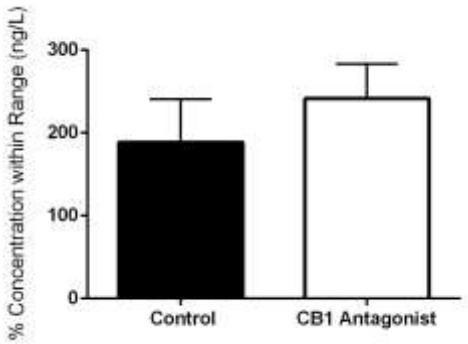
Leptin levels were significantly less in the CB_1 antagonist group (Figure 3.5.A, p=0.018) following six weeks of IP injection of AM251 daily. Glucagon was significantly reduced in the AM251 treated group (Figure 3.5.B, p=0.04 and lower circulating levels of Ghrelin were detected in the CB_1 Antagonist AM251 group (Figure 3.5.D, p=0.003). No difference between groups was observed in GLP-1 (Figure 3.5.C, p=0.09).

Cytokine detection

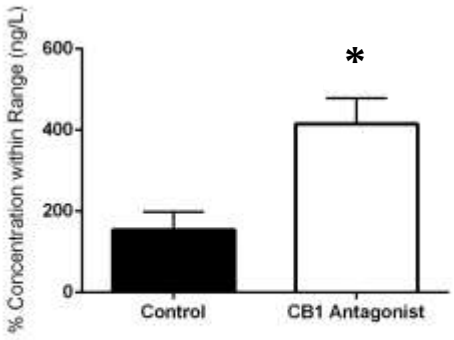


A; EPO

B; G-CSF

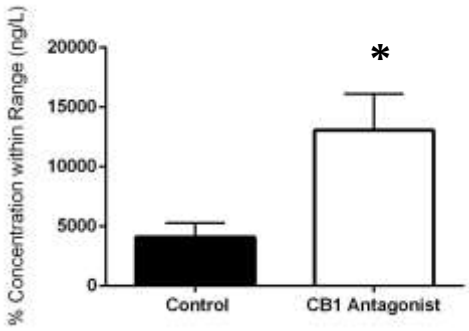


C; GRO/KC

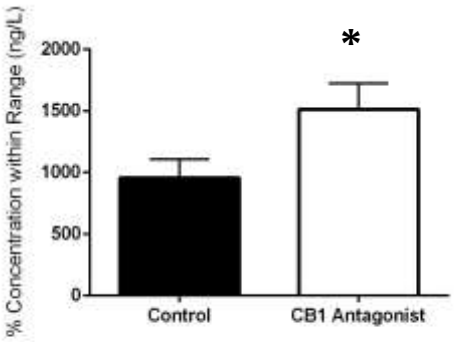


D, IL-1α

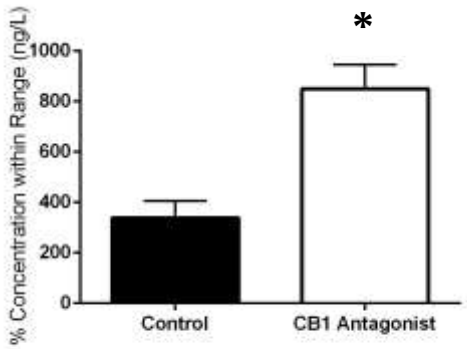
Figure 3.7.A *Plasma levels of EPO of high fat fed rats following six weeks of CB_1 antagonist AM251 for six weeks.* ***B*** *G-CSF plasma levels of DIO rats fed a high fat diet and treated for six weeks with CB_1 Antagonist AM251.* ***C*** *Plasma levels of GRO/KC of obese rats following six weeks of treatment with CB_1 antagonist AM251.* ***D*** *IL-1α plasma levels of rats fed a high fat diet and treated for six weeks with CB_1 antagonist AM251 (n=9).*



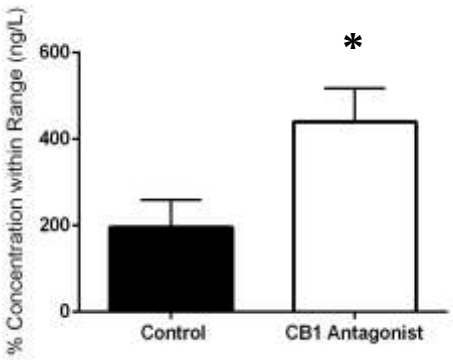
E; IL-1β



F; MCP-1

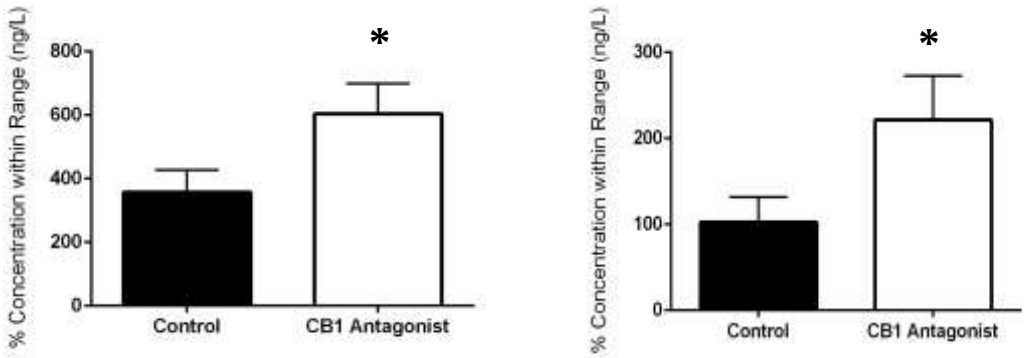


G; IL-2



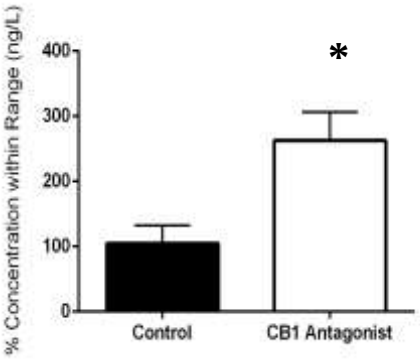
H; IL-4

Figure 3.7.E *Plasma levels of IL-1β of obese rats following six weeks of CB_1 antagonist AM251 for six weeks.* ***F*** *MCP-1 plasma levels of obese rats fed a high fat diet and treated for six weeks with either CB_1 antagonist AM251.* ***G*** *Plasma levels of IL-2 of high fat fed rats following six weeks of CB_1 antagonist AM251 for six weeks.* ***H*** *IL-4 plasma levels of obese rats fed a high fat diet and treated for six weeks with CB_1 antagonist AM251(n=9).*

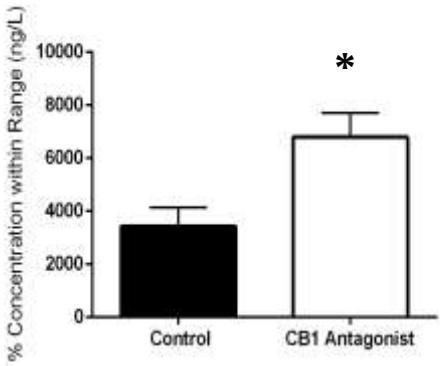


I; IL-5

J; IL-13

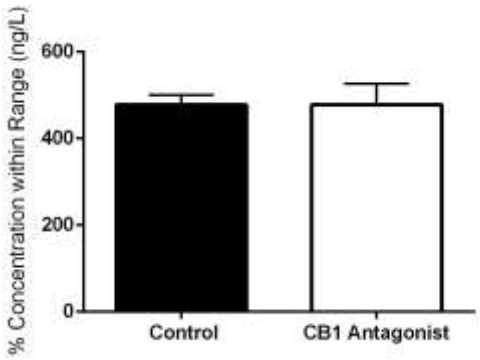


K; IL-17α

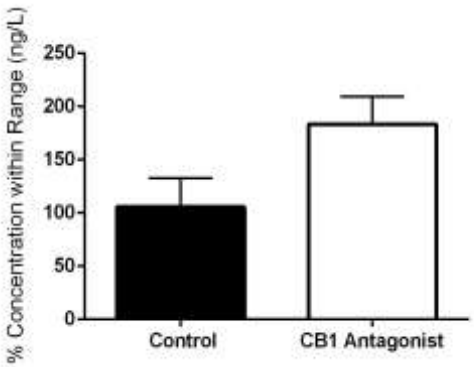


L; IL-18

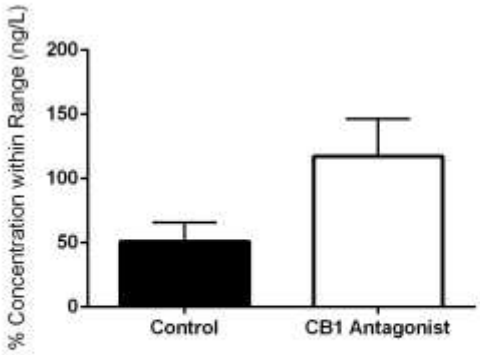
Figure 3.7.I *Plasma levels of IL-5 of high fat fed rats following six weeks of CB_1 antagonist AM251 for six weeks.* ***J*** *IL-13 plasma levels of obese rats fed a high fat diet and treated for six weeks with CB_1 Antagonist AM251.* ***K*** *Plasma levels of IL-17α of high fat fed rats following six weeks of treatment with CB_1 antagonist AM251.* ***L*** *IL-18 plasma levels of obese rats fed a high fat diet and treated for six weeks with CB_1 antagonist AM251 (n=9).*



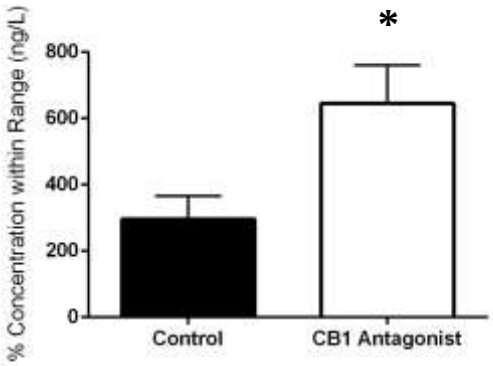
M; MCSF



N; MIP-3α

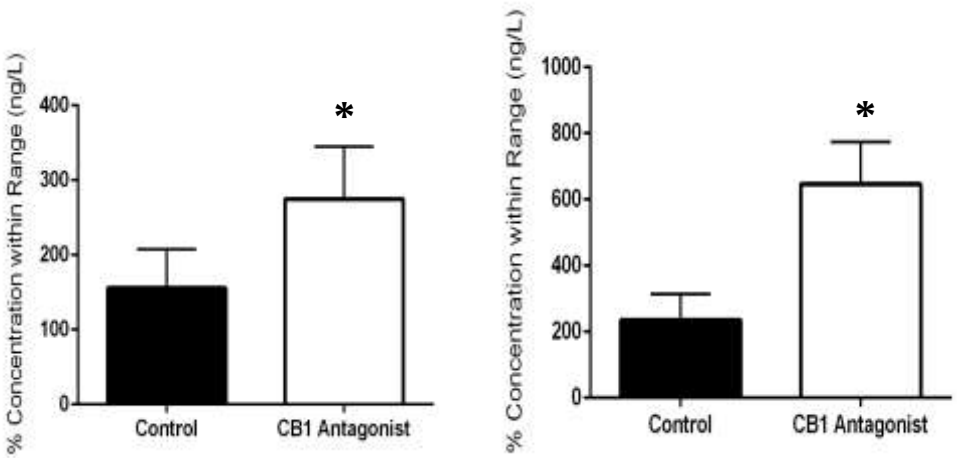


O; VEGF



P; RANTES

Figure 3.7.M *Plasma levels of MCSF of high fat fed rats following six weeks of CB_1 antagonist AM251 for six weeks.* ***N*** *MIP-3α plasma levels of obese rats fed a high fat diet and treated for six weeks with CB_1 Antagonist AM251.* ***O*** *Plasma levels of VEGF of high fat fed rats following six weeks of treatment with CB_1 antagonist AM251.* ***P*** *RANTES plasma levels of obese rats fed a high fat diet and treated for six weeks with CB_1 antagonist AM251 (n=9).*



Q; TNF-α **R; IL12p70**

Figure 3.7.Q *Plasma levels of TNF-α of high fat fed rats following six weeks of treatment with CB_1 antagonist AM251.* ***R*** *IL12p70 plasma levels of obese rats fed a high fat diet and treated for six weeks with CB_1 antagonist AM251 (n=9).*

Adiponectin

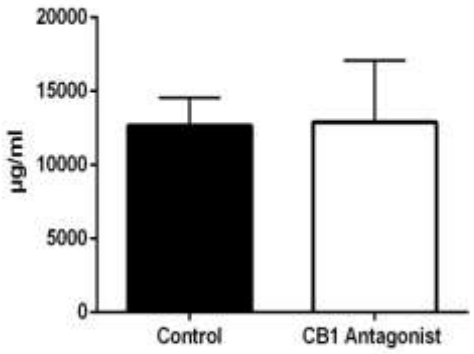


Figure 3.8 *Adiponectin concentration (µg/ml) in plasma in obese rats fed a HF diet for 9 weeks and treated with AM251 (CB_1 Antagonist) for six weeks compared to a HF saline treated control group.*

Cytokine levels in plasma in obese rats fed a HF diet for 9 weeks and treated with AM251 mRNA significantly increased in **EPO** (Figure 3.7.A, p=0.01), **IL-1α** (Figure 3.7.D, p=0.01)**,** **IL-1β** (Figure 3.7.E, p=0.05), and **MCP-1** (Figure 3.7.F, *p=0.03*), IL-4 (Figure 3.7.H, p=0.04), and **IL-5** (Figure 3.7.I, p=0.03)**, Il-17α** (Figure 3.7.K, p=0.05)**, Il-18** (Figure 3.7.L, p=0.02) and **MCSF** (Figure 3.7.M, p=0.05), **RANTES**; (Figure 3.7.P, p=0.01), **TNF-α** (Figure 3.7.P, p=0.01) and **IL12p70** (Figure 3.7.R, p=0.005). The circulating levels of **IL-2** significantly decreased (Figure 3.7.G, p=0.04). In obese rats administered with AM251 treatment for six weeks following a HF diet for 9 weeks, cytokine levels in plasma in obese rats fed a high fat diet the mRNA levels did not alter in **G-CSF** (Figure 3.7.B, p=0.39), **GLP-1** (Figure 3.7.C, p=0.21) and **GRO/KC** (Figure 3.7.D, p=0.21), **MIP-3α** (Figure 3.7.N, p=0.09), **VEGF** (Figure 3.7.O, p=0.12), or **IL-13** levels when compared to obese controls.

Adiponectin assay results which looked at all isoforms in circulating plasma levels at death showed that there were no significant differences *(Figure 3.8, p=0.42)* between the high fat control and AM251 treatment groups.

*Cytokines GM-CSF, IL-6, IL-10 and MIPIα were all tested as part of the Bioplex multiple cytokines kit however these analytes were too low to be detected in the majority of samples and as such cannot be included in the data presented.

3.9 Discussion

The weight loss effects that occur in the obese state following CB_1 antagonist treatment has been discussed at length earlier in this thesis (*see chapter 1*). The exact mechanisms and physiological changes that occur in a variety of research modes, is required to fully elucidate the reasons for the weight loss effects of CB_1 antagonism. In this study, chronic CB_1 antagonism via AM251 treatment resulted in decreased weight gain, a reduction in body fat content, and a transient decrease in food intake. Plasma samples collected from the animals indicated that there was a decrease in circulating leptin, ghrelin and glucagon levels, however it also resulted in an increase in a number of circulating pro-inflammatory markers. These data provide further information on the effects of chronic CB_1 antagonism and the effects on plasma hormone and the cytokine markers.

Collectively the results of this study show that in this animal model of obesity, chronic CB_1 antagonist treatment with AM251 successfully reduced the animal's body weight. This is in support of AM251 administered in obese Zucker rats resulting in weight loss and a decrease in leptin levels (Merroun et al. 2013). The animals had decreases in fat pad mass and decreased leptin levels. The significant decrease in the leptin levels in the AM251 treated group in this research is indicative of lipolysis and the dissolution of fat cells (Merroun et al. 2013). Reduced food intake, and a decrease in circulating leptin levels in weight loss occurs in both animals and humans (Maffei et al. 1995).

Ghrelin, a hormone associated with increasing both appetite and food intake (Wren et al. 2001), was reduced in animals chronically treated with AM251. Ghrelin levels have been found to be decreased in obesity (Tschöp et al. 2001). Glycogen is the main store of carbohydrates in the liver and skeletal muscle. Glucagon is a polypeptide hormone released from the pancreas that

stimulates the conversion of glycogen to glucose by the liver. Increases in glucagon levels are induced by decreases in circulating blood glucose levels. The glucagon levels in this study were significantly reduced in the AM251 treatment group, despite no alteration observed in blood glucose levels. The current study observed a transient decrease only in food intake at the onset of treatment, which has been replicated in other interventions with pharmaceutical agonists/antagonist's studies, investigating CB receptors (Colombo et al. 1986, Cota et al. 2003, Verty et al, 2004, Liu et al. 2005, Thornton-Jones et al. 2006, Wang et al. 2007). The decreases seen in ghrelin and leptin therefore appear to be independent of food intake. Despite being a DIO model of obesity prior to treatment, no changes in glucose and insulin sensitivity were detected following treatment with AM251. This indicates that it is possible that there was an increased reliance on fatty acids as a metabolic fuel. This finding is consistent with the decrease in plasma leptin levels, and this in turn is consistent with the reduction in adipose tissue stores.

The animals in this study that were treated for six weeks with a CB_1 antagonist had significantly less epididymal and brown fat pads. White adipose tissue is stored in both large pads and smaller areas that reside in isolation next to organs such as the lymph nodes, the heart and the liver (Goodpaster et al. 2003).

The epididymal fat pad is one such white fat pad which sits in the lowest part of the abdomen. Brown fat, unlike white fat, plays a significant role in energy metabolism and thermogenesis assisted via its high concentration of mitochondria and rich vascularisation (Ghorbani et al. 1997). In contrast, rats receiving CB_1 antagonist treatment for 21 days resulted in an increase in energy expenditure, and thermogenesis which was attributed to brown fat activation, the weight of the fat pad mass however was not recorded (Verty et al. 2009). The current study

was not able to determine if the decrease in the brown fat pads resulted in altered mitochondrial function. Brown and white adipose tissue has been found to be affected by leptin (Siegrist-Kaiser et al. 1997). In transgenic mice (ob/ob, UCP-DTA, MSG), body fat storage levels have been shown tocorrelate with increasing leptin levels in obesity, this occurs without affecting the food intake of a high fat diet, suggesting that in the obese state leptin's influence on appetite and food intake becomes disrupted (Frederich et al. 1995). It is established that insulin resistance has a yet to be elucidated cause and effect relationship with obesity (Preis et al. 2010). It is also well established that adiponectin levels decline in obesity (Eglit et al. 2013). Moreover a decrease in circulating adiponectin has been shown to correlate with an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines (Jalovaara et al. 2008). If these physiological changes are effected by cannabinoid receptor modulation is yet to be elucidated.

It is also well established that adiponectin levels decline in obesity (Eglit et al. 2013). Moreover a decrease in circulating adiponectin has been shown to correlate with an increase in pro-inflammatory cytokines and a decrease in anti-inflammatory cytokines (Jalovaara et al. 2008). Whether these physiological changes are influenced by cannabinoid receptor modulation is yet to be elucidated.

It has been established that CB_1 antagonism in humans and animals has resulted in an increase in both insulin sensitivity and plasma adiponectin levels (Stefan et al. 2002, Hotta et al. 2001). In this study however, neither hormone was altered with chronic administration of AM251 in a DIO model. However the decrease in fat pad weight, glucagon levels and the decrease in leptin are all indicative of increased fat pad lipolysis.

Obesity is associated with low grade chronic inflammation (Bruun et al. 2006, Maachi et al. 2004). When energy intake exceeds that of energy expenditure, circulating levels of glucose and fatty acids increase which are then converted to fat and are stored as triglycerides in adipose tissue (Byrne et al. 1984, Yki-Jarvinen et al., 1998). In diet induced obesity, this action is continuous, resulting in expanding adipocytes which release a continuous flow of cytokines and cytokine mediators), the released cytokines have been shown to be pro-inflammatory (Bruun et al. 2006, Maachi et al. 2004

Considering this increase in pro-inflammatory markers in the accumulation of fat pad mass, it seems possible then, that a reduction in fat pad mass may result in a decrease in these pro-inflammatory cytokines (Yki-Jarvinen et al. 2013). As discussed, weight loss did occur with the animals within this study, however the plasma inflammation levels did not have the anticipated effect.

The DIO animals in this study that were chronically treated for six weeks with CB_1 antagonist, AM251, were detected as having a physiological inflammatory response. The data showed that there was an increased production of circulating plasma cytokines at the time of death. Pro-Inflammatory cytokines: TNF-α, IL-1α, IL-1β, IL-2, IL-5, Il-17α, Il-18, IL12p70, MCSF, G-CSF were all significantly increased in the plasma.TNF-α is involved in the regulation of a wide spectrum of biological processes including cell proliferation, differentiation, apoptosis, lipid metabolism, and coagulation (Hotamisligil et al. 1993). The effects of CB_1 antagonism on TNF-α is conflicting. In an epithelial cell culture model, CB_1 antagonism with rimonabant resulted in a down regulation in the TNF-α inflammatory pathways (Huang et al. 2010). In support of this, CB_1 agonism in an animal model has been shown to increase TNF-α levels (Karmaus et al. 2013). In support, however of the increase in TNF-α seen in this study,

treatment with CB_1 antagonist rimonabant increased the production of TNF-α in a mouse model of autoimmune disease (de Lago et al. 2012). In contrast, a blood cell culture model found that modulation with either CB_1 agonist or CB_1 antagonist was ineffective in affecting TNF-α or IL-2 signalling (Cencioni et al. 2010). IL-2 is a cytokine important for the proliferation of T and B lymphocytes in human blood cells. Treatment with a CB_1 agonist has however been shown to have no specific effect on IL-2 (Ihenetu, et al. 2003), which is in contrast to the current study which demonstrated an increase in IL-2 following 6 weeks of blocking CB_1 with AM251.

IL-1α is produced by monocytes and macrophages and is released in response to cell injury, and thus induces apoptosis (Cohen et al. 2015). In a cell culture model, CB_1 agonism via synthetic cannabinoids down regulated the pro-inflammatory effects of IL-1α (Mbvundula et al. 2006). The current study is the first to show modulation of IL-1 and IL-2 by CB_1 antagonism in a DIO animal model. Moreover, this study is the first to show that chronic CB_1 antagonism significantly increased both IL-5 and IL-18 circulating levels. IL-5 acts as a growth and differentiation factor for both B cells and eosinophils. IL-18 is known to stimulate interferon gamma production in T-helper type I cells. While this study is the first to demonstrate a modulation of these cytokines following treatment with a CB_1 antagonist, it is unable to determine whether this is a direct effect of modulation of this system or an indirect effect. As such further research into the effects and regulation of these cytokines and the endocannabinoid system is required.

IL-17α is a pro-inflammatory cytokine that regulates the activities of NF-kappaB and mitogen-activated protein kinases and IL-1β is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation, differentiation, and

apoptosis (Numasaki et al. 2013). Circulating plasma levels of IL-17α and IL- 1β increased in this study which is in line with research showing that CB_1 antagonism increases Il-17α and IL-1β in the CNS of a mouse model (Lou et al. 2011). Circulating levels of IL12p70 were also significantly increased, this cytokine has been found to be important for sustaining a sufficient number of memory/effector Th1 cells (Schulz et al. 2000). Moreover, IL12 and MCP-1 were found to have increased in this study, which is in opposition to findings in other research such as an obese mouse model. Treatment with CB_1 antagonist rimonabant caused a reduction in plasma levels of the pro-inflammatory cytokines IL12 and MCP-1 (Dol-Gleizes et al. 2009). Moreover, this study found that MCP-1 and RANTES were both significantly increased and both have been found to be involved in immunoregulatory and inflammatory processes (Conti & DiGioacchino et al. 2001). Interestingly RANTES (CCL5) is responsible for the induction of pro-inflammatory cytokines such as macrophages and the promotion of human adipose tissue cell survival (Keophiphath et al. 2010, Fischer et al. 2003).

Increases in both MCSF and G-CSF were recorded in the plasma of the CB_1 antagonist treatment groups.MCSF controls the production, differentiation, and function of macrophages and G-CSF controls the production, differentiation, and function of granulocytes. This is the first study that has indicated an influence of expression in MCSF and G-CSF following chronic CB_1 antagonist treatment.

Similarly the present study found that CB_1 antagonist treatment with AM251 resulted in increased circulating plasma levels of EPO. EPO is released into the blood stream in response to anoxia (local or systematic lack of oxygen to tissues) the effects of CB modulation has on EPO has not yet been established, reinforcing these data as novel findings EPO is a glycoprotein hormone that stimulates the production of erythrocytes which increases the

oxygen carrying capacity of blood plasma. Given the potential positive therapies associated with an increase in circulating oxygen capacity, further research into establishing the role of chronic CB_1 antagonist on EPO modulation is recommended.

In contrast to the increase in pro-inflammatory markers seen in this study, the anti-inflammatory cytokine IL-4 was found to have significantly increased in the AM251 treatment group. This cytokine investigated in a cell culture model, has shown that the modulation of CB_1 gene expression was found influenced by IL-4 (Borner et al. 2007). The data in this study when compared to current literature can be difficult to interpret. Previous studies with CB_1 antagonism investigating the implications of inflammation show variable results. (Jourdan et al. 2010, Mukhopadhyay et al. 2010). Moreover, the effects of blocking CB_1 signaling in acute pancreatitis were investigated in rats, with markers of inflammation (serum amylase, white blood cells, haematocrit and IL-6) tested following the administration with AM251 (Matsuda, 2005). Although the AM251 antagonist treatment had no effect on the systematic inflammation in the animals, it did result in an increased survival rate and dramatically improved blood pressure (Matsuda, 2005). This highlights the fundamental importance of establishing further research into the modulation of cannabinoid receptors.

Until now such an extensive overview of the effects of chronic AM251 CB_1 antagonism treatment on DIO parameters and the adaptations that occur in circulating cytokine and metabolic hormone levels in a DIO model had not been conducted. This study resulted in the animals having a transient decrease in food intake, a common marker indicating the effectiveness of CB_1 blockade. The animals had significant weight loss and the fat pad mass was significantly decreased. Despite the anticipated beneficial effects of a decrease in fat accumulation and food intake, the animals were in a pro-inflammatory state with evidence of

significant increases in a range of cytokines. AM251 has previously has been associated with causing food avoidance and behaviours associated with nausea in rats. The concentration of AM251 recorded in which behaviours first start to alter were 4 mg/kg/bw (McLaughlin et al. 2005). The concentration of AM251 in this study was administered daily at 3mg/kg/bw to avoid the possible induction of malaise. Further research into the physical, biological and possible psychological effects of chronic AM251 CB_1 antagonism treatment in a DIO model is highly recommended.

3.10 Conclusion

In conclusion the animals in the CB_1 antagonism (AM251) treatment group, lost weight and reduced fat pad stores. The theory that there may have been an increased reliance on fatty acids is supported by the reduction seen in the plasma levels of leptin and glucagon. Moreover, a reduction in fat pad weight was seen despite a recovery in animals' daily food intake. Despite presenting as an obese model of obesity prior to treatment, no alterations of glucose and insulin sensitivity were detected following treatment with AM251, similarly circulating levels of adiponectin did not alter. Finally, the effects of weight loss and thesuspected lipolysis in the animals treated with CB_1 antagonism may well be a positive one, however more investigation is needed to determine this outcome. Especially with the results showing an increase in the circulating plasma levels of cytokines, the majority playing pivotal roles in mediating inflammation. The exact cause of the increase in inflammatory markers following chronic CB_1 AM251 antagonist treatment is yet to be elucidated. Therfore the null hypothese is accepted as the chronic administration with CB_1 antagonism reduced circulating plasma hormone and cytokine markers in a diet induced model of obesity.

A major limitation of this study is the inability to target the exact time point during treatment when an up regulation of inflammation occurred. Whether a chronic low dose of CB_1 antagonism induces exacerbated lipolysis and therefore induces an increase in inflammation is not known and to date no studies have investigated the spectrum of cytokines that have been studied within this thesis, however it should be acknowledged that the results are in contrast to what others in the field have found. Determining if the same incidence of lipolysis can occur at a lower dose of AM251 without inflammation should be established. This would assist in the understanding of the modulating affect that CB_1 antagonism plays in the expression of plasma hormone and cytokine markers in obesity.

Chapter 4 –The effects of CB_1 Antagonism and adiponectin on fatty acid oxidative pathways in skeletal muscle.

4.0 Abstract

The endocannabinoid system has received much recent interest as a target for anti-obesity pharmacological intervention. The effects of chronic CB_1 antagonist treatment with AM251 in a DIO rodent model on the mRNA markers of cellular signalling in skeletal muscle and whole muscle mitochondria is not yet determined. This study therefore investigated the effects of a chronic treatment with AM251, in a model of DIO on oxidative markers and adiponectin signalling. Male Sprague–Dawley rats were fed a high-fat diet (HFD: 40% digestible energy from lipids) for 9 weeks to elicit DIO. After 9 weeks consuming the HFD, rats were injected daily for 6 weeks with 3mg/kg AM251 (n=9) or saline via IP injection (n=9) while continuing to consume the HFD. The animals were deeply anaesthetised and skeletal muscle (soleus, EDL, red and white gastrocnemius) was removed before death to preserve cytokine activity. Immediately after their removal, longitudinal strips of the soleus and EDL were incubated in an oxygenated organ bath (37ºC pre-gassed at 95% O2 -5% C02). Each longitudinal strip was placed in an individual chamber of Krebs-Henseleit buffer with either 0.1 µg/ml of adiponectin (AD+) or the contralateral hind limb muscle exposed to control (AD-) for a period of 30 minutes. Citrate synthase activity (CS) was measured in a whole muscle preparation of (red and white) gastrocnemius. Chronic blockade of CB_1 with AM251 resulted in no change to CS; acute exposure to adiponectin resulted in decreased mRNA expression of AMPK in the EDL muscle. ADIPOR1 mRNA expression decreased in soleus muscle when exposed to

adiponectin. ADIPOR2 gene expression was significantly decreased, equally PGC1-α levels decreased in the EDL following acute adiponectin exposure.

4.1 Introduction

CB_1 has been identified to be involved in a variety of functions including, cell growth and differentiation, neurotransmission, cardiovascular function, immune function and cellular metabolism (Howlett et al. 2010). CB_1 influences a number of cellular signaling pathways, inhibiting voltage-gated Ca^{2+} channels, activating K^+ currents, inhibiting adenylyl cyclase, activating mitogen-activated protein kinases (MAPK), and increasing nitric oxide (NO) signalling (Mackie et al. 1992). Modulation of which effects both CNS and peripheral tissues. CB_1 has been identified in skeletal muscle; however, the downstream signalling pathways in this tissue have not been thoroughly investigated (Cavuoto et al. 2007, Eckardt et al. 2009, Mackie et al. 1992). CB_1 agonism has been shown to increase insulin resistance in skeletal muscle (Lipina et al. 2010). Similarly, CB_1 antagonism in skeletal muscle results in an increase in glucose uptake and an increase in the expression of genes involved in oxidative metabolism. Blockade of CB_1 was shown to increase fatty acid oxidation in skeletal muscle myotubes (Esposito et al. 2008, Pagotto et al. 2005, Liu et al. 2005, Espositi et al. 2008). CB_1 antagonism increases energy expenditure through increased fatty acid oxidation, which may be a contributor to the body weight reduction that occurs with CB_1 antagonism, as the reduction in food intake that occur with antagonist treatment only appear to be transient in nature (Herling et al. 2008).

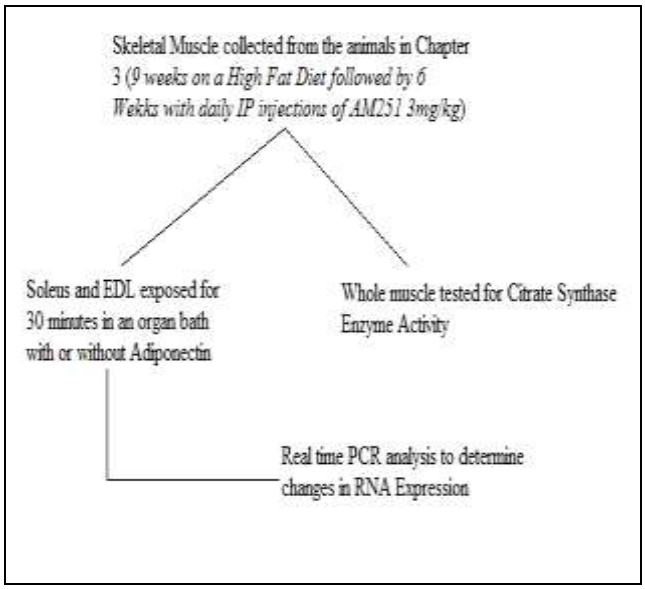
Skeletal muscle plays an important role in whole body energy metabolism such as fatty acid oxidation and glucose control (Andersson et al. 2002). The molecular changes that occur when the CB_1 signaling pathway is blocked may inhibit the influx of intracellular fatty acids,

allowing an up regulation in expression of the genes involved in fatty acid oxidation and glucose control. It has been established that in lean muscle there are predominately two muscle fibre types: type I which is highly oxidative and type II which is less oxidative (Kelley et al. 2002). In obesity and diabetes, the ratio between the fiber types alter from that seen in skeletal muscle from lean individuals (He et al. 2001). The increased type II muscle fibres causes a decrease in fatty acid oxidation and an increase in intramyocellular fatty acids (Turner et al. 2007). This accumulation of fatty acids is thought to inhibit cell functionality resulting in cellular stress and in turn low grade inflammation (Mizunoya et al. 2013). FAT/CD36, AMPK, PDK4, PGC-1α are proteins which have been shown to be in some way modulated by the adiponectin signaling pathway (Benton et al. 2010). Investigating the mechanisms of intramyocellular pathways requires some insight into the proteins known to be regulators of cellular communication in fatty acid clearance and increased mitochondrial activity.

Adiponectin plays a role in anti-inflammatory, anti-diabetic and insulin sensitizing in obesity (Waki et al. 2004, Goto et al. 2014, Lihn et al. 2005; Bruce et al. 2005). Adiponectin concentrations in the plasma are negatively correlated with the level of adipose tissue in the body (Yang et al. 2006; Kopp et al. 2005; Bruce et al. 2005). CB_1 antagonism has however been shown to increase plasma adiponectin levels independent of weight loss (Flamment et al. 2009). Whether this increase in plasma adiponectin also results in improved adiponectin signalling in skeletal muscle is not known. There are two cell receptors known to interact with adiponectin, AdipoR1 and AdipoR2. Adiponectin must attach to these adiponectin specific receptors to initiate cellular signalling. Following this interaction with the adiponectin receptors, the first proteins known to interact with adiponectin in intracellular communication are APPL proteins. APPL1 functions as a positive mediator in the adiponectin pathway and plays a role in insulin stimulated glucose transporter signalling (Michael et al. 2001, Mao et al.

2006). APPL1 expression increases adiponectin signalling whereas suppression reduces adiponectin signalling (Wang et al. 2009). APPL2 shares up to 54% identity in protein sequences with APPL1 (Mao et al. 2006; Wang et al. 2009). APPL2 may be involved in an opposing role to APPL1 however more research into this area is required to determine the mechanisms mediated by APPL2 (Wang et al. 2009).

It is yet to be determined if chronic CB_1 antagonism influences cellular adiponectin resistance in skeletal muscle. Skeletal muscle is paramount in glucose clearance, fatty acid oxidation and energy production. The exploration into the role that the endocannabinoid system plays in adiponectin resistance in skeletal muscle will provide possible pharmacological targets for the treatment of obesity and diabetes**.** The effect of chronic treatment with the CB_1 antagonist AM251 on adiponectin signalling in skeletal muscle is not known and will be a focus of this study. Moreover, this study will look at the effects of chronic CB_1 antagonism on the fatty acid oxidative pathways in skeletal muscle. Based the evidence presented, it is hypthesised that chronic CB_1 antagonism will modulate adiponectin resistance by influencing intracellular signalling via fatty acid oxidative pathways in skeletal muscle.



4.2 Materials and Methods

4.3 Animals and Experimental Protocol

The vehicle animals used in this study are the same animals used as the control group in chapter 3. The animals underwent the same treatment, environmental and euthanasia protocol as those detailed in Chapter 3.2.1. This group of animals however were maintained on the HFD and treated daily with either vehicle control 0.9% isotonic saline solution containing 0.75% Tween 80 or 3 mg/kg of AM251 dissolved in vehicle solution via IP injection.

4.4 Muscle Sample Preparations

Following anaesthesia induced by 5% isofluorane inhalation, each animal underwent surgical removal. Each muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. Following dissection, the gastrocnemius muscle was separated into red and white muscle and snap frozen for subsequent measurement of citrate synthase activity.

Immediately after their removal longitudinal strips of the soleus and EDL were incubated in an oxygenated organ bath (37ºC pre-gassed at 95% O_2 -5% $C0_2$). Each longitudinal strip was placed in an individual chamber of Krebs-Henseleit buffer with either 0.1 µg/ml of adiponectin (AD+) or the contralateral hind limb muscle exposed to control (AD-) for a period of 30 minutes. The muscles were taken from opposing limbs from each animal to ensure that alternate muscles were exposed to AD treatment for example: rat one- right then left hind limb muscle; rat 2- left and then right limb muscle was treated with the adiponectin or control and this sequence was then switched for each subsequent rat. Following the treatment muscle samples were snap frozen and stored in liquid nitrogen for subsequent analysis.

4.5 mRNA analysis

Total RNA was isolated from the soleus and EDL muscle by using TRIzol Reagent (Invitrogen, Carlsbad, CA) as previously described (McAinch et al., 2006). In short, rodent muscle tissue extracts (approximately 15 mg) were dissociated using 1000 mg of ceramic/silica beads and RNA was extracted in TRIzol and treated with the RQ1 RNase–free DNase kit (Promega Corporations, Madison, WI). RNA concentration of 1µg/9µl total volume. First strand cDNA was then generated from 0.3 µl of template RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) using random hexamers and oligo dTs. cDNA was stored at -20 ºC for subsequent analysis. 'Real-time' PCR was conducted using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and the MyiQ™ single colour 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA). Forward and reverse oligonucleotide primers are shown in Table 4.1. 'Real-time' PCR reactions were run for 50 cycles at 95 ºC for 15 s and 60 ºC for 60 s. The PCR was normalized to housekeeping genes, GAPDH and Cyclophilin. Relative changes in mRNA abundance were quantified using the Delta Delta Ct method as previously detailed (Livak and Schmittgen, 2001) and data is reported in arbitrary units and expressed as Mean ± SEM.

Table 4.1 Oligonucleotide primers for 'real-time' PCR primers

Genes	Accession number	Forward primer	Reverse primer
Cyclophilin	NM_017101.1	CTGATGGCGAGCCCTTG	TCTGCTGTCTTTGGAACTTTGTC
GAPDH	NM_017008.3	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA
AdipoR1	NM_207587.1	TGAGGTACCAGCCAGATGTC	CGTGTCCGCTTCTCTGTTAC
AdipoR2	NM_001037979.1	TCCATGGAGTCTCAACCTG	GGAGAGTATCACAGCGCATC
APPL1	XR_007603	TCACTCCTTCCCCATCTTTC	TAGAGAGAGGGCAGCCAAAT

APPL2	NM_001108741	TGCTCGGGCTATTCACAA	AAACAGGCCCGTGACACT
FAT/CD36	NM_031561.2	GACCATCGGCGATGAGAAA	CCAGGCCCAGGAGCTTTATT
AMPK	NM_023991.1	ACTCTGCTGATGCACATGCT	AGGGGTCTTCAGGAAAGAGG
PDK4	NM_053551.1	GGGATCTCGCCTGGCACTTT	CACACATTCACGAAGCAGCA
PGC-1α	NM_013261.3	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC

GAPDH: *glyceraldehyde-3-phosphate dehydrogenase;* ***AdipoR1:*** *Adiponectin receptor 1;* ***AdipoR2:*** *Adiponectin receptor 2;* ***APPL1*** *adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1;* ***APPL2****: adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2;* ***FAT/CD36:*** *fatty acid translocase/CD36;* ***AMPK:*** *5'adenosine monophosphate-activated protein kinase a-2;* ***PDK4:*** *pyruvate dehydrogenase kinase 4;* ***PGC1a:*** *peroxisome proliferator-activated receptor gamma co-activator 1 alpha.*

4.6 Citrate Synthase

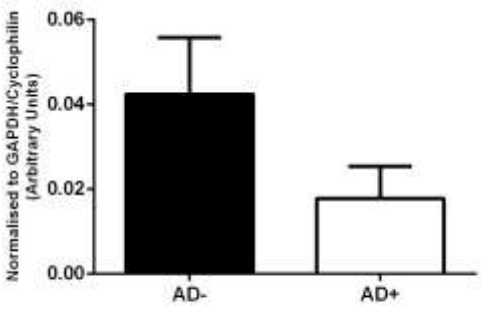
Citrate Synthase (CS) was assayed according to the protocol of Srere (1969). Briefly, after the addition of 5 µl of muscle homogenate (0.175 KCl & 2mM EDTA, PH-7.4), 230 µl of reagent cocktail [3mM Acetyl CoA (Sigma A-2056), 100 mM Tris buffer (BioRad) 161-0719; pH 8.3), 1 mM DTNB (Sigma D-8130)] and 15 µl of 10 mM Oxalacetate (Sigma 0-4126). CS activity was measured at room temperature by recording the increases in the colorimetric compound (5, 5-dithiobis-2-nitrobenzoate), which is linked to citrate production, at 412 nm (xMark™ Microplate Spectrophotometer, BioRad). Readings of CS activity were recorded every 15 seconds for 3 minutes. All enzyme activity was expressed relative to wet weight (Betik et al. 2008). The change in absorbance/min was calculated to measure CS activity. All muscle samples were measured in duplicates, and the average activity used to characterize CS activity.

4.7 Statistical Analysis

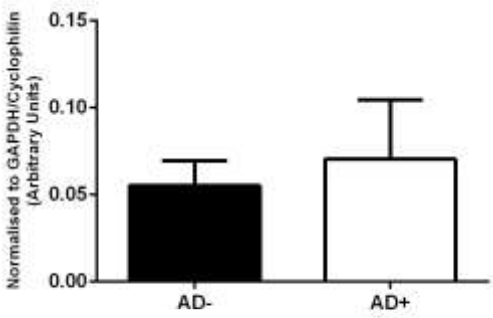
SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group direct analysis or mixed model ANOVA with Tukey's post-hoc tests. * Significance was accepted when $p < 0.05$.

4.8 Results

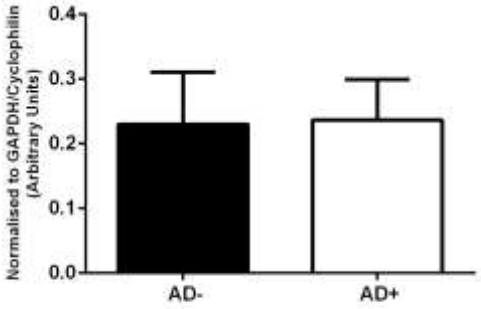
mRNA expressions of intramyocellular genes known to modulate adiponectin in skeletal muscle fibre types of DIO rats, chronically treated with CB_1 antagonist AM251 and acutely exposed to adiponectin organ bath treatment.



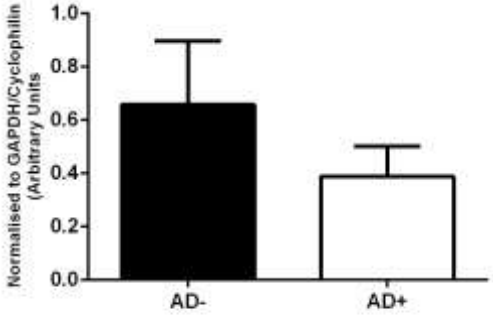
A; AdipoR1



B; AdipoR1

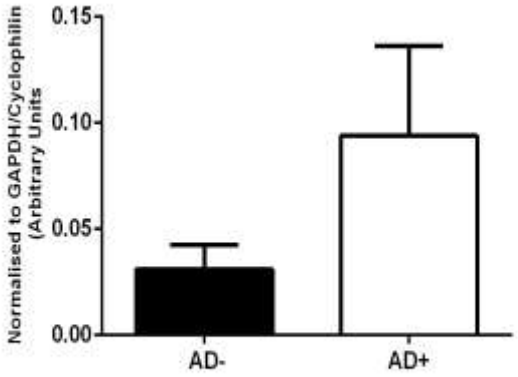


C; AdipoR2

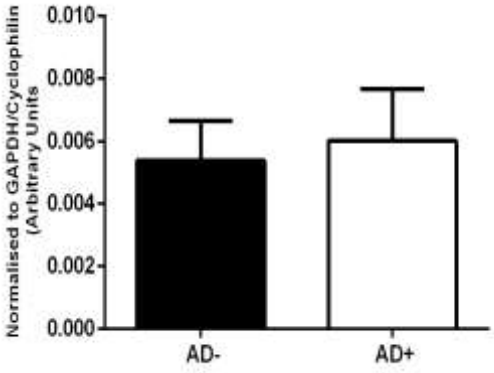


D; AdipoR2

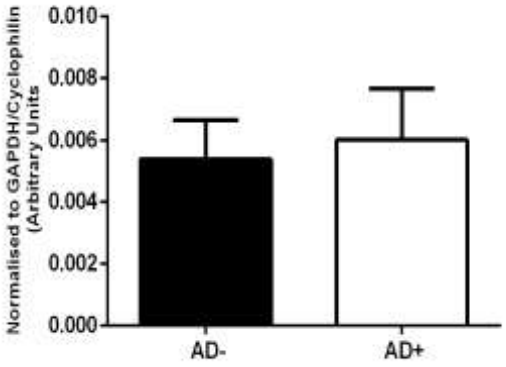
Figure 4.1.A *the gene expression of AdipoR1 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***B*** *the effects of AdipoR1gene expression the EDL following adiponectin exposure.* ***C*** *Gene expression of AdipoR2 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***D*** *the effects of adiponection on AdipoR2 gene expression in the EDL. Muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks then treated for 6 weeks with AM251. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*



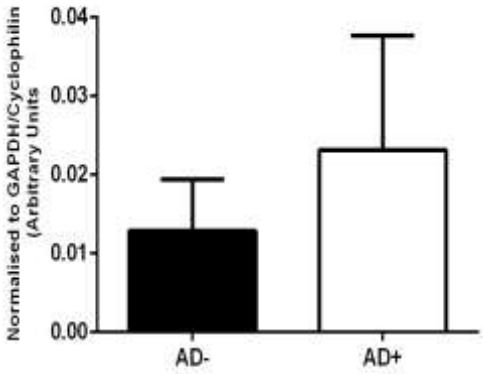
E; APPL1



F; APPL1



G; APPL2



H; APPL2

Figure 4.1.E *Gene expression of APPL1 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***F*** *the effects of APPL1 gene expression the EDL following adiponectin exposure.* ***G*** *Gene expression of APPL2 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***H*** *the effects of APPL2 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks then treated for 6 weeks with AM251. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*

The mRNA gene expression of muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in no difference in AdipoR1 mRNA expression in the soleus (Figure 4.1.A, p=0.06) or EDL (Figure 4.1.B, p=0.32) between the control (AD-) and adiponectin exposed (AD+) muscle of the DIO control animals. AdipoR2 mRNA expression did not alter between the control AD- and control AD+ Soleus (Figure 4.1.C, p=0.07) and EDL (Figure 4.1.D, p=0.35). The mRNA gene expression of muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in not change in APPL1 in the soleus AD- control (Figure 4.1.E, p=0.07) and EDL (Figure 4.1.F, p=0.35). Similarly, no change was seen in the controls of APPL2 prior to treatment in the soleus (Figure 4.1.G, p=0.36) and EDL (Figure 4.1.H, p=0.21) compared to obese controls (n = 9).

mRNA Expression of adiponectin receptors and downstream adapter proteins in skeletal muscle fiber types, the effects on DIO rats chronically treated with CB_1 antagonist AM251 following acute exposure to adiponectin.

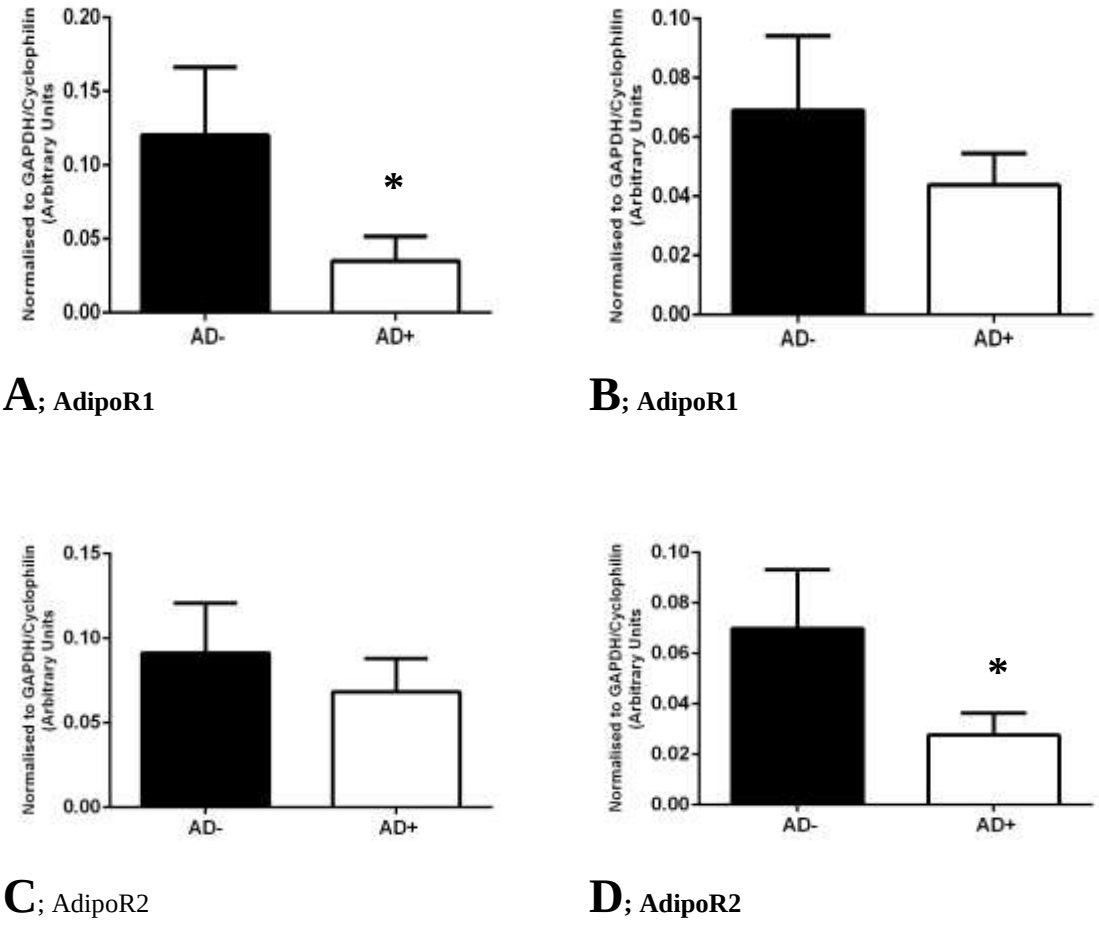
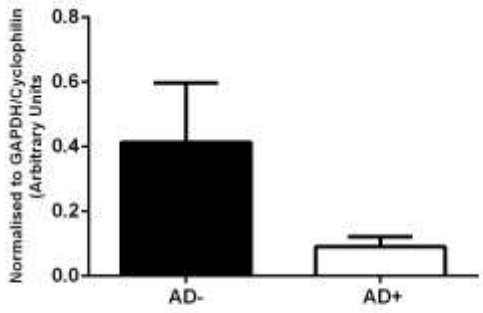
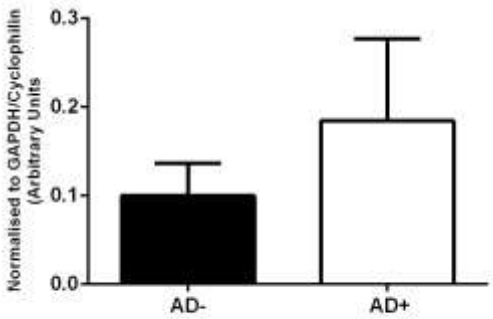


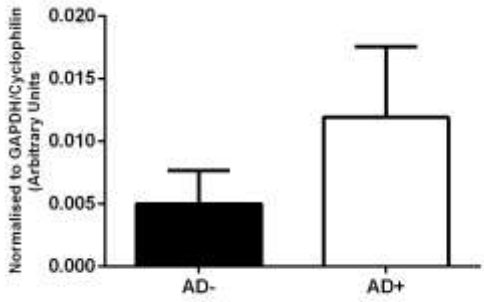
Figure 4.2.A *Gene expression of AdipoR1 exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***B*** *the effects of AdipoR1 gene expression the EDL following adiponectin exposure.* ***C*** *Gene expression of AdipoR2 following exposer to adiponectin negative or adiponectin positive in the soleus muscle.* ***D*** *the effects of AdipoR2 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks then treated for 6 weeks with AM251. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*



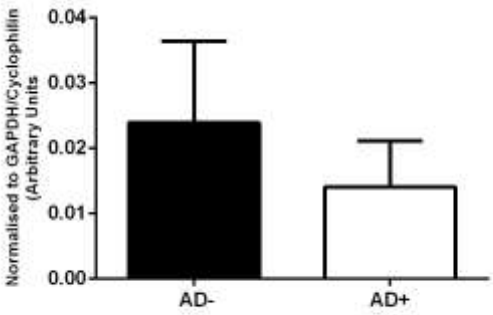
E; APPL1



F; APPL1



G; APPL2

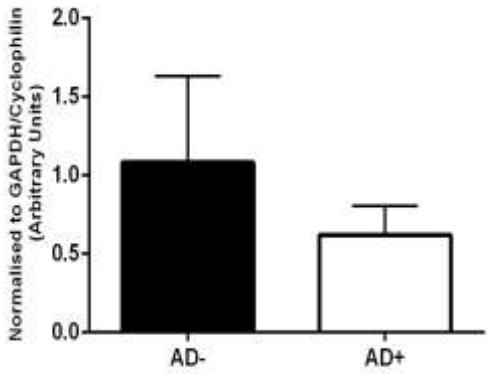


H; APPL2

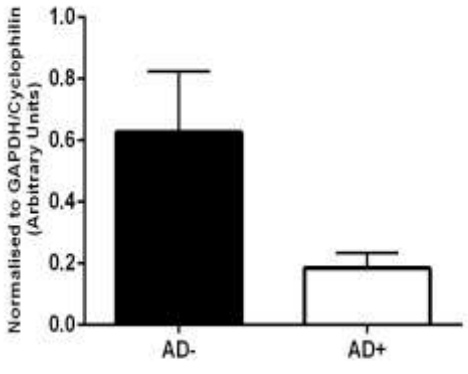
Figure 4.2.E *Gene expression of APPL1 exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***F*** *the effects oAPPL1 gene expression the EDL following adiponectin exposure.* ***G*** *Gene expression of APPL2 following exposer to adiponectin negative or adiponectin positive in the soleus muscle.* ***H*** *the effects of APPL2 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks then treated for 6 weeks with AM251. Data* ***(****n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*

The mRNA gene expression of muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in no difference in a significant decrease in AdipoR1 in the soleus muscle (Figure 4.2.A, $p<0.05$) but not in the EDL (Figure 4.2.B, $p=0.32$). No change was seen in AdipoR2 in the soleus muscle (Figure 4.2.C, $p=0.36$) in the EDL (Figure 4.2.D, $p=0.01$) there was a significant decrease. The mRNA gene expression of muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in no change in APPL1 in APPL1 mRNA expression in the soleus (Figure 4.2.E, $p=0.21$) or EDL (Figure 4.2.F, $p=0.36$). No change was recorded in APPL2 mRNA expression in either the soleus (Figure 4.2.G, $p=0.23$) or EDL (Figure 4.2.H, $p=0.36$).

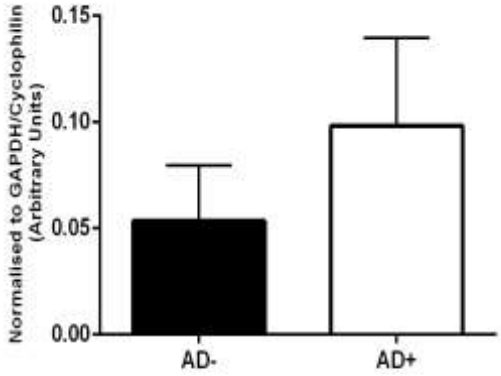
mRNA Expression of genes involved in fatty acid oxidation and adiponectin modulation in skeletal muscle fiber types, the effects on DIO rats chronically treated with CB_1 antagonist AM251 following acute exposer to adiponectin.



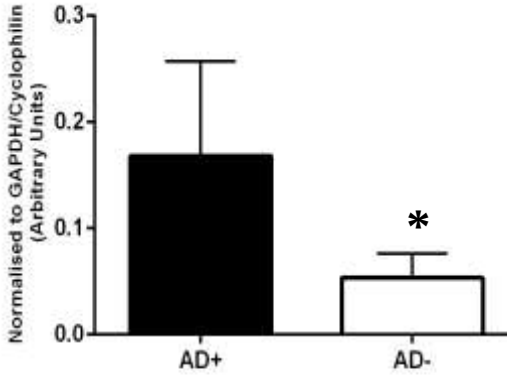
A; FAT/CD36



B; FAT/CD36

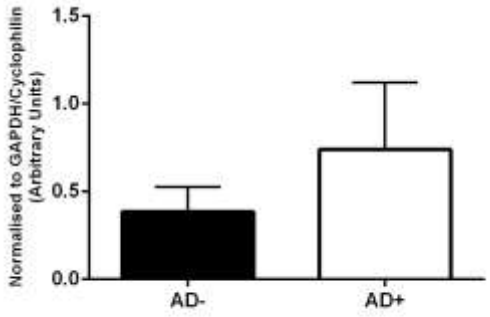


C; AMPK

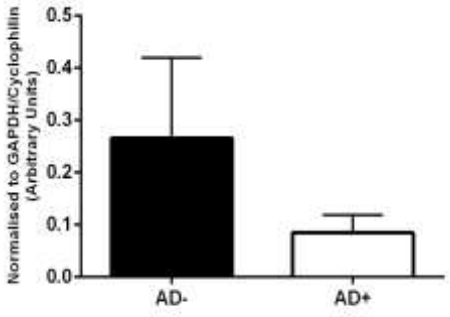


D; AMPK

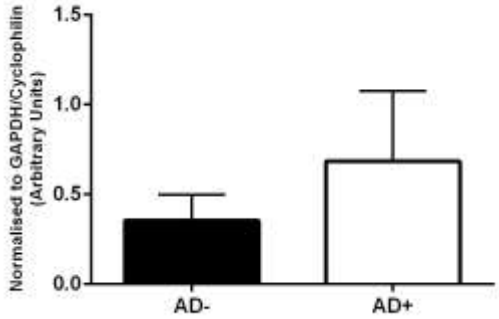
Figure 4.3.A *Gene expression of FAT/CD36 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***B*** *the effects of FAT/CD36 gene expression the EDL following adiponectin exposure.* ***C*** *Gene expression of AMPK control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***D*** *the effects of AMPK gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks then treated for 6 weeks with AM251. Data* ***(****n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*



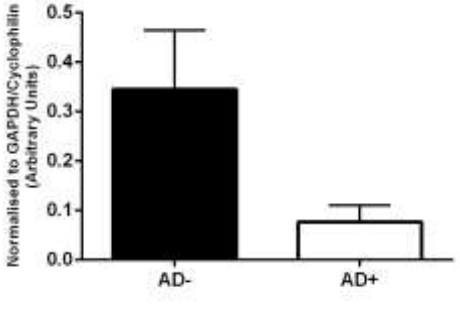
E; PDK4



F; PDK4



G; PGC1-α

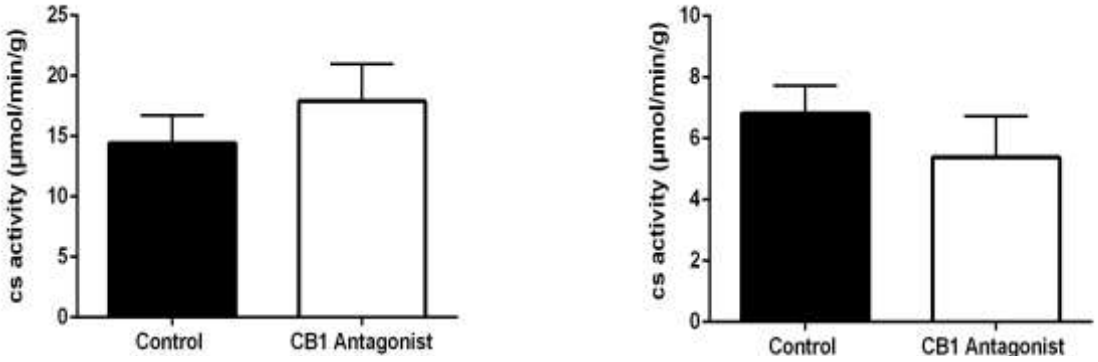


H; PGC1-α

Figure 4.3.E *Gene expression of PDK4 exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***F*** *the effects of PDK4 gene expression the EDL following adiponectin exposure.* ***G*** *Gene expression of PGC1-α following exposer to adiponectin negative or adiponectin positive in the soleus muscle.* ***H*** *the effects of PGC1-α gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks then treated for 6 weeks with AM251. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*

The mRNA gene expression of muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in no difference in FAT/CD36 soleus ($p=0.15$) or EDL ($p=0.32$) mRNA expression between the control AD- and control AD+. For AMPK, no change was seen in the soleus ($p=0.06$), however levels significantly decreased in EDL ($p=0.03$). The mRNA gene expression of muscle tissue exposed to 0.1ug/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in no change in PDK4 mRNA expression between the control AD- and AD+ Soleus ($p=0.10$) and EDL ($p=0.41$). There was no change detected in PGC-1α gene expression in the soleus ($p=0.23$) or the EDL (0.50) compared to obese controls ($p < 0.05$, $n = 9$).

Citrate Synthase Activity



A; Red Gastrocnemius **B; White Gastrocnemuis**

Figure 4.4.A *Citrate synthase activity in red gastrocnemius muscle of the control animals and animals chronically administered with CB_1 antagonist AM251.* ***B*** *Citrate synthase expression in white gastrocnemius muscle of animals chronically administered with CB_1 antagonist.*

The measured concentration of citrate synthase in the red and white gastrocnemius muscle following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks resulted in no change in the red gastrocnemius (Figure 4.4.A, $p=0.36$) or white gastrocnemius skeletal muscle (Figure 4.4.B, $p=0.08$) in animals chronically administered with CB_1 antagonist AM251.

4.9 Discussion

CB_1 antagonist treatment has been shown to induce weight loss and reduce the amount and size of body fat stores (Bensaid et al. 2003; Yang et al. 2005). These results have been replicated in the animals in this thesis (*see chapter 3*). There was a transient decrease in food intake (*For results see chapter 3, Section: 3.3*) however it appears the changes in body composition occurred independently of nutrient intake. To elucidate if the weight-loss occurred through changes in fatty acid oxidation which further resulted in the modulation of adiponectin in obesity, the plasma levels of adiponectin were tested. Adiponectin is known to increase following CB_1 antagonism in human studies (Despres et al. 2006). While this did not occur in the obese animals administered AM251 (chapter 3) it remains unclear in both human and animal work whether CB_1 antagonism improves adiponectin signalling. The animals in this study following chronic CB_1 antagonism exhibited fat loss however there was no increase in the circulating adiponectin levels (Chapter 3). Therefore, although the results in this study provide valuable evidence of the obese physiological state it may not be a true representation of the modulating effects of circulating adiponectin levels that would usually occur in a DIO model (Poirier et al. 2004).

The results of chronic blockade of CB_1 with AM251 in the skeletal muscle resulted in no change to citrate synthase. The ex-vivo treatment with adiponectin (organ bath treatment) resulted in decreased expression of AMPK in EDL muscle following adiponectin treatment. AdipoR1 mRNA expression was decreased in the soleus muscle when exposed to adiponectin, AdipoR2 was also significantly decreased in the EDL muscle as was the expression of PGC1-α, following acute exposure of the skeletal muscle tissue to adiponectin treatment in an organ bath, to mimic an *In-vivo* physical environment.

The optimal exposure time and concentrations for specific treatments to measure changes in mRNA expression are yet to be determined. For example, following organ bath exposure, adipocytes from obese humans exposed to insulin for two hours became unresponsive to the effect of insulin, exhibiting self-induced insulin resistance. The concentration of insulin had to be increased at 2h exposure time to have an effect (Stevens et al. 2009). At the onset of adiponectin resistance there is an increase in the circulating levels of adiponectin. The biological changes that occur in adiponectin signaling appear to have a negative feedback effect; adaptive changes caused by the exposure to increased adiponectin results in a potential 'saturation' within the cell. This then initiates a down regulation in receptors causing tissue resistance. Whether this resistance contributes to an increase in the inflammation that is known to occur in obesity is yet to be determined.

AdipoR1 and AdipoR2 damage by inflammation decreases adiponectin binding and reduces its functional mechanisms (Yamauchi et al. 2007). However, the downregulation of AdipoR1 mRNA expression but not AdipoR2 was detected following AM251 administration in a DIO rat model for 14 days (Crespillo et al. 2011). Levels of circulating inflammatory markers of the animals in this study by Crespillo et al., (2011) were not tested. The results in the current study (Chapter 3) showed an increase in circulating inflammation markers in the plasma of the current obese rat model. Therefore, when interpreting the ex vivo results of the mRNA gene changes following acute exposure to adiponectin in the skeletal muscle of these animals, the effect of the biological changes that occur in inflammation should be considered.

The mRNA expression of AdipoR1 is down regulated in primary skeletal muscle myotubes collected from individuals with chronic heart failure (CHF) a condition associated with adiponectin resistance (Sente et al. 2016). Moreover, when exposed to TNF-α myotubes

displayed evidence of a down regulation in adiponectin signalling, myogenesis and mitochondrial biogenesis (Sente et al. 2016). The results in the present study recorded a down regulation of AdipoR1 in the highly oxidative soleus muscle with acute exposure to adiponectin. As these animals had increased levels of circulating TNF-α and other inflammatory markers (see chapter 3), it may be that the down regulation of AdipoR1 gene expression is a result of the biological alterations that have occurred in the presence of these cytokines. The acute exposure to adiponectin in this study is done to replicate the conditions of adiponectin resistance in skeletal muscle. Although these results provide evidence that an increase in pro-inflammatory cytokines such as TNF-α may cause a down regulation of AdipoR1 in an ex-vivo DIO model following chronic AM251 treatment. Further studies are needed to fully elucidate the relationship between CB_1 antagonism and adiponectin resistance in skeletal muscle.

To fully assess if acute adiponectin exposure in the skeletal muscle of DIO animals treated chronically with a CB_1 antagonist altered the fatty acid oxidative potential of the muscle, the mRNA expression of key proteins involved in fatty acid oxidation was measured. The mRNA expression of FATCD/36 and PDK4 in this study did not alter. The AMPK mRNA expression in the CB_1 antagonist group however was significantly decreased in EDL following acute exposure to adiponectin. AMPK consists of heterotrimeric complexes of α-subunit and regulatory β- and γ-subunits (α1, α2, β1, β2, γ1, γ2 and γ3). Skeletal muscle is known to express all subunits of AMPK (Johansson et al. 2004). Phosphorylation occurs via upstream kinase and increased cellular ATP concentrations (Hawley et al. 1995, Davies et al. 1995). The activation of AMPK has favorable results in glucose resistant skeletal muscle (Fryer et al. 2002). AMPK is activated in muscle by adiponectin (Yamauchi et al. 2012) and leptin (Minokoshi et al. 2002).

Fatty acid oxidative capacity in skeletal muscle is reduced in obesity (Gaster et al. 2004), and these effects may be related to a reduction in skeletal muscle AMPK activity (Bandyopadhyay et al., 2006). Increased inflammatory markers such as TNF-α contribute to adiponectin resistance in skeletal muscle, resulting in a down regulation of differentiation, growth, and function (Sente et al. 2016). Chronic CB_1 antagonism caused an increase in inflammation (*see chapter 3*) when compared to high fat controls, despite having no changes in adiponectin. The decrease in AMPK in this study is a different outcome to AM251 administration in C2C12 cell culture model where mRNA expression of AMPK increased (Cavuoto et al. 2006). However, in a cell culture model, results are reflective of mechanisms that occur without the influences of environmental factors such as circulating hormones eg. leptin that may influence the outcome. Leptin levels in the CB_1 antagonist group in this study were significantly decreased (*see Chapter 3*). Leptin suppresses the activity of ACC, thereby stimulating the oxidation of fatty acids in skeletal muscle. Blocking AMPK inhibits the phosphorylation of ACC stimulated by leptin thereby decreasing oxidation of fatty acids (Minokoshi et al. 2002). Moreover, an increase in levels of TNF-α induces insulin resistance in skeletal muscle by down regulating AMPK (Steinberg et al. 2002). CB_1 antagonism in this study resulted in an increase in circulating TNF-α which may explain the decrease in AMPK mRNA expression in response to the adiponectin treatment.

Further results revieled that the mRNA expression of PGC-1α was reduced in the EDL after CB_1 antagonism.This decrease is in conflict of what others have found to be beneficial findings in the skeletal muscle, for example in lean and insulin-resistant obese Zucker rats, PGC-1α improved lipid utilization, insulin signaling and glucose transport (Benton et al. 2010). In skeletal muscle, the direct phosphorylation of PGC-1α has been identified to be initiated by

AMPK (Jager et al. 2007). As AMPK levels were decreased in the EDL of the animals in this study it is an expected finding that PGC-1α levels were also reduced following acute adiponectin exposure. In isolated muscle cells, PGC-1α has been found to reduce local inflammation by suppressing pro-inflammatory cytokine interleukin 12 (IL-12) (Eisele et al. 2015).

PGC-1α is down regulated in obesity and diabetes (Mootha et al. 2003) with expression decreasing as a result of a high fat diet (Koves et al. 2005). Despite the relationship between PGC-1α and highly oxidative muscle fibre types (Koves et al. 2005), changes in PGC-1α in this study occurred in the low oxidative fibre types only. This is interesting because an increase in low oxidative fibre types is associated with the obese state (Mizunoya et al. 2013). These data are in line with previous findings that support a relationship between PGC-1α expression and CB modulation in a DIO model of obesity (Cavuoto et al., 2007). Discovering if these changes occurred as a result of a down regulation of AMPK or as an independent target will give a better understanding of CB modulation and its role in oxidative metabolism in skeletal muscle.

It is not known if the down regulation of adiponectin receptors, AMPK, or PGC-1α mRNA expression was influenced by the inflammation experienced in vivo, or in the ex vivo environment following acute exposure to adiponectin. On the other hand, it gives further evidence to support a relationship between CB_1 modulation and adiponectin resistance associated with increases in inflammation.

4.10 Conclusion

In summary adiponectin assists the body to produce intracellular energy at a rate that is commensurate with energy intake. In obesity and diabetes, the levels of adiponectin decline and skeletal muscle characteristics alter, with the amount of the less oxidative muscle fibre type increasing. CB_1 antagonism has been found to strongly influence weight loss and metabolism. In this study, chronic blockade of CB_1 with AM251 resulted in a decrease in the detected mRNA expression of AdipoR1 in the soleus muscle when it was exposed to adiponectin. In the EDL muscle however following acute exposure to adiponectin, it was detected that there was a significant decrease in the detected amounts of AdipoR2 expression. Similarly the levels of PGC-1α detected had decreased in the skeletal muscle following acute adiponectin exposure. These data highlight the need for further research to be undertaken to fully elucidate if skeletal muscle adaption is the result of inflammation and if these adaptions then affect the functionality of adiponectin receptors and the intramyocellular signaling targets: if this damage can be improved through CB_1 antagonism, this would allow new avenues for pharmacological targeting of obesity.

Chapter 5 – The effects of chronic CB_2 agonism and antagonism on plasma hormone and cytokine markers in obesity.

5.0 Abstract

The effects of chronic CB_2 modulation metabolic markers in a DIO model have not yet been fully established. Therefore, the aim of this study was to characterise the effects of chronic treatment with the CB_2 agonist AM1241 or the CB_2 antagonist AM630 on physiological and plasma markers of obesity and circulating plasma cytokine levels in a model of DIO. Male Sprague–Dawley rats were fed a high-fat diet (HFD: 40% digestible energy from lipids) for 9 weeks to elicit DIO. After 9 weeks consuming a HFD, rats were injected daily for 6 weeks with either 3mg/kg of the CB_2 agonist AM1241 or 0.3 mg/kg of the CB_2 antagonist AM630 (n=9) or saline via IP injection (n=9) while continuing to consume the HFD. Chronic modulation of CB_2 in a DIO model via CB_2 agonist or antagonist treatment resulted in no change in weight, food intake or body fat percentage. Despite the lack of changes seen in body weight and body fat percentage, CB_2 agonism decreased epididymal, peri-renal and brown fat pad mass and increased plasma levels of the inflammatory marker IL-12p70. CB_2 antagonism also decreased brown fat pad mass and decreased the plasma levels of the metabolic hormones, ghrelin and leptin.Further research is required to understand the effect of this modulation on specific tissues in a DIO model.

5.1 Introduction

In 2014 the World Health Organisation reported that there were more than 600 million obese individuals on the planet, 6.8 percent of these were children under the age of five (World Health

statistics 2014). The rate of increase is 30% faster in poorer and non-affluent countries than in developed countries (World Health statistics 2014). The current rate of death from being overweight or obese is reported to be 8 million people annually (WHO, 2010). The burgeoning universal problem of obesity and the consumption of a high fat diet are associated with insulin resistance and chronic inflammation, causing abnormal cytokine production (Arkan et al. 2005, Valerio et al. 2006).

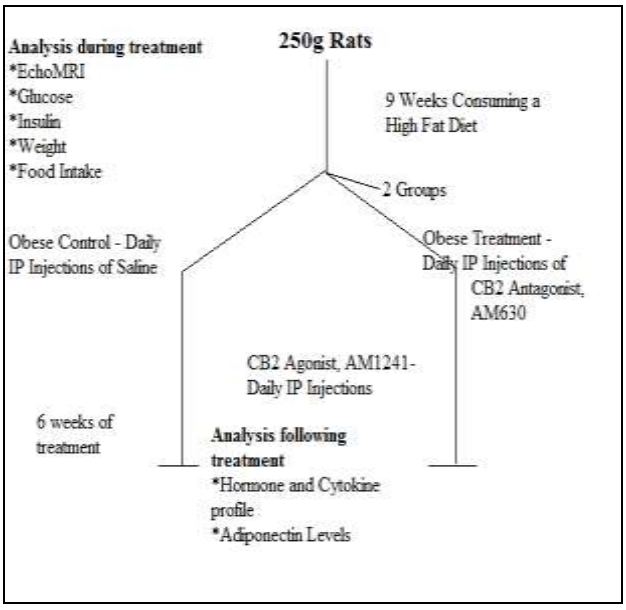
The endocannabinoid system plays a role in the regulation of metabolism and inflammation with modulation of this system having substantial biological effects on obesity (Pagotto et al. 2012, Ziring et al. 2006). The two main recognized cannabinoid receptors are known as CB_1 and CB_2, with CB_2 expressed in numerous tissues such as skeletal muscle, white and brown adipose tissue, bone marrow, kidneys, brain, spleen, thymus, leukocytes and cells involved in immune system (Cavuoto et al. 2007, Jenkin et al. 2013, Persidsky et al. 2015, Krott et al. 2006, Xie et al. 2016, Sugiura et al. 2002).

CB_2 function has previously been mainly associated with alterations in the immune system and pain research (Bermudez-Silva et al. 2007). To date, research investigating the impact on metabolism of chronic CB_2 modulation in obesity is not well-studied. As a receptor, CB_2 is a single chained polypeptide that contains seven transmembrane α-helices, with an extracellular glycosylated N-terminus and an intracellular C-terminus (Cabral et al. 2009). In CB_2 knockout mice it has been shown that their immune response is down regulated (Ziring et al. 2006; Ostanin et al. 2005; Elmes et al. 2004).

Cytokines are involved in a variety of cell inflammation and immune responses (Ziring et al. 2006). They are responsible for cell maintenance and the regulation of various communications within a cell (Valerio et al. 2006). Pro-inflammatory cytokine levels significantly increase in

the obese state (Spyridaki et al. 2014, Ziring et al. 2006, Bastard et al. 2000). As such, obesity is associated with low grade inflammation (Souza et al. 2005, Spyridaki et al. 2014).

Pro-inflammatory cytokines are involved in the induction of an immune response. Anti-inflammatory cytokines are involved in inhibiting the actions of inflammation. Chronic inflammatory cytokine production decreases insulin sensitivity (Arkan et al. 2005, Valerio et al. 2006). Similarly, obesity influences metabolic hormones such as leptin and ghrelin which are known to play a role in hunger, fat storage and food intake (Pasquali et al. 1991, Lustig et al. 2004). The modulating effects of chronic CB_2 agonist or CB_2 antagonist treatment in a DIO model have yet to be established. As such the aim of this study is to determine the effect of chronic CB_2 agonism and CB_2 antagonism in a DIO rodent model on circulating plasma hormone and cytokines. While there is a yet to determined relationship between obesity induced inflammation and CB_2 function, it is hypothesised that activating CB_2 receptors via agonist administration will increase plasma hormone and cytokine markers in an obese model. In contrast blocking CB_2 receptors will result in a decrease in plasma hormone and cytokine markers in an obese model.



5.2 Materials and Methods

5.3 Animals and Experimental Protocol

Seven-week old male Sprague Dawley rats were individually housed in an environmentally controlled laboratory (ambient temperature 22-24 °C) with a 12-hour light/ dark cycle (7:00 - 19:00). Rats were fed a HFD ad libitum (containing 21% fat by weight, sourced from Specialty Feeds, Glen Forrest, Australia) for nine weeks to induce obesity. Obesity was determined when the treatment animals were compared to chow fed only animals at week 9; there was significant weight gain between the high fat and the chow treatment groups (*data not shown*). Rats were then matched using several physiological parameters and put into either obese control or CB_2 agonist or antagonist treatment groups (n = 9 per group). For six weeks, rats were maintained on the HFD and treated daily, via IP injection, with either vehicle control 0.9% isotonic saline solution containing 0.75% Tween 80, 3 mg/kg of AM1241 or 0.3 mg/kg AM630 dissolved in vehicle solution. Following the conclusion of the experimental protocol, rats were deeply anesthetized and killed via cardiac puncture. Organs were removed, weighed and stored for further analysis, as outlined in Section 2.6. Experimental procedures were approved by the Howard Florey Animal Ethics Committee (AEC 11-036).

5.4 Biological Measurements

Rat weight and food was recorded daily throughout the experimental period. An IPGTT (intraperitoneal glucose tolerance test) and IPIST (intraperitoneal insulin sensitivity test) were performed to determine the effects on whole body glucose homeostasis and was measured a week prior to drug treatment (week nine) and a week prior to euthanizing of the animal (week

15). The IPGTT was performed at the start of week nine and week fifteen. The IPIST was performed at the end of week nine so that there was less impact of stress on the animal.

5.5 Hormone and Cytokine assay

Cardiac blood was extracted at the time of death using an EDTA vacutainer system. Samples were immediately spun at 4000 RCF at 4 °C for 10 minutes. Plasma was then aspirated and checked for turbidity. The plasma was then stored at –80 °C. Prior to analysis, samples were thawed and spun at 1792 RCF at 4 °C for 10 minutes and 25 µl of plasma removed for analysis using the Diabetes 4-Plex panel and the rat cytokine 23-Plex panel multiplex protein arrays (Biorad, Biorad Laboratories GmgH, Munich, Germany), enabling the quantification of all parameters across a single sample. The Diabetes 4-Plex Panel contained: 1. Ghrelin, 2. Leptin, 3. GLP-1 (*Glucagon-like peptide-1*), 4. PAI-1 (*Plasminogen activator inhibitor-1*) (Biorad, Biorad Laboratories GmgH, Munich). The Cytokine Panel – The Rat Cytokine 23-Plex panel kit contained: 1. EPO (*Erythropoietin*), 2. G-CSF (*Granulocyte Colony Stimulating Factor*), 3. GM-CSF (*Granulocyte Macrophage Colony Stimulating Factor*), 4. GRO/KC (*Growth-related oncogene*), 5. IFN-γ (*Interferon Gamma*), 6. IL-α and IL-β (*Interleukin 1- alpha and beta*), 7. IL-2 (*Interleukin 2*), 8. IL-4 (*Interleukin 4*), 9. IL-5 (*Interleukin 5*), 10. IL-6 (*Interleukin 6*), 11. IL-7 (Interleukin 7), 12. IL-10 (*Interleukin 10*), 13. IL-12p70 (*Interleukin 12*), 14. IL-13 (Interleukin 13), 15. IL-17α (*Interleukin 17*), 16. IL-18 (*Interleukin 18*), 17. M-CSF (*Macrophage colony-stimulating factor*), 18. MCP-1 (*Monocyte Chemotactic Protein 1*), 19. MIP-1α (*Macrophage Inflammatory Protein 1α*), 20. MIP-3 (*Macrophage Inflammatory Protein 3α*), 21. RANTES *(Chemokine (C-C motif) ligand 5*), 22. TNF-α (*Tumor Necrosis Factor Alpha*), 23. VEGF (*Vascular Endothelial Growth Factor*) (Biorad, Biorad Laboratories GmgH, Munich, Germany).Germany).

5.6 Adiponectin

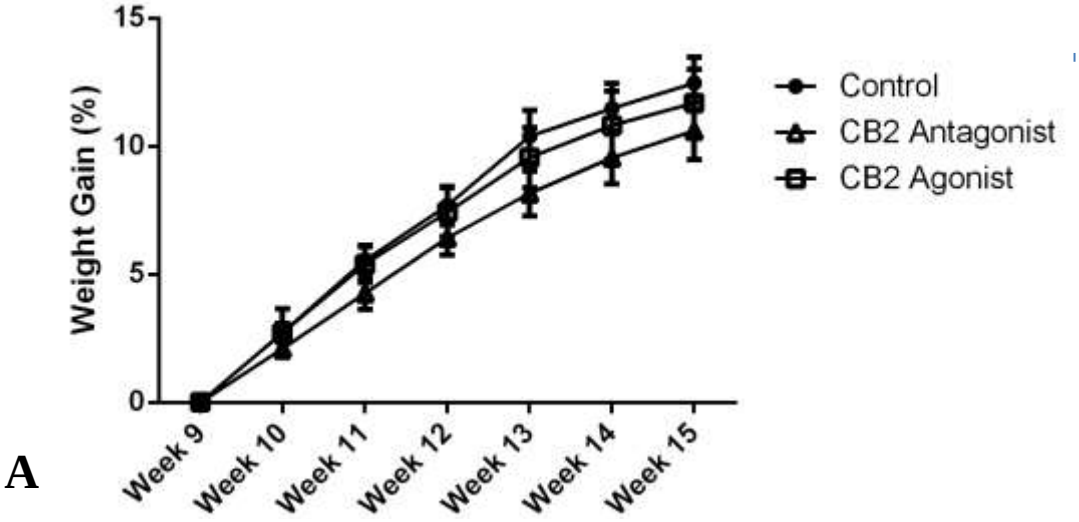
Cardiac blood was extracted and stored as detailed above in section 3.2.3. Plasma samples were thawed on ice and plasma adiponectin was analysed using an enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical, Tokushima, Japan). The kit measures total adiponectin amount including all native isoforms (high, medium and low-molecular-weight-adiponectin). The test was carried out following the manufactures instructions; absorbance was read using a microtitreplate reader at 450 nm.

5.7 Statistical Analysis

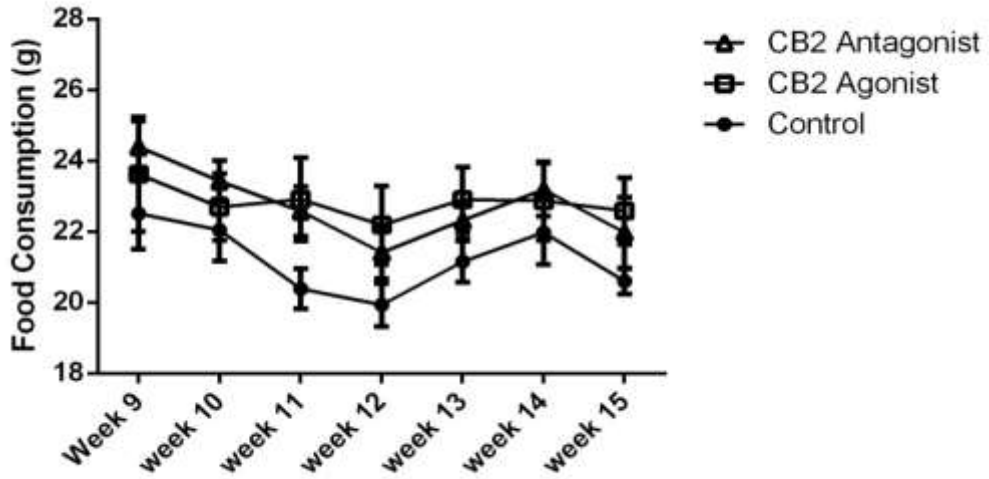
SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group direct analysis or mixed model ANOVA with Tukey's post-hoc tests for analysis of pre-treatment and post-treatment measurements between obese control, AM1241 and AM630 treated groups. Significance was accepted when $p < 0.05$.

5.8 Results

Obesity Markers

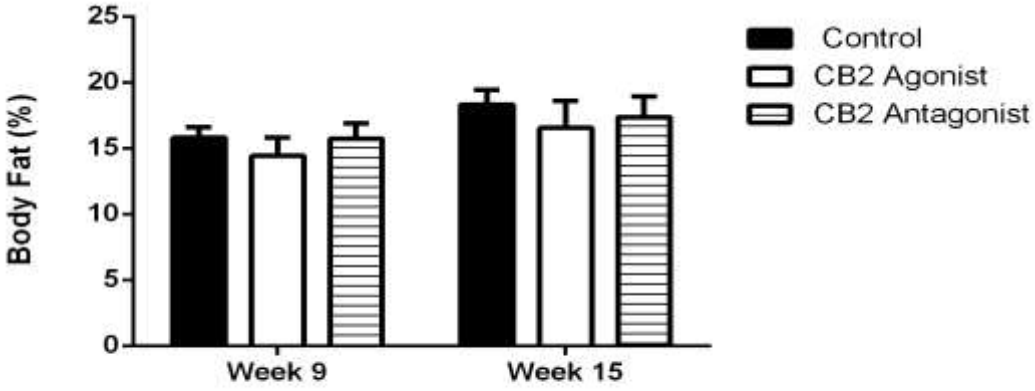


A

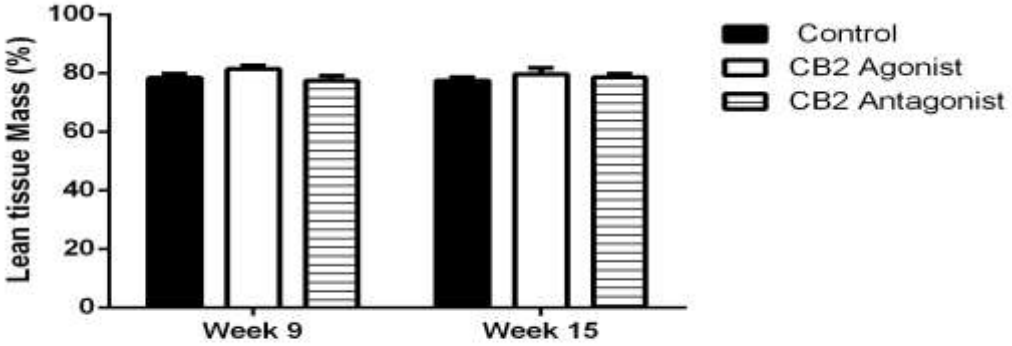


B

Figure 5.1.A *Percentage weight gain following 9 weeks of HF diet, prior to treatment for six weeks with either CB_2 Agonist AM1241 or CB_2 Antagonist AM630 compared to obese controls.* ***B*** *Percentage weight gain compared to weight following 9 weeks and treated with CB_2 Agonist AM1241 or CB2 Antagonist AM630 for six weeks compared to obese controls. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*



A

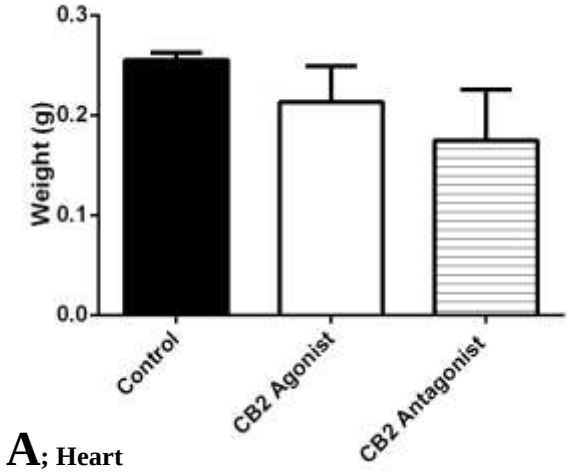


B

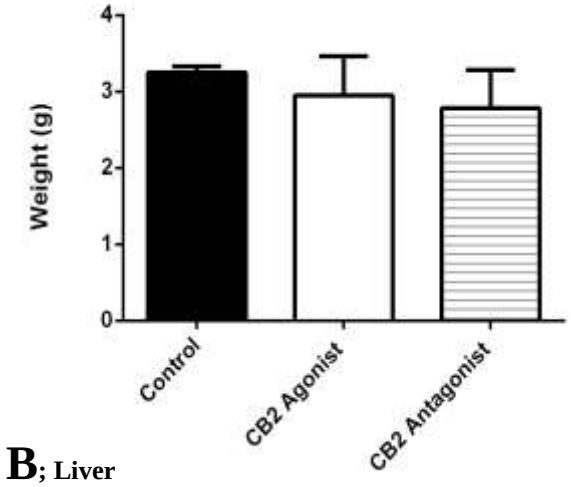
Figure 5.2.A *Percentage weight gain during 6 weeks of treatment with either CB_2 Agonist AM1241 or CB_2 Antagonist AM630 following 9 weeks of HF diet.* ***B*** *Percentage body fat in obese rats fed a HF diet for 9 weeks and treated with AM251 (CB1 Antagonist) for six weeks compared to DIO obese controls. Figure 3.2.B Displays the change in lean tissue mass that occurred during six-week treatment of CB_2 Agonist AM251 or CB_2 Antagonist AM630 (n=9).*

No difference in weight gain was detected throughout the six-week treatment period (Figure 5.1.A). Food consumption (g/day) was also not different between the obese CB_2 agonist, AM1241, or CB_2 antagonist, AM630, treated groups and the obese control group (*Figure 5.1.B*). There was no difference in percentage body fat (Figure 5.2.A, p=0.71) or lean tissue changes (Figure 5.2.B, p=0.33) detected following Echo MRI at week 9 and week 15 in either the AM1241 or AM630 treatment group compared to the obese vehicle control animals.

Organ weight



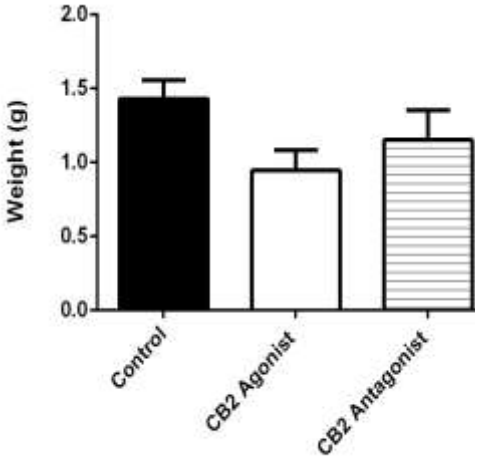
A; Heart



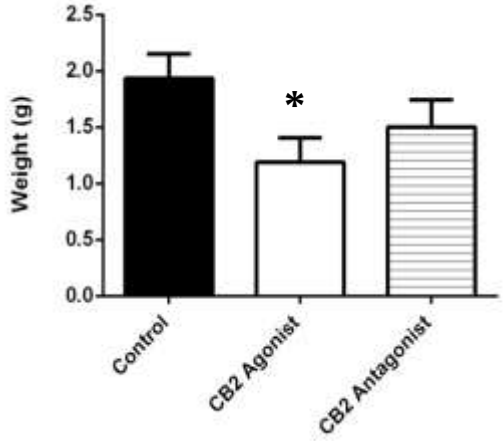
B; Liver

Figure 5.3.A *Heart weight (g) following treatment of obese rats fed a HF diet for 9 weeks and treated with either the CB_2 AM1241 agonist or CB_2 AM630 antagonist for six weeks, compared to obese controls.* ***B*** *Indicates the Liver weight (g) of obese rats fed a HF diet for 9 weeks and treated with either CB_2 AM1241 agonist or CB_2 AM630 antagonist for six weeks compared to control.*

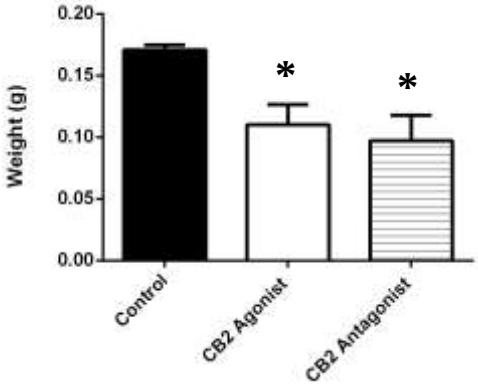
Fat Pad Weights



A; Epididymal Fat



B; Peri-Renal Fat Pad

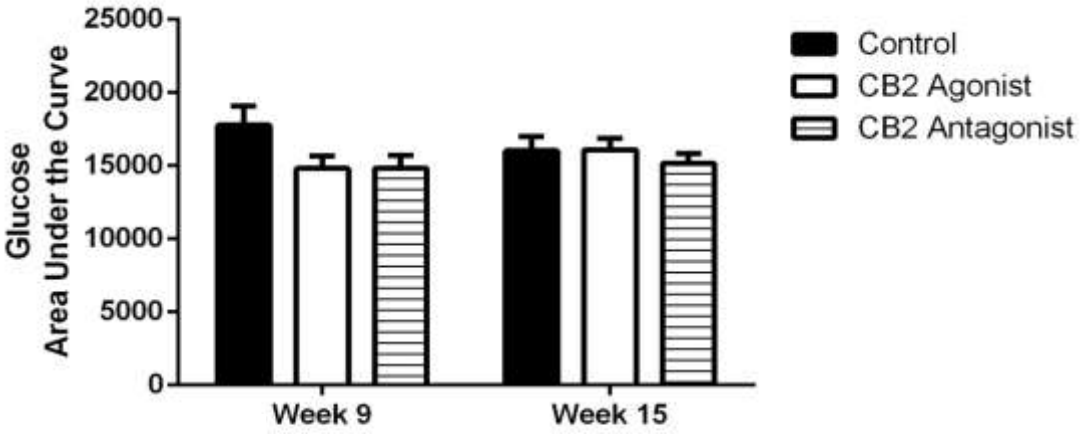


C; Brown Fat Pad

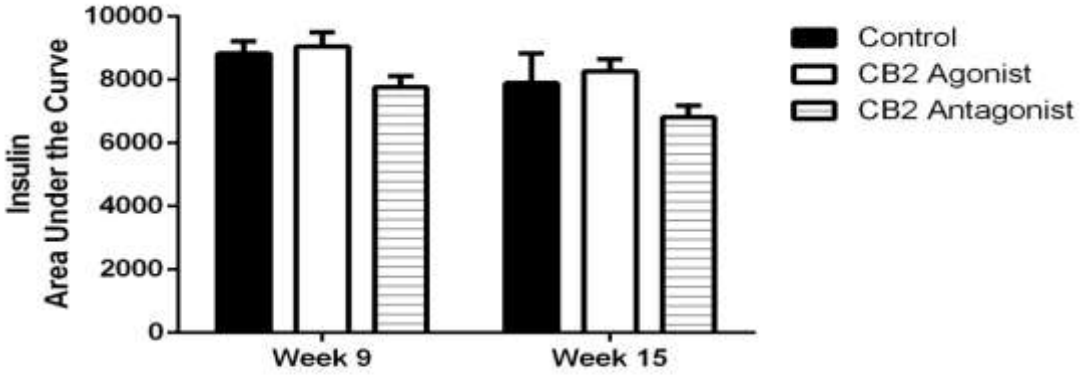
Figure 5.4.A *Epididymal Fat Pad (g) at death of obese rats fed a HF diet for 9 weeks and treated with CB_2 agonist AM1241 or CB_2 antagonist AM630 for six weeks, compared to obese control.* ***B****. Peri-Renal Fat Pad (g) at death of obese rats fed a HF diet for 9 weeks and treated with either the CB_2 agonist AM1241 or CB_2 AM630 antagonist for six weeks.* ***C*** *Brown Fat Pad from the high fat of either the CB_2 agonist AM1241 or CB_2 antagonist AM630 group compared to control. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

There was no difference in the weight of the heart between the AM1241 and AM630 (Figure 5.3.A, p=0.41) compared to the obese control animals. Liver size between the AM1241 (Figure 5.3.B, p=0.56) or AM630 groups and the obese control group showed no significant difference in weight. Compared to the high fat control group the AM1241 treatment group showed a significant decrease in the weight of the epididymal (Figure 5.3.A, p=0.01), peri-renal (Figure 5.3.B, p=0.02) and brown fat pads (Figure 5.3. C, p=0.002). AM630 treatment however only resulted in a decrease (Figure 5.3.D, p=0.001) in the weight of the brown fat pad.

Glucose and Insulin



A; Glucose



B; Insulin

Figure 5.5.A *Glucose tolerance test area under the curve following six weeks of treatment with either CB_2 agonist AM1241 or CB_2 antagonistAM630 compared to obese control.* ***B*** *Insulin tolerance test area under the curve following six weeks of treatment with either the CB_2 agonist AM1241 or CB_2 antagonistAM630 compared to obese control. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

There were no differences between the CB_2 AM1241 agonist (Figure 5.5.A, p=0.30, week 9) or CB_2 AM630 antagonist (Figure 5.4.A, p=0.58, week 9) group in glucose levels following an intraperitoneal glucose tolerance test. Following an insulin sensitivity test glucose was not different following six weeks of treatment with AM1241 Figure 5.4.B, p=0.71, week 15) or AM630 (Figure 5.4.B, p=0.29, week 15).

Hormone detection

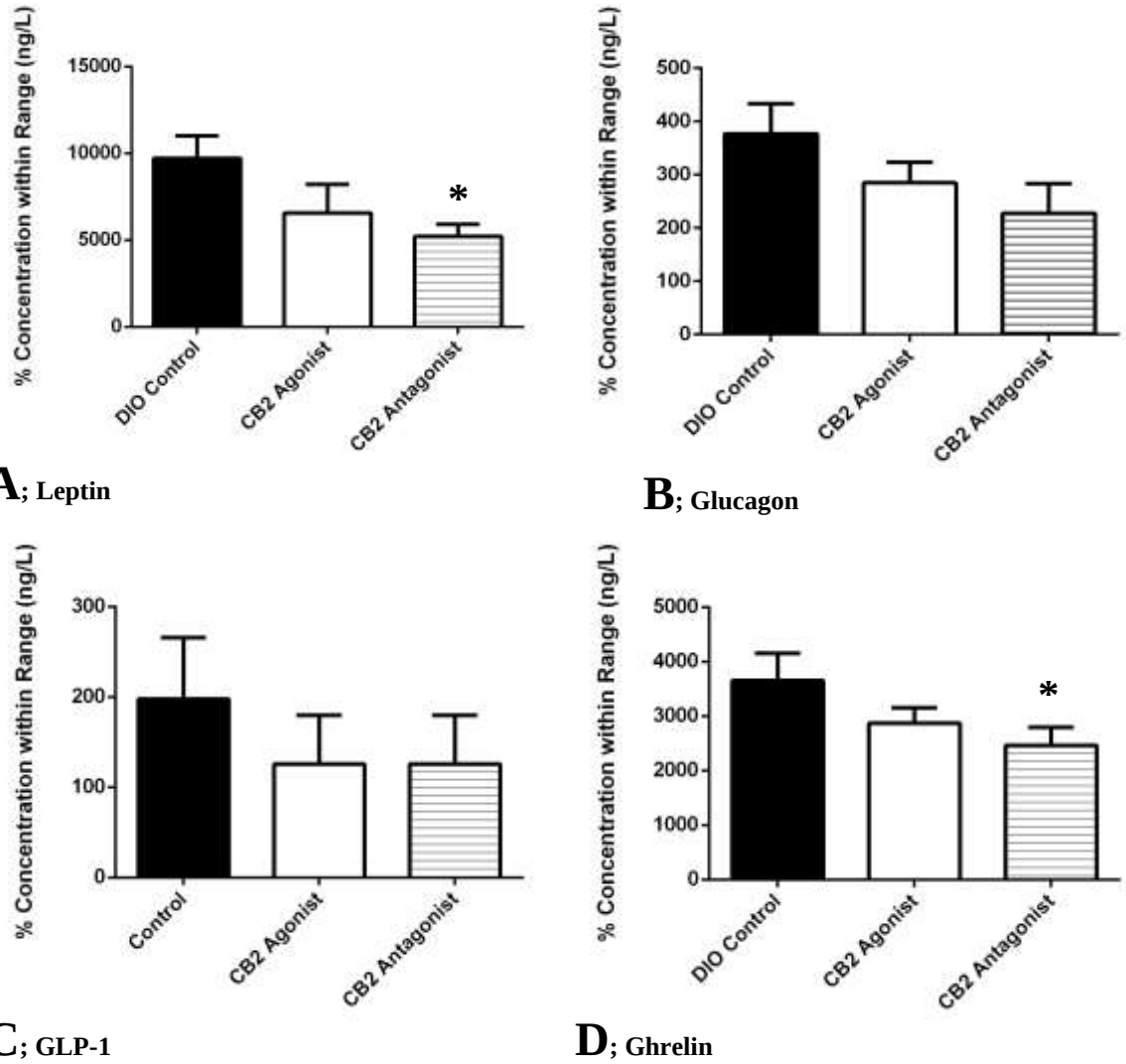


Figure 5.6.A *Leptin levels in plasma in obese rats fed a HF diet for 9 weeks and treated with either CB_2 agonist AM1241 or CB_2 antagonist AM630.* ***Figure B*** *Glucagon levels in plasma in obese rats fed a HF diet for 9 weeks and treated with either CB_2 agonist AM1241 or CB_2 antagonist AM630.* ***C*** *GLP-1 levels in the plasma of obese rats fed a HF diet for 9 weeks and treated with either CB_2 agonist AM1241 or CB_2 antagonist AM630.* ***D*** *Ghrelin levels following treatment witheither CB_2 agonist AM1241 or CB_2 antagonist AM630 .Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

Leptin levels were significantly less in the CB_2 antagonist group (Figure 5.6.A, p=0.001) whereas CB_2 agonist treatment did not alter (Figure 5.6.A, p=0.11) following six weeks of IP injection. Glucagon was not significantly reduced in either AM1241 (Figure 5.6.B, p=0.19) or AM630 (Figure 5.6.B, p=0.06) groups compared to control. GLP-1 did not alter between CB_2 AM630 antagonist (Figure 5.6.C, p=0.20) or the agonist treatment (Figure 5.6.C, p=0.20). Similar to the leptin results, ghrelin levels were lower in CB_2 AM630 antagonist (Figure 5.5.D, p=0.03) but not the agonist treatment (Figure 5.6.D, p=0.10).

Cytokine detection

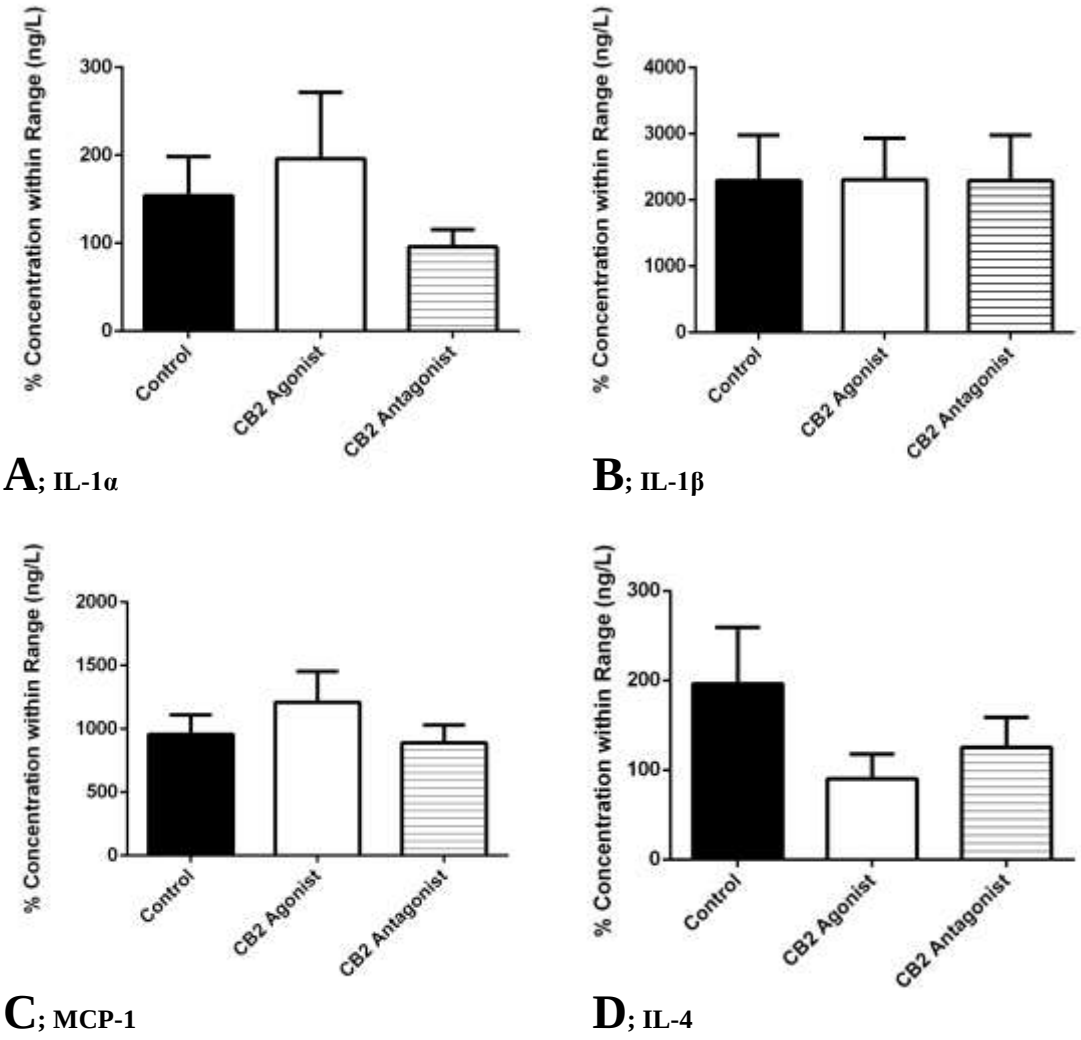
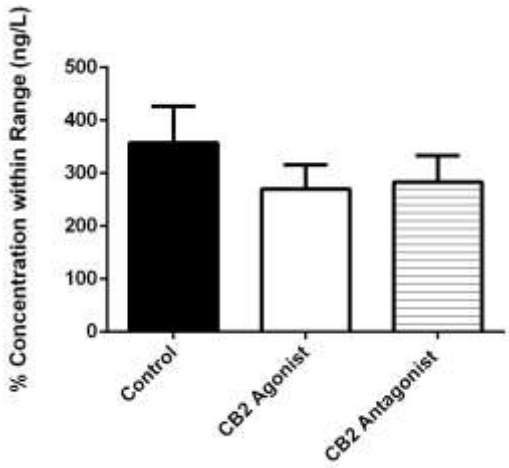
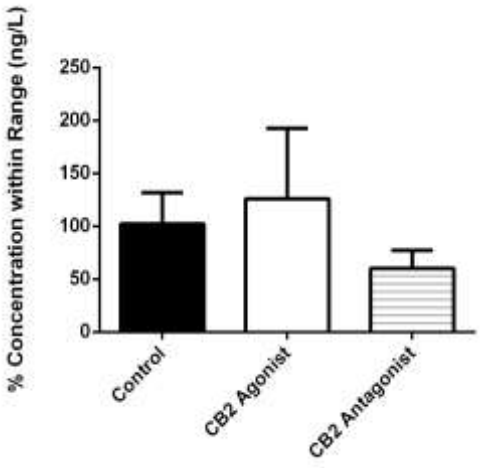


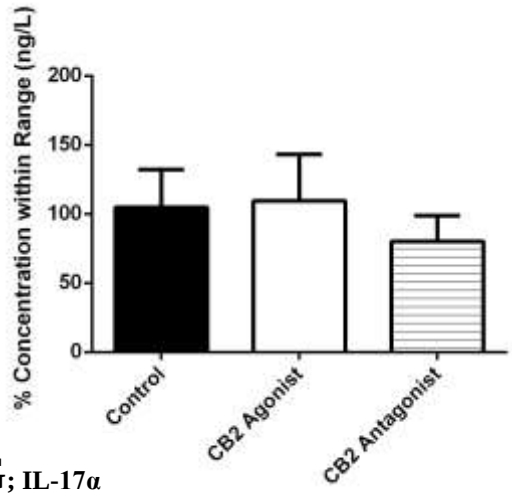
Figure 5.7.A *Plasma levels of 1L-1α of high fat fed rats following six weeks of CB_2 agonist AM1241 or CB_2 antagonist AM630 for six week.* ***B*** *IL-1β plasma levels of obese rats fed a high fat diet and treated for six weeks with either AM1241 or AM630.* ***C*** *Plasma levels of MCP-1 following six weeks of CB_2 AM1241 agonist or CB_2 antagonist AM630 for six weeks .****D*** *IL-4β plasma levels of obese rats treated for six weeks with either CB_2 AM1241 agonistAM1241 or CB_2 AM630 antagonist AM630 (n=9).*



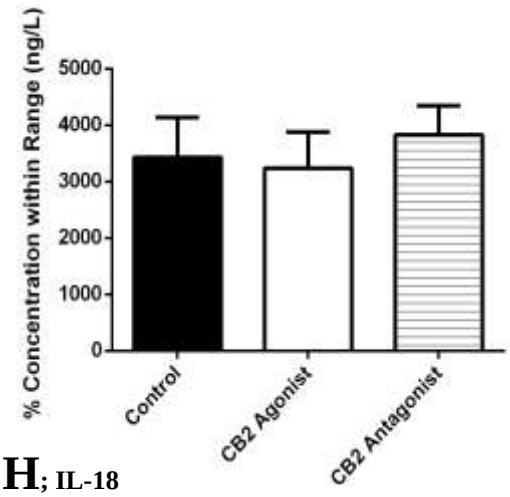
E; IL-5



F; IL-13



G; IL-17α



H; IL-18

Figure 5.7.E *Plasma levels of 1L-5 of obese rats following six weeks of treatment with either CB_2 agonist AM1241or CB_2 antagonist AM630 for six weeks.* ***F*** *IL-13 plasma levels of obese rats fed a high fat diet and treated for six weeks with either CB_2 agonist AM1241 or CB_2 antagonist AM630.* ***G*** *Plasma levels of IL-17α of DIO rats following six weeks of CB_2 agonist AM1241or CB_2 antagonist AM630 for six weeks.* ***H*** *IL-18 plasma levels of obese rats fed a high fat diet and treated for six weeks with either CB_2 AM1241 agonist or CB_2 antagonist AM630 (n=9).*

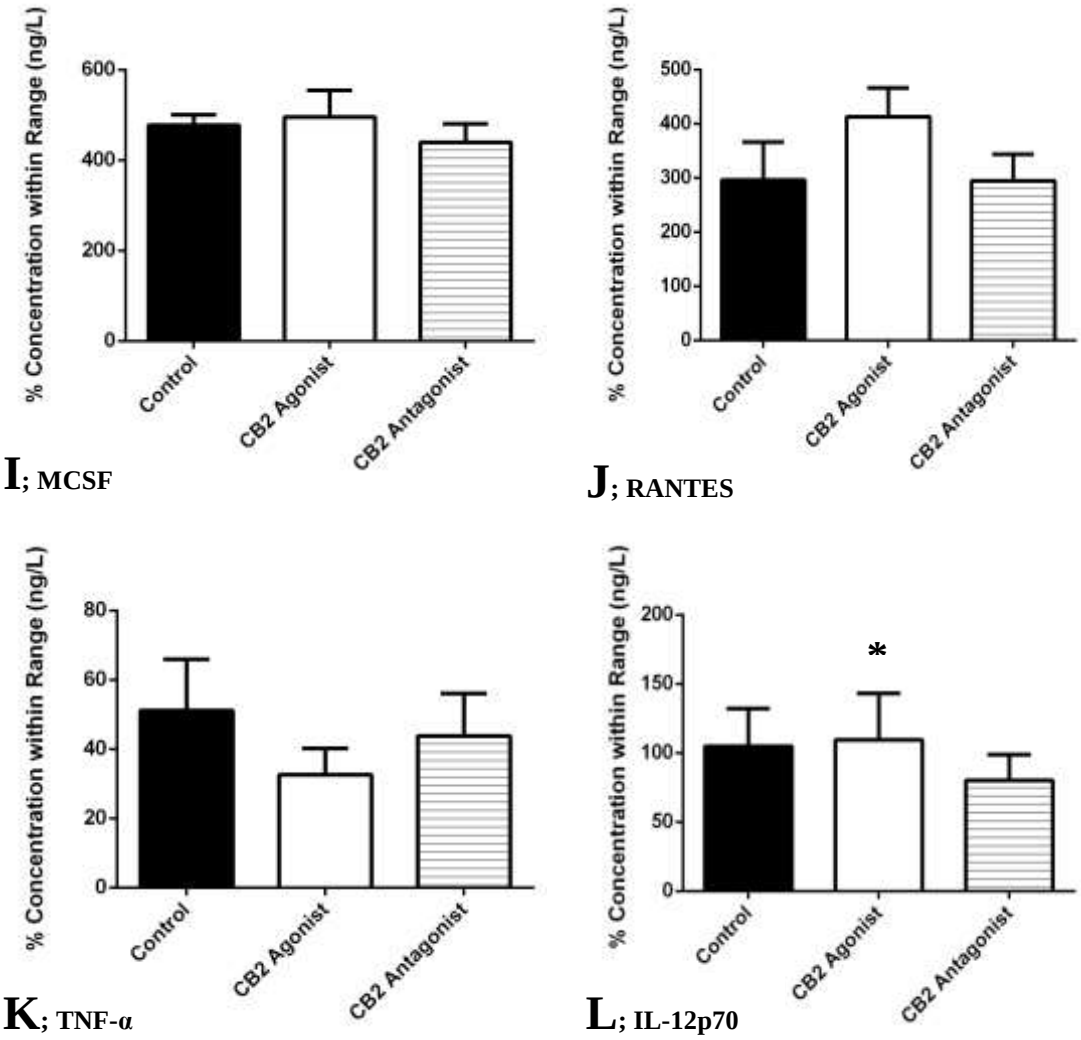


Figure 5.7.I *Plasma levels of MCSF of obese rats following six weeks of treatment with either CB_2 agonist AM1241 or CB_2 antagonist AM630.* ***J*** *RANTES plasma levels of obese rats fed a high fat diet and treated for six weeks with either AM1241 or AM630.* ***K*** *Plasma levels of TNF-α of obese rats following six weeks of treatment with either CB_2 agonist AM1241 or CB_2 antagonist AM630.* ***L*** *IL-12p70 plasma levels of DIO rats fed a high fat diet and treated for six weeks with either CB_2 agonist AM1241 or CB_2 antagonist AM630 (n=9). Significance is indicated by * p=0.05.*

Adiponectin

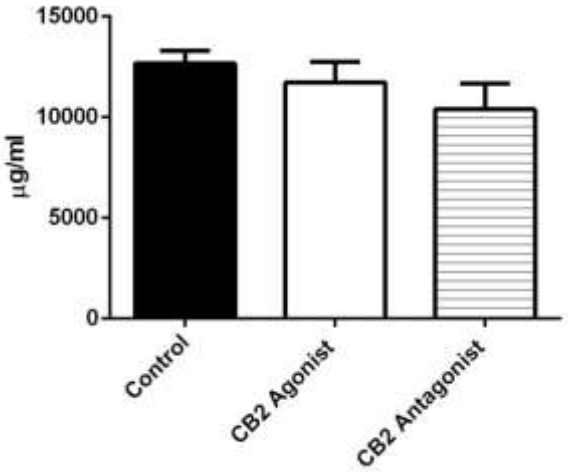


Figure 5.8 *The adiponectin concentration (µg/ml) in the plasma of obese rats fed a HFD for 9 weeks, did not alter when treated for six weeks with either the CB_2 agonist AM1241 or CB_2 antagonist AM631.*

Cytokine levels in plasma of obese rats fed a HFD for 9 weeks and treated with AM1241 mRNA did not alter **IL-1α** (Figure 5.7.A, p=0.39), **IL-1β**(Figure 5.7.B, p=0.68)**, MCP-1** (Figure 5.7.C, p=0.21), **IL-4** (Figure 5.7.D, p=0.18), **IL-5** (Figure 5.7.E, p=0.23), **IL-13** (Figure 5.7.F, p=0.43), **IL-17α** (Figure 5.7.G, p=0.89), **IL-18** (Figure 5.7.H, p=0.42), **MCSF** (Figure 5.7.I, p=0.41), **RANTES** (Figure 5.7.J, p=0.06), or **TNF-α** (Figure 5.7.K, p=0.29) when compared to obese controls. AM1241 treatment however did significantly increase **IL12p70** concentrations compared to obese controls (Figure 5.7.L, p=0.01).

In obese rats administered with AM630 treatment for six weeks following a HF diet for 9 weeks, cytokine levels in plasma in obese rats fed mRNA did not alter **IL-1α** (Figure 5.7.A, p=0.20), **IL-1β** (Figure 5.7.B, p=0.13), **MCP-1** (Figure 5.7.C, p=0.42), **IL-4** (Figure 5.7.D, p=0.17), **IL-5** (Figure 5.7.E, p=0.43), **IL-13** (Figure 5.7.F, p=0.41), **IL-17α** levels between the groups (Figure 5.7.G, p=0.25), **IL-18** (Figure 5.7.H, p=0.34), **MCSF** (Figure 5.7.I, p=0.24), **RANTES** (Figure 5.7.J, p=0.39), **TNF-α** (Figure 5.7.K, p=0.39), or **IL12p70** (Figure 5.7.L, p=0.32).

Adiponectin concentration in the plasma of obese rats fed a HF diet for 9 weeks did not alter when treated with either the AM1241 (Figure 5.8, p=0.21) or AM630 (Figure 5.8, p=0.10) for six weeks compared to a HF saline treated control group.

**Cytokines GM-CSF, IL-6, IL-10, VEGF, EPO, G-CSF, GLP-1, GRO/KC, IL-2 IL-4 and MIPIα were all tested as part of the Bioplex multiple cytokines kit however these analytes were too low to be detected and as such there was not enough power to conduct statistical analysis.*

5.9 Discussion

Obesity is associated with chronic inflammation, resulting in abnormal cytokine production. CB_2 is known to play a role as a modulator in immune responses. (Valerio et al. 2006, Ziring et al. 2006; Ostanin et al. 2005). The results of this study have shown that chronic CB_2 agonist treatment resulted in an increase in circulating plasma levels of IL12p70 and a decrease in the size of epididymal, peri-renal and brown fat pads. CB_2 antagonism however, resulted in a decrease in only the brown fat pad mass and a decrease in the circulating levels of plasma leptin and ghrelin.

The significant decreases seen in fat pad mass indicating whole body lipolysis in the current study provides further evidence to support CB_2 as a potential therapeutic metabolic target. CB_2 agonism resulted in a decrease in epididymal, peri-renal and brown fat pads. This is in support of research that found that in a DIO mouse model the impact of CB_2 receptor agonist (JWH-015) at several doses (0.0, 1.0, 5.0 and 10.0 mg kg-1) and administered for 21 days (Verty et al. 2015). In contrast to the results in the present study the DIO mouse model treated with (10.0 mg Kg-1) had significantly reduced body weight compared to vehicle. Also in contrast, food intake in the DIO mice was significantly reduced following JWH- 015 treatment (Verty et al. 2015). The fat loss by JWH-015 treatment induced a reduction in plasma levels of free fatty acids, triglycerides and insulin (Verty et al. 2015) in comparison with the present study six weeks of treatment with AM1241 in a DIO model did not result in anychange in insulin.

Interestingly both the DIO mouse model discussed and the DIO rats in this study treated for six weeks with AM1241 both had a significant reduction in white adipose tissue. There was a significant reduction on the fat pad mass of both epididymal and peri-renal fat pads. In support of this the DIO mice treated for 21 days with JWH- 015 had a reduction in inguinal white fat

pad by 33% and retroperitoneal fat pad mass by 40% (Verty et al. 2015). The JWH-015 treatment however did not affect the brown fat pad whereas in the present study, a six-week treatment with AM1241 resulted in a significant decrease in the brown fat pad. In both CB_2 agonist studies in DIO animal models, the activation of CB_2 resulted in modulation of the levels of cytokines in different tissues. JWH-015 treatment produced a significant increase in IL-10 and a significant decrease in TNF-α expression in the protein extracted from white and brown fat (Verty et al. 2015). Six weeks of AM1241 treatment in the DIO animals in this study resulted in an increase in circulating plasma levels of IL12p70. Further research is required to determine the mechanisms behind these inflammatory responses. Regardless there is evidence in both CB_2 agonist treatments in DIO models to suggest that CB_2 activation results in lipolysis.

Limited information exists on the *in vivo* role of CB_2 antagonism in the obese state. Evidence of mechanistic roles in genetically modified mice shows that blocking CB_2 signalling in $CB_2^{-/-}$ knockout mice, results in a significant increase in food intake and body weight with age compared to $CB_2^{+/+}$ mice (Agudo et al. 2010). The $CB_2^{-/-}$ knockout mice failed however to develop insulin resistance and showed evidence of improved insulin-stimulated glucose uptake in skeletal muscle (Agudo et al. 2010). Chronic CB_2 antagonist treatment with AM630 in this study resulted in a decrease of the brown fat pad only. The leptin levels in the CB_2 antagonist AM630 group were also reduced. Leptin levels are known to reflect adipose tissue storage with obesity resulting in elevated levels (Frederich et al. 1995). Despite the reduction in plasma leptin, the decrease in the weight of the brown fat pad mass was not reflected with any change seen in the body fat percentage or lean tissue mass percentage. Determining the relationship between CB_2 modulation and leptin will provide further evidence in understanding the role of CB_2 in metabolism and as such further work into the changes in the adipose tissue mRNA

expression of key signalling pathways is being undertaken, but is beyond the scope of this thesis.The results in this study showed few significant outcomes, if the research was repeated and a working hypothesis established based on the results of the current study, an increase in the dosage of both CB_2 agonism and antagonism would be advised. The exact concentration would be decided by a dose response protocol on a sample of animals. Furthermore, as this research investigated the skeletal muscle response, it would beneficial to have a group that was obese and underwent exercise during the treatment period. This would investigate if the increase in expended energy required for exercise training results in an influence on the effects of CB_2 agonism and antagonism.CB_2 agonist treated animals showed no changes in the measured hormones at the time of death. CB_2 antagonism however caused a decrease in both leptin and ghrelin levels. Leptin and CB_2 have been shown to have a synergistic partnership in neuroprotection (Lopez-Rodriguez et al. 2016). Leptin is made by adipocytes and plays a regulatory role in hunger and satiety and fat metabolism (Yildiz et al. 2004). Ghrelin is released by the stomach and duodenum and plays a role in appetite regulation by stimulating hunger (Levin, 2006).

Circulating plasma levels of ghrelin are increased prior to meal-times and circulating levels modulate intake of food to satiety (Date et al. 2000, Cummings et al. 2001). In freely fed conscious rats, an intraperitoneal (IP) injection of different doses of selective CB_2 receptor antagonist AM630 increased acute food intake via dose response (Ting et al. 2014). In humans, obesity negatively correlates with low levels of circulating ghrelin (Tschop et al. 2001). In the current study, the plama levels of ghrelin were significantly reduced with CB_2 antagonism without changes in food intake. A high fat diet in mice has been demonstrated to alter appetite regulatory actions of both leptin and ghrelin (Kentish et al. 2013). Compared to a high fat

control group, the rats in this study that were chronically treated with the CB_2 antagonist showed decreased plasma levels of leptin and ghrelin, however food intake and weight did not change. This is in support of research suggesting that acute AM630 administration does not have an acute effect on food intake in rats (Ting et al. 2014). This is the first study to establish a relationship between chronic CB_2 antagonist treatment and ghrelin in a DIO model, albeit without the concomitant reduction in food intake and as such why these animals had a reduction in ghrelin levels without changes in food intake and body weight requires further research.

The link between CB_2 and immune cells is supported through clinical study results showing that CB_2 agonist treatment results in an anti-inflammatory state (Zoppi et al. 2014). The chronic state of inflammation associated with obesity has been shown to be correlated with an increase in circulating glucose and insulin resistance (Ziring et al. 2006, Ostanin et al. 2005, Elmes et al. 2004). The animals in the current study did not change in either their fasting glucose or insulin levels, indicating that they were not insulin resistant. Obesity results in the production of low grade chronic inflammation resulting in metabolic disturbance (Ziring et al. 2006). Therefore, to determine the effects of CB_2 modulation on markers of obesity proinflammatory cytokine concentrations were tested in circulating plasma levels of the animals following six weeks of CB_2 agonist AM1241 treatment or CB_2 antagonist AM630 treatment.

Cytokines investigated in this study when exposed to chronic CB_2 antagonism did not alter circulating plasma levels of pro-inflammatory cytokines - IL-1α, IL-1β, MCP-1 , IL-4 , IL-5 , IL-13 , IL-17α , IL-18 , MCSF, RANTES, TNF-α, IL-12p70. However, CB_2 agonist treatment for 6 weeks did result in a significant increase in the circulating plasma levels of IL-12p70. Two subunits IL12p35 and IL12p40 when expressed together make up the bioactive cytokine IL12p70 (IL-12). The limited research available on the relationship between IL-12p70 and CB_2

shows anandamide (AEA) decreases expression of IL-12p70 (Correa et al. 2011). THC, a natural endocannabinoid agonist, has been shown to inhibit IL-12p40 in an animal model in a dose-dependent manner (Lu et al. 2006). IL-12p70 is a cytokine predominately produced by macrophages and monocytes and is known to initiate Th1 and IFN-$_\gamma$ cell responses (Pflanz et al. 2002). In an obese leptin deficient (ob/ob) mouse model of acute pancreatitis, inflammation was induced by IL-12 (Sennello et al. 2008). The results were also reproduced and supported in the same model of inflammation in DIO mice (Pini et al. 2010). This suggests that the effects of IL-12 are independent of leptin. This study is the first to show that chronic treatment with CB_2 agonist, AM1243, up regulates IL-12 p70 in a DIO rat model. Conversely the mechanisms behind this increase may not be as simple and as deleterious as an increase in inflammation. The CB_2 agonist, AM1243, suppressed neurodegeneration in an animal model of stroke by indirectly suppressing inflammation through the proposed reduction of cell necrosis mechanisims (Seong-Jin et al. 2015).

5.10 Conclusion

CB_2 has been to shown, to play a number of roles in a variety of biological systems including metabolism and inflammation. This study provides further evidence that CB modulation can have an effect on the decrease of fat pad mass. Chronic CB_2 agonist treatment with AM1241 decreased the weight of epididymal, peri-renal and brown fat pad mass, moreover administration resulted in increased plasma levels of the inflammatory marker IL-12p70. CB_2 antagonsim decreased the brown fat pad mass while decreasing plasma levels of the metabolic hormones of ghrelin and leptin. Following CB_2 agonism the null hypothesis of this study is accepted, as the data showed that stimulation of CB_2 receptors increased some inflammatory

markers in an obese model. In contrast, CB_2 antagonism administration failed to modulate the plasma hormone and cytokine markers measured in an obese model.

Further research is required to understand the effect of this modulation on specific tissues in a DIO model, further exploration should be undertaken to elucidate the mechanisms underpinning the reduction in fat pad mass and the increase in circulating levels of IL-12p70.

Chapter 6 – The effects of chronic CB_2 agonism and antagonism and acute adiponectin treatment on fatty acid oxidative pathways in skeletal muscle.

6.0 Abstract

This chapter describes the ex-vivo work performed to measure the effect of adiponectin treatment on markers of fatty acid oxidation in muscle collected from the DIO animal studies described in chapter 5. Soleus and extensor digitorum longus (EDL) muscles were carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. Immediately after their removal longitudinal strips were incubated in an oxygenated organ bath (37ºC pregassed at 95% 0^2 -5% $C0^2$). Each muscle was placed in an individual chamber of Krebs-Henseleit buffer with either adiponectin - 0.1µg/ml (AD+) or no adiponectin (AD-) for a period of 30 minutes of treatment. Acute adiponectin exposure on skeletal muscle from a DIO model following chronic CB_2 agonist treatment AM1241 resulted in an increase in AdipoR1 and PGC-1α mRNA expression and a significant decrease in AMPK mRNA expression in the EDL muscle. Chronic AM630 treatment in DIO model, however, resulted in increased levels of citrate synthase expression in the highly oxidative red gastrocnemius skeletal muscle, which could not be explained by any alterations in mRNA expression of key markers of oxidative metabolism.

6.1 Introduction

Obesity results in an increase in circulating fatty acids, an increase in the activity of the endocannabinoid system and a decrease in physiological levels of adiponectin (Bensaid et al. 2003). The upregulation of the endocannabinoid system in obesity results in an increase in appetite and a number of metabolic changes (Blüher et al. 2006). CB_2, the less studied of the endocannabinoid receptors is a single chained polypeptide that contains seven transmembrane α-helices, with an extracellular glycosylated N-terminus and an intracellular C-terminus (Cabral et al. 2009). CB_2 was first identified and cloned from HL60 cells and since has held strong association with immune system and pain research (Munro et al. 1993, Bermudez-Silva et al. 2007). It has also been found to be expressed in white and brown adipose tissue, bone marrow, kidneys, brain, spleen, thymus and leucocytes and cells involved in immune system and skeletal muscle (Persidsky et al. 2015, Krott et al. 2006, Jenkin et al. 2013, Xie et al. 2016, Sugiura et al. 2002, Cavuoto et al. 2007).

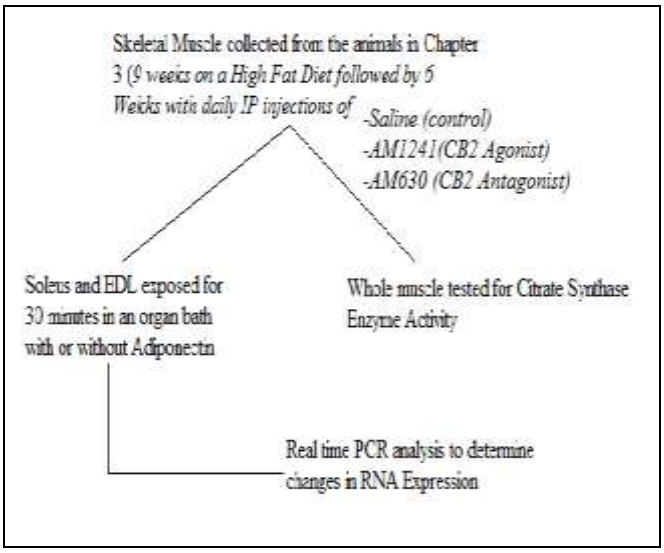
CB_2 function in skeletal muscle has not been determined in *in vitro* studies, however CB_2 was detected in a rodent myogenic cell line (L6) and in lean human skeletal muscle myotubes collected from the rectus abdominus muscle (Cavuoto et al. 2007). It was later confirmed that CB_1 and CB_2 were involved in the regulation of fatty acid oxidative pathways in skeletal muscle (Zhao et al. 2010). There's currently very little information regarding the role of CB_2 and its possible mechanisms in obesity. CB_2 knockout mice (-/-) maintained on a high fat diet, showed an improvement in insulin sensitivity despite unchanged body weight and food intake (Agudo et al. 2010). Administration of the CB_2 agonist HU210 in C57BL/6J mice (which are biologically susceptible to diet-induced obesity and diet induced diabetes) fed a high fat diet prevented changes in body weight, body fat percentage and food intake however CB_2 agonism

accompanied with a HF diet resulted in diet induced glucose intolerance in skeletal muscle (Guerts et al. 2013). In contrast, CB_2 knockout mice fed a high fat diet display no obesity-induced inflammation and did not develop insulin resistance (Deveaux et al. 2009).

In skeletal muscle a relationship between CB_2 activation and acute inflammation has been established (Tian-Shiuet et al. 2010). An increase in macrophages and almost a twofold increase per hour of CB_2 expression has been found to occur in localized skeletal muscle of Sprague Dawley rats following acute localised physical trauma; the increased expression remained for up to 14 days (Tian-Shiuet et al. 2010). Moreover, a follow up study using localised acutely damaged skeletal muscle of Sprague Dawley rats who were injected at the damaged site; every second day for 14 days with either CB_2 agonist JWH-133 or CB_2 antagonist AM630 that the localized immune response in the JWH-133 group showed a significant decrease in myofibroblasts whereas the AM630 group showed an up-regulation in fibrogenic markers (Tgfb1, Fn1 and Acta2) (Tianshiu et al. 2015). This supports a role between CB2 activity and inflammation in skeletal muscle during acute damage of skeletal muscle. The whole body response to the effects of chronic CB_2 modulation on skeletal muscle signalling pathways however is not yet known.

Adiponectin plays a role in anti-inflammatory, anti-diabetic and insulin sensitizing in obesity (Waki et al. 2004, Goto et al. 2014, Lihn et al. 2005; Bruce et al. 2005). Adiponectin enters the cell via AdipoR1 and AdipoR2 where they are thought to communicate with the adapter proteins APPL1 and APPL2, and subsequent downstream signalling pathways to elicit positive effects on mitochondrial respiration (Liu et al. 2010). Research determining the effects of chronic treatment with the CB_2 agonist, AM1241, or CB_2 antagonist, AM630, on adiponectin resistance in skeletal muscle has yet to be undertaken. Skeletal muscle plays a major role in

glucose clearance, fatty acid oxidation and energy production (Cavaliere et al. 2016). Understanding the role adiponectin receptors play in adiponectin resistance in skeletal muscle will provide possible pharmacological targets for the treatment of obesity and diabetes. Therefore, the aims of this study will be to determine the effects on skeletal muscle oxidative markers and adiponectin signalling following 6 weeks of treatment with a CB_2 agonist or antagonist. Moreover, this study will also investigate the effects following this treatment on the ex vivo effects of acute adiponectin treatment on skeletal muscle oxidative markers and adiponectin signalling. It is hypothesised that the activation of CB_2 receptors via agonist administration will result in a reduction in mRNA expression of key genes associated with adiponectin signalling and fatty acid oxidation. In contrast blocking CB_2 receptors will result in an increase in mRNA expression associated with improving adiponectin sensitivity and fatty acid oxidation in skeletal muscle.



6.2 Materials and Methods

6.3 Animals and Experimental Protocol

The animals used in this study are the same animals used as a control group in chapter 5. The animals underwent the same treatment, environmental and euthanasia protocol, as those detailed in Chapter 5.3. This group of animals however were maintained on the HFD and treated daily with either vehicle control 0.9% isotonic saline solution containing 0.75% Tween 80 or 3 mg/kg of AM251 dissolved in vehicle solution via IP injection.

6.4 Muscle sample preparation

Following anaesthesia induced by 5% isoflorane administration each animal underwent bilateral surgical removal of soleus, EDL and gastrocnemius. Each muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. Following dissection, the gastrocnemius muscle was separated into red and white muscle and snap frozen for subsequent measurement of citrate synthase activity.

Immediately after their removal longitudinal strips of the soleus and EDL were incubated in an oxygenated organ bath (37ºC pre-gassed at 95% O2 -5% C02). Each longitudinal strip was placed in an individual chamber of Krebs-Henseleit buffer with either 0.1 µg/ml of adiponectin (AD+) or the contralateral hind limb muscle exposed to control (AD-) for a period of 30 minutes. The muscles were taken from alternating limbs from each animal to ensure that alternate muscles were exposed to AD treatment for example: rat one- right then left hind limb muscle then rat 2 left and then right limb muscle was treated with the adiponectin or control this was then rotated for each subsequent rat. Following the treatment muscle samples were snap frozen and stored in liquid nitrogen for subsequent analysis.

6.5 mRNA

Total RNA was isolated from the soleus and EDL muscle by using TRIzol Reagent (Invitrogen, Carlsbad, CA) as previously described (McAinch et al., 2006). In short, rodent muscle tissue extracts (approximately 15 mg) were dissociated using 1000 mg of ceramic/silica beads and RNA was extracted in TRIzol and treated with the RQ1 RNase–free DNase kit (Promega Corporations, Madison, WI) RNA concentration of 1µg/11µg total volume. First strand cDNA was then generated from 0.3 µl of template RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) using random hexamers and oligo dTs. cDNA was stored at -20 ºC for subsequent analysis. 'Real-time' PCR was conducted using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and the MyiQ™ single colour 'real-time' PCR detection system (Bio-Rad Laboratories, Hercules, CA). Forward and reverse oligonucleotide primers are shown in Table 4.1. 'Real-time' PCR reactions were run for 50 cycles of 95 ºC for 15 s and 60 ºC for 60 s. The PCR normalized to housekeeping genes, GAPDH and Cyclophilin. Relative changes in mRNA abundance was quantified using the 2(-Delta Delta Ct) method as previously detailed (Livak and Schmittgen, 2001) and data is reported in arbitrary units and expressed as Mean ± SEM.

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; AdipoR1: Adiponectin receptor 1; AdipoR2: Adiponectin receptor 2; APPL1 adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1; APPL2: adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2; FAT/CD36: fatty acid translocase/CD36; AMPK:

1 5'adenosinemonophosphate-activated protein kinase a-2; PDK4: pyruvate dehydrogenase
2 kinase 4; PGC1a: peroxisome proliferator-activated receptor gamma co-activator 1 alpha.

3 **Table 6.1: Oligonucleotide primers for 'real-time' PCR primers**

Genes	Accession number	Forward primer	Reverse primer
Cyclophilin	NM_017101.1	CTGATGGCGAGCCCTTG	TCTGCTGTCTTTGGAACTTTGTC
GAPDH	NM_017008.3	AGTTCAACGGCACAGTCAAG	GTGGTGAAGACGCCAGTAGA
AdipoR1	NM_207587.1	TGAGGTACCAGCCAGATGTC	CGTGTCCGCTTCTCTGTTAC
AdipoR2	NM_001037979.1	TCCATGGAGTCTCAACCTG	GGAGAGTATCACAGCGCATC
APPL1	XR_007603	TCACTCCTTCCCCATCTTTC	TAGAGAGAGGGCAGCCAAAT
APPL2	NM_001108741	TGCTCGGGCTATTCACAA	AAACAGGCCCGTGACACT
FAT/CD36	NM_031561.2	GACCATCGGCGATGAGAAA	CCAGGCCCAGGAGCTTTATT
AMPK	NM_023991.1	ACTCTGCTGATGCACATGCT	AGGGGTCTTCAGGAAAGAGG
PDK4	NM_053551.1	GGGATCTCGCCTGGCACTTT	CACACATTCACGAAGCAGCA
PGC-1α	NM_013261.3	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC

GAPDH: *glyceraldehyde-3-phosphate dehydrogenase;* ***AdipoR1:*** *Adiponectin receptor 1;* ***AdipoR2:*** *Adiponectin receptor 2;* ***APPL1*** *adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1;* ***APPL2****: adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 2;* ***FAT/CD36:*** *fatty acid translocase/CD36;* ***AMPK:*** *5'adenosine monophosphate-activated protein kinase a-2;* ***PDK4:*** *pyruvate dehydrogenase kinase 4;* ***PGC1a:*** *peroxisome proliferator-activated receptor gamma co-activator 1 alpha.*

4 **6.6 Citrate Synthase**

5 Citrate Synthase (CS) was assayed according to the protocol of Srere (1969). Briefly, after the
6 addition of 5 µl of muscle homogenate (0.175 KCl & 2mM EDTA, PH-7.4), 230 µl of reagent
7 cocktail [3mM Acetyl CoA (Sigma A-2056), 100 mM Tris buffer (BioRad) 161-0719; pH 8.3),
8 1 mM DTNB (Sigma D-8130)] and 15 µl of 10 mM Oxalacetate (Sigma 0-4126). CS activity
9 was measured at room temperature by recording the increases in the colorimetric compound

(5, 5-dithiobis-2-nitrobenzoate), which is linked to citrate production, at 412 nm (xMark™ Microplate Spectrophotometer, BioRad). Readings of CS activity were recorded every 15 seconds for 3 minutes. All enzyme activity was expressed relative to wet weight (Betick et al. 2008). The change in absorbance/min was calculated to measure CS activity. All muscle samples were measured in duplicates, and the average activity used to characterize CS activity.

6.7 Statistical Analysis

SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group direct analysis or mixed model ANOVA with Tukey's post-hoc tests. * Significance was accepted when $p < 0.05$.

6.8 Results

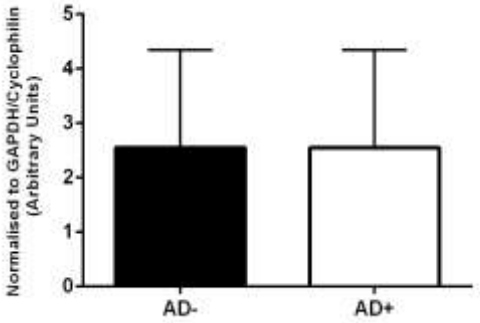
To determine if the AD treatment in isolation had an effect once introduced to the organ bath the control group of high fat fed obese rats (n=9) were treated and the mRNA expression of this tissue was tested (see Chapter 3). As the same control tissue was used in this study it is relevant to present changes that occurred in this group (please table 6.1 below)

Table 6.2 Tabular results of control AD- and AD+

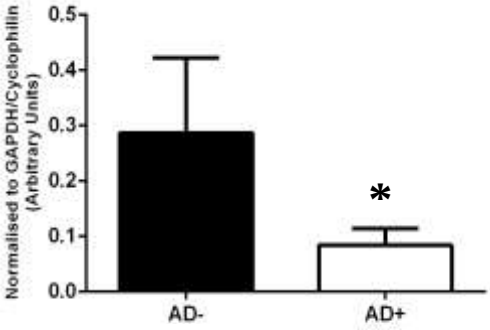
Control AD- compared to AD +	Skeletal Muscle	±SEM	*P Value*
AdipoR1	Soleus	0.01-0.007	0.12
AdipoR1	EDL	0.01-0.03	0.36
AdipoR2	Soleus	0.08-0.06	0.36
AdipoR2	EDL	0.23-0.11	0.37
APPL1	Soleus	0.01-0.04	0.17
APPL1	EDL	0.02-0.008	0.31
APPL2	Soleus	0.001-0.001	0.36
APPL2	EDL	0.006-0.01	0.21
FATCD	Soleus	1.03-0.98	0.24
FATCD	EDL	0.24-0.34	0.32
AMPK	Soleus	0.01-0.13	*0.03
AMPK	EDL	0.97-0.10	0.06
PDK4	Soleus	0.24-0.33	0.35
PDK4	EDL	0.24-0.25	0.26
PGC1a	Soleus	0.29-0.13	0.07
PGC1a	EDL	0.20-0.15	0.48

AD- stands for exposure to an organ bath with no treatment added, AD+ stands for sample exposed to an organ bath chamber treated with adiponectin. Significance is indicated by p < 0.05, n = 9)*

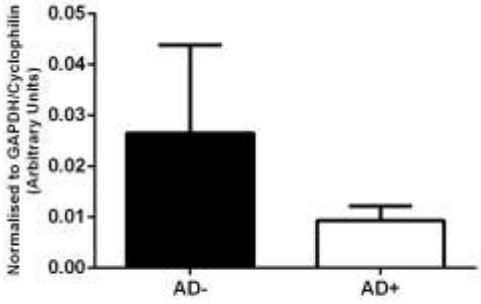
mRNA expressions of intramyocellular genes known to modulate adiponectin in skeletal Muscle fiber types of DIO rats chronically treated with CB_2 agonist AM1241 or CB_2 antagonist AM630 and acutely exposed to adiponectin.



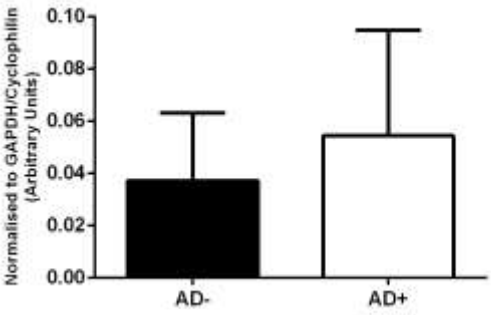
A; AdipoR1



B; AdipoR1

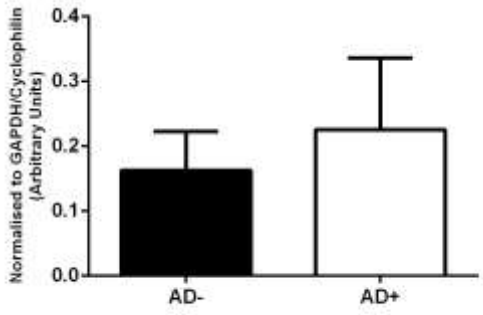


C; AdipoR1

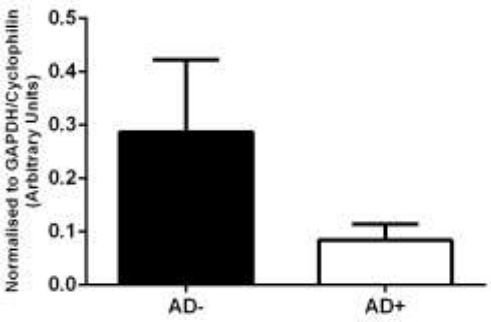


D; AdipoR1

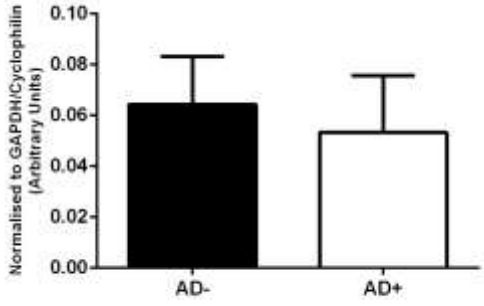
Figure 6.1.A *Gene expression of AdipoR1control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***B*** *the effects of AdipoR1gene expression the EDL following adiponectin exposure.* ***C*** *Gene expression of AdipoR1 control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***D*** *the effects of AdipoR1 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control, CB_2 agonist AM1241 or CB_2 antagonist AM630 for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*



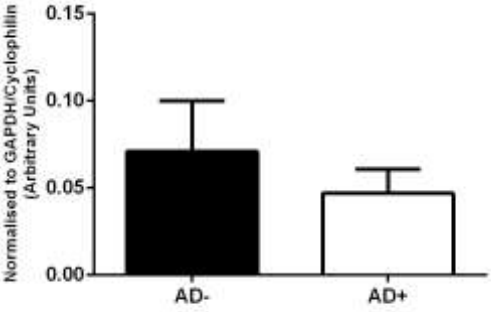
E; AdipoR2



F; AdipoR2

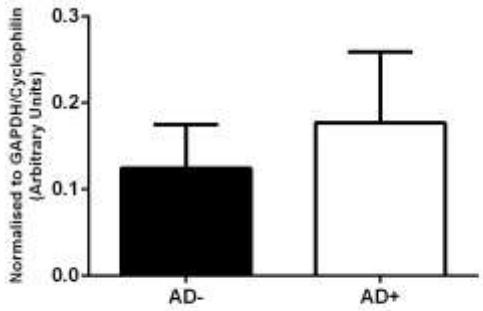


G; AdipoR2



H; AdipoR2

Figure 6.1.E *Gene expression of AdipoR2 control samples exposed to vehicle (AD-) or adiponectin (AD+) positive in the soleus muscle.* ***F*** *the effects of AdipoR2 gene expression the EDL following adiponectin exposure.* ***G*** *Gene expression of AdipoR2 control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***H*** *the effects of AdipoR2 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml l of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control, CB_2 agonist AM1241 or CB_2 antagonist AM630 for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*



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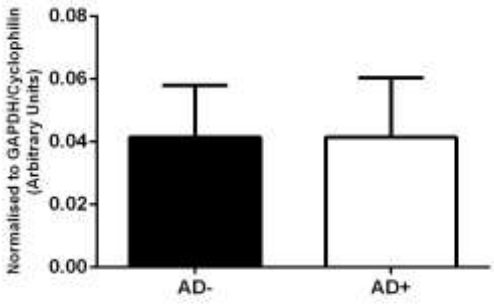

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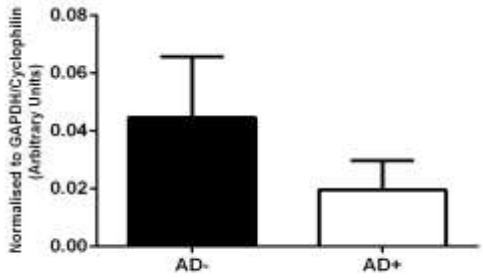
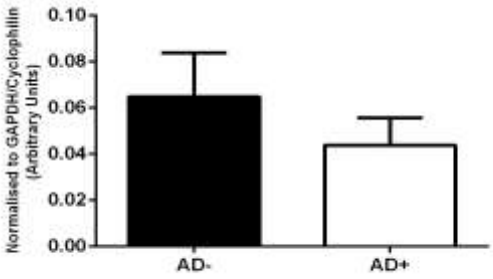
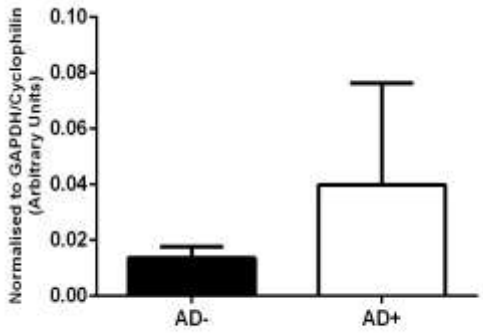


K, **APPL1**

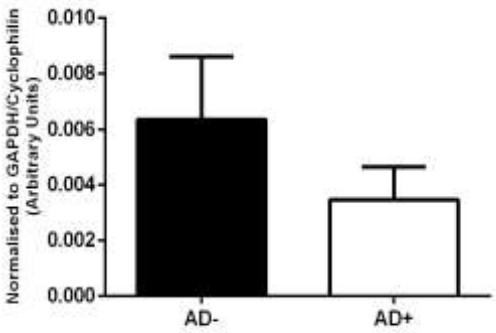


L, **APPL1**

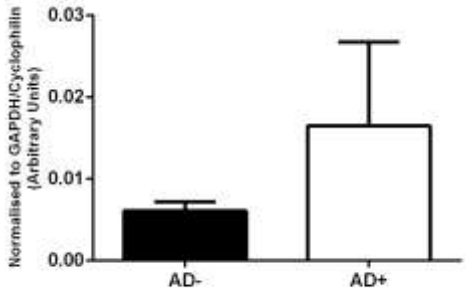
Figure 6.1.I *Gene expression of APPL1control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***J*** *the effects of APPL1gene expression the EDL following adiponectin exposure.* ***K*** *Gene expression of APPL1 control samples exposed toadiponectin negative or adiponectin positive in the soleus muscle****. L*** *the effect of APPL1 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control, CB2 agonist AM1241 or CB2 antagonist AM630 for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*



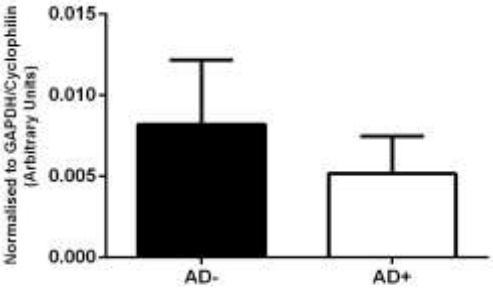
M; A PPL2



N; A PPL2



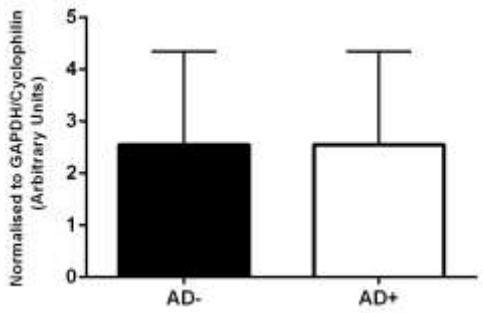
O; APPL2



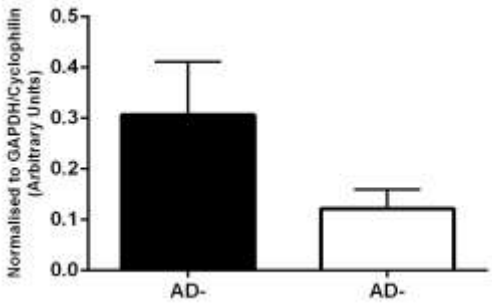
P; APPL2

Figure 6.1.L *Gene expression of APPL2 control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***M*** *the effects of APPL2 gene expression the EDL following adiponectin exposure.* ***N*** *Gene expression of APPL2 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***O*** *the effect of APPL2 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control, CB_2 agonist AM1241 or CB_2 antagonist AM630 for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin.*

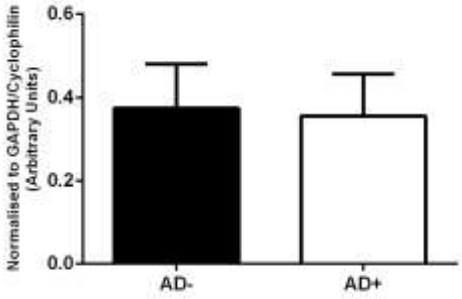
mRNA expression of intramyocellular genes in skeletal muscle fiber types of DIO rats chronically treated with CB_2 agonist AM1241 or CB_2 antagonist AM630 following acute exposure to adiponectin



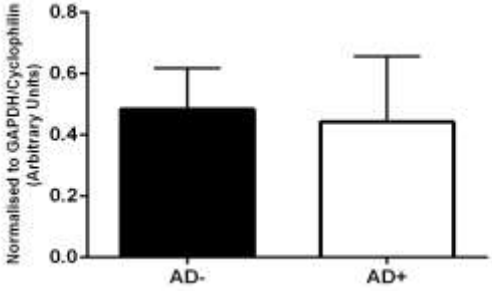
A; FAT/CD36



B; FAT/CD36

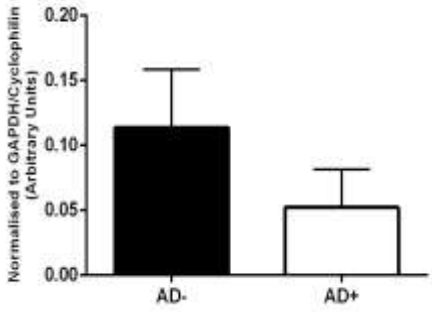


C; FAT/CD36

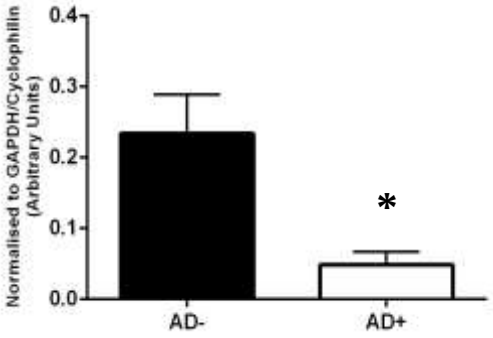


D; FAT/CD36

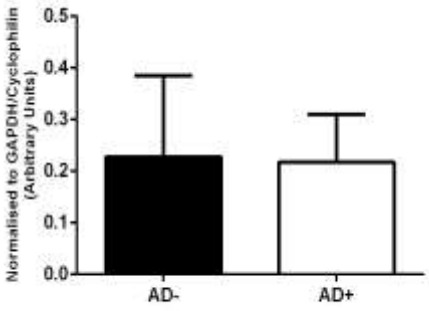
Figure 6.2.A *Gene expression of FAT/CD36 control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***B*** *the effects of FAT/CD36 gene expression the EDL following adiponectin exposure.* ***C*** *Gene expression of FAT/CD36 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***D*** *the effects of FAT/CD36 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin. Significance is indicated by * ($p < 0.05$).*



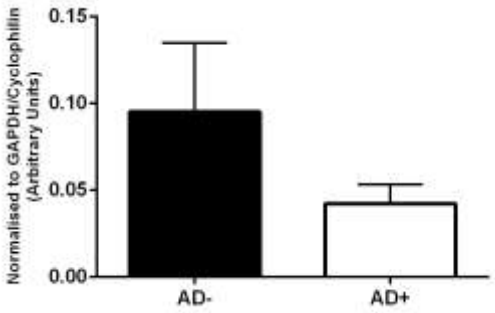
E; AMPK



F; AMPK

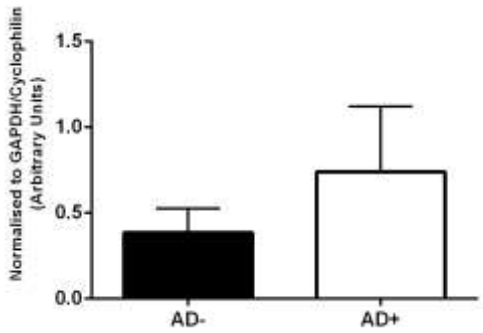


G; AMPK

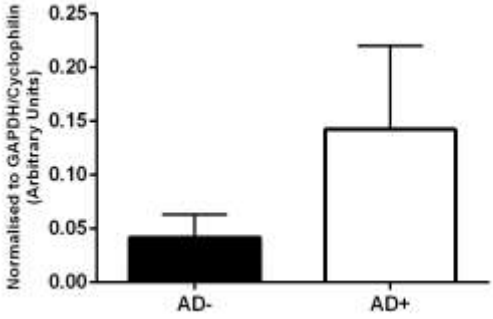


H; AMPK

Figure 6.2.E *Gene expression of AMPK control samples exposed to control (AD-) or adiponectin (AD+) in the soleus muscle.* ***F*** *the effects of AMPK gene expression the EDL following adiponectin exposure.* ***G*** *Gene expression of AMPK control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle. The effects of AMPK gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin. Significance is indicated by * ($p < 0.05$).*



I; PDK4



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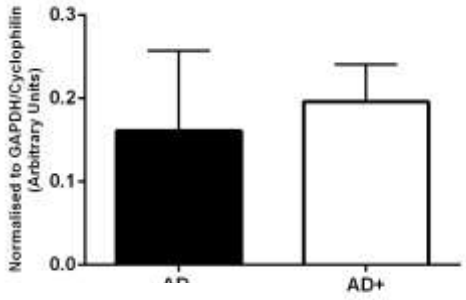
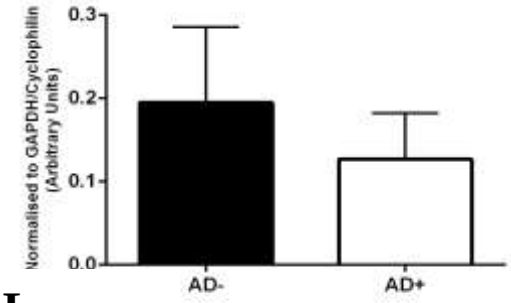
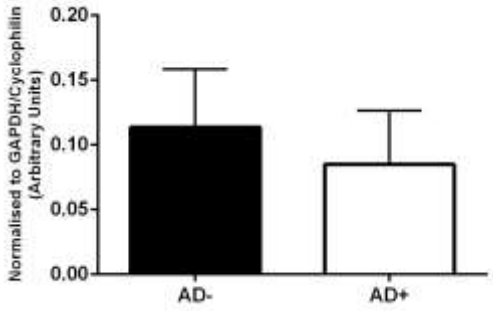


K; PDK4

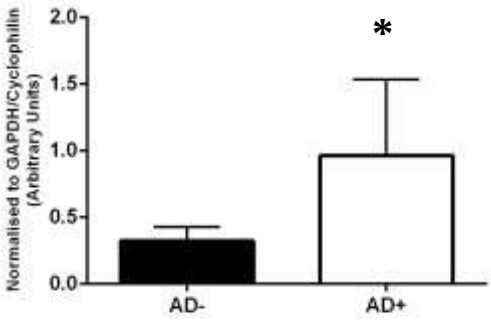


L; PDK4

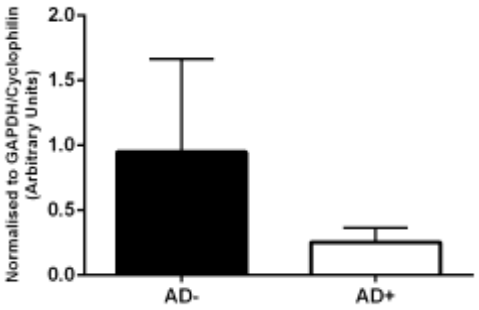
Figure 6.2.I *Gene expression of PDK4 control samples exposed to control (AD-) or adiponectin (AD+) in the soleus muscle.* ***J*** *the effects of PDK4 gene expression the EDL following adiponectin exposure.* ***K*** *Gene expression of PDK4 control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***L*** *the effects of PDK4 gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin. Significance is indicated by * ($p < 0.05$).*



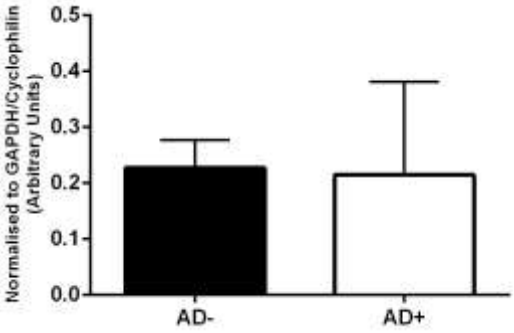
M; PGC1-α



N; PGC1-α



O; PGC1-α



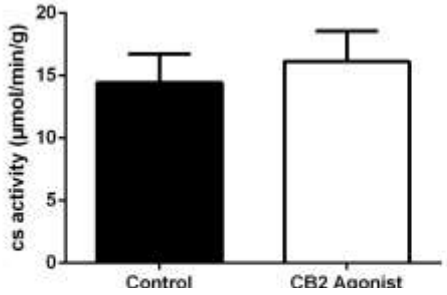
P; PGC1-α

Figure 6.2.M *Gene expression of PGC1-α control samples exposed to vehicle (AD-) or adiponectin (AD+) in the soleus muscle.* ***N*** *the effects of PGC1-α gene expression the EDL following adiponectin exposure.* ***O*** *Gene expression of PGC1-α control samples exposed to adiponectin negative or adiponectin positive in the soleus muscle.* ***P*** *the effects of PGC1-α gene expression the EDL following adiponectin exposure. Muscle tissue exposed to 0.1 µg/ml of adiponectin (AD+) or control (AD-) for 30min following extraction from animals fed a HFD for 9 weeks to induce DIO and then injected with a saline control for 6 weeks. Data (n = 9) is expressed as Mean ± SEM in arbitrary units normalized to housekeeping genes, GAPDH and Cyclophilin. Significance is indicated by * ($p < 0.05$).*

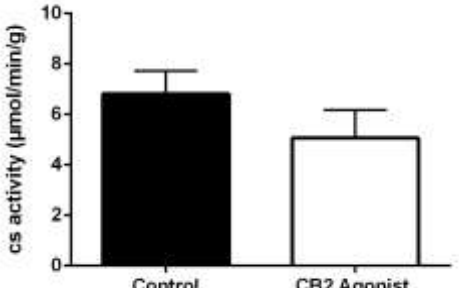
Acute adiponectin (0.1ug/ml) exposure following 6 weeks of treatment with the CB_2 agonist AM1241 in a DIO model did not alter the soleus muscle mRNA expression of AdipoR1 (Figure 6.1.A, p=0.38), AdipoR2 (Figure 6.1.E, p=0.42), APPL1 (Figure 6.1.I, p=0.84) or APPL2 (Figure 6.1.M, p=0.13). In the EDL of the DIO animals treated for six weeks with the CB2 agonist AM1241, acute exposure of adiponectin resulted in a significant increase in mRNA expression of AdipoR1 (Figure 6.1.B, p=0.04), but no alterations in AdipoR2 (Figure 6.1.F, p=0.35) or APPL1 (Figure 6.1.J, p=0.42) or APPL2 (Figure 6.N.A, p=0.07). Acute adiponectin exposure following 6 weeks of treatment with CB_2 agonist AM1241 in a DIO model also did not alter soleus muscle mRNA expression of FAT/CD36 (Figure 6.1.A, p=0.50), AMPK (Figure 6.1.C, p=0.09), PDK4 (Figure 6.1.E, p=0.18) or PGC1-α (Figure 6.1.G, p=0.18). In the EDL of the DIO animals treated for six weeks with CB_2 agonist AM1241, acute exposure of adiponectin did not alter mRNA expression of FAT/CD36 (Figure 6.1.B, p=0.10), or PDK4 (Figure 6.1.K, p=0.13), but decreased AMPK (Figure 6.1.H, p=0.03) and increased PGC1-α (Figure 6.1.P, p=0.01).

Acute adiponectin exposure following 6 weeks of treatment with the CB_2 antagonist AM1241 in a DIO model did not alter soleus muscle mRNA expression of AdipoR1 (Figure 6.2.C, p=0.38), AdipoR2 (Figure 6.2.G, p=0.16), APPL1 (Figure 6.2.K, p=0.18) or APPL2 (Figure 6.2.O, p=0.13). In the EDL of the DIO animals treated for six weeks with the CB_2 antagonist AM630, acute exposure of adiponectin did not alter the mRNA expression of AdipoR1 (Figure 6.2.D, p=0.32), AdipoR2 (Figure 6.2.H, p=0.32), APPL1 (Figure 6.2.L, p=0.18) or APPL2 (Figure 6.2.P, p=0.07). Acute adiponectin exposure following 6 weeks of treatment with the CB_2 antagonist AM630 in a DIO model also did not alter soleus muscle mRNA expression of FAT/CD36 (Figure 6.2.C, p=0.50), AMPK (Figure 6.2.E, p=0.54), PDK4 (Figure 6.2.I, p=0.07) or PGC1-α (Figure 6.2.M, p=0.26). In the EDL of the DIO animals treated for six weeks with the CB_2 antagonist AM630, acute exposure of adiponectin also did not alter the mRNA expression of FAT/CD36 (Figure 6.1.D, p=0.23), AMPK (Figure 6.1.F, p=0.63), PDK4 (Figure 6.1.J, p=0.38) or PGC1-α (Figure 6.1.N, p=0.26).

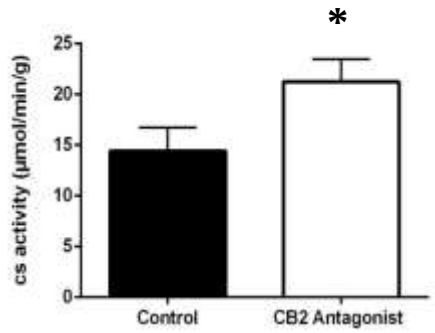
Citrate Synthase tested in whole Muscle



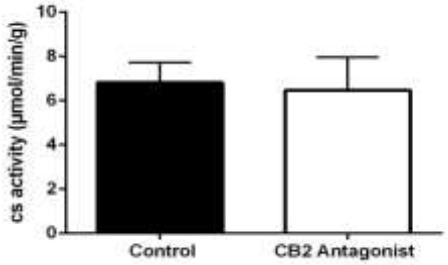
A; **Red Gastrocnemius**



B; **White Gastrocnemius**



C; **Red Gastronemius**



D; **White Gastronemius**

Figure 6.3.A *Citrate synthase expression in red gastrocnemius muscle in animals chronically administered with CB_2 agonist AM1241.* ***B*** *Citrate synthase expression in white gastrocnemius muscle of animals chronically administered with CB_2 agonist AM1241 treatment.* ***C*** *Citrate synthase expressions in red gastrocnemius muscle in animals chronically administered with CB_2 antagonist.* ***D*** *Citrate synthase expression in white gastrocnemius muscle of animals chronically administered with chronic CB_2 antagonist treatment in a DIO model. Significance is indicated by * compared to obese controls ($p < 0.05$, $n = 9$).*

The citrate synthase activity following six weeks of treatment with AM1241 did not change in the red gastrocnemius (Figure 6.5.A, p=0.35) however this activity was increased following six weeks of treatment with CB_2 antagonist AM630 (Figure 6.5.B, p=0.02). Citrate synthase activity in white gastrocnemius did not alter following six weeks of treatment with either AM1241 (Figure 6.5.C, p=0.14) or AM630 (Figure 6.5.D, p=0.18).

6.9 Discussion

CB_2 agonist treatment has previously resulted in weight loss and altered metabolic markers of obesity in a DIO mouse model (Verty et al. 2015). The effects of chronic CB_2 agonism and antagonism on skeletal muscle oxidative genes and adiponectin signalling are unknown. The results in this study show acute adiponectin ex vivo exposure on skeletal muscle from a DIO model following chronic CB_2 agonism treatment resulted in an increase in AdipoR1 and PGC-1α mRNA expression and a decrease in AMPK mRNA expression in the EDL muscle. Despite no alterations in the mRNA expression of key markers measured in this study, chronic treatment with CB_2 antagonist, AM630 increased levels of citrate synthase activity in the highly oxidative red gastrocnemius muscle.

This study is the first to show that chronic activation of CB_2 and acute adiponectin exposure resulted in an increase in AdipoR1 mRNA expression in skeletal muscle. In the muscle of diabetic humans, AdipoR1 levels have been associated with insulin by positively correlating with first-phase insulin secretion, and plasma triglyceride levels (Staiger et al 2004). In mice fed a high fat diet, exercise has been linked with improvement in insulin sensitivity and improvements in AdipoR1/APPL1 protein levels in the skeletal muscle (Farias et al. 2012). AdipoR1 expression is associated with improvements in skeletal muscle metabolic changes (Van Berendoncks et al. 2010). The mRNA expression in primary skeletal muscle myotubes, derived from human donors after exposure to globular adiponectin (gAd) resulted in an increase in AdipoR1 mRNA expression in lean tissue but not obese or diabetic tissue (McAinch et al. 2006). This finding suggests that increases in AdipoR1 expression indicates that the skeletal muscle of the chronic CB_2 agonist group may be adaptable towards resembling that of lean skeletal muscle. Obesity and a high fat diet are associated with an increase in low oxidative fibre types and equally an increase in insulin resistance. In hepatic tissue CB_2 expression

positively correlates with adiponectin-induced lipid metabolism (Auguet et al. 2004). Therefore, the observed increase in AdipoR1 in low oxidative fiber type when exposed ex vivo to adiponectin following six weeks of treatment to CB_2 agonism presents a possible novel pharmacological strategy in obesity.

An increase in AdipoR1 has also been found to result in a down regulation of an adiponectin-mediated increase in intracellular Ca^{2+} and a decrease in AMPK (Iwabu et al. 2010). AdipoR1 modulation results in changes to adiponectin induced AMPK activation (Yamauchi et al. 2007). Interestingly the AMPK levels in the CB_2 agonist group were significantly reduced in the EDL. AMPK plays a key role in increasing fatty acid oxidation and skeletal muscle functionality in diabetes (Yamauchi et al. 2007). In the skeletal muscle of humans with metabolic syndrome, the mRNA expression of AMPKα1 and AMPKα2 was found to be down regulated (Van Berendoncks et al. 2015). The decrease in AMPK in the DIO animals chronically treated with AM1241 in this study had no evidence of a change in circulating levels of either glucose or adiponectin (chapter 5). Evidence exists to suggest a relationship between CB_2 and AMPK. The CB_2 agonist, trans-caryophyllene (TC) ameliorated ischaemic injury in cultures of rat cortical neurons/glia, potentially through modulation of AMPK/CREB signalling pathways (Choi et al. 2013). AMPK has been shown to phosphyralate PGC-1α in skeletal muscle (Jäger et al. 2007).

This is in contrast to the results in this study that found chronic CB_2 agonism resulted in a significant increase in the PGC-1α mRNA expression in the EDL muscle. PGC-1α is strongly associated with adaptations of fibre type changes in skeletal muscle and increasing mitochondrial biogenesis and function (Iwabu et al. 2010). The AMPK/PGC-1α signaling pathway has been established in skeletal muscle (Cantó et al. 2010, Lira et al. 2010). Adiponectin increases the AMPK/PGC-1α signaling pathway and blocking adiponectin results

in the down regulation of AMPK/PGC- 1α signalling in a rat diabetic heart model (Yan et al. 2013). However, in this study the acute exposure to adiponectin resulted in a decrease in AMPK gene expression but an increase in PGC-1 α gene expression in skeletal muscle. Therefore, the increase in PGC-1α may be the result of other mechanisms, for example PGC-1α has been found to have a relationship with suppressing the pro-inflammatory cytokine interleukin 12 (IL-12) in isolated muscle cells. (Eisele et al. 2015). Further research should be undertaken to determine if the increase in PGC-1α was a mechanism in some way to counter the increase in IL-12p70. There was a significant increase in the circulating levels of inflammatory marker IL-12p70 in these animals when chronically exposed to CB_2 activation (Chapter 5).

There were no changes seen in FAT/CD36 or PDK4 mRNA expression upon acute adiponectin exposure following chronic CB_2 modulation. FAT/CD36 has a role in the regulation of fatty acid uptake by skeletal muscle (Joost et al. 2002). Previous research has suggested that in humans with either obesity or type II diabetes there is a fourfold increase of FAT/CD36 in muscle tissue (Bonen et al. 2004). This increase has also been shown to occur in the muscles of rats fed a high fat diet for two weeks resulting in an increase in FAT/CD36 and increased levels of fatty acid oxidation (Mullens et al. 2009). PDK4 is predominantly expressed in muscle (skeletal and heart) and plays a role in metabolism and mitochondrial oxidation (Pilegaard et al. 2004).

Intracellular APPL1 activation by adiponectin receptors is thought to be necessary for positive adiponectin induced effects of AMPK (Zhou et al. 2009). There were no changes seen in APPL1 or APPL2 mRNA expression upon acute ex vivo adiponectin exposure in the muscles of DIO animals chronically treated with a CB_2 agonist or antagonist in the present study. In rats fed a high fat diet for 10 weeks before 14 days treatment with AM251, the mRNA expression of AdipoR1 and CB_2 were reduced significantly (Crespillo et al. 2011).

Chronic treatment with CB_2 antagonist treatment did not result in changes to the mRNA expression of skeletal muscle when acutely exposed to adiponectin. In the whole muscle of the DIO model chronically treated with AM630 resulted in a significant increase in citrate synthase (CS) expression in the whole muscle of highly oxidative red gastrocnemius. CS is localized in the mitochondrial matrix and is used as a quantitative marker of enzyme content of intact mitochondria (Holloszy et al 1970). The results of the current study support those observed in CB_2 knockout mice fed a high fat diet which displayed markers of improved insulin sensitivity (Agudo et al. 2010). The animals in the current study were not insulin resistant (Chapter 5), however an increase in citrate synthase is associated with an increase in oxidative capacity and is down regulated in highly oxidative type one muscle fibre types in diet-induced diabetes (Simoneau et al. 1985). An increase in mitochondrial substrate citrate synthase was seen in the highly oxidative type one muscle following chronic CB_2 antagonist treatment; therefore, despite no alterations observed in mRNA expression of key markers of oxidative metabolism and adiponectin treatment, further work is warranted on whether CB_2 antagonism is a viable pharmacological target in a DIO model.

As previously discussed limited studies have been conducted on CB_2 modulation in the obese state. This study showed that CB_2 antagonist treatment had no effect on mRNA expression in skeletal muscle when acutely exposed to adiponectin in an ex vivo model. There was an increase in citrate synthase activity in the whole muscle which could have major implications in the adaptation of skeletal muscle in a DIO model. Increasing the number of mitochondria, increases the likelihood of elevating oxidative capacity, therefore resulting in increased fatty acid and glucose clearance, possibly decreasing the obesity related effects on skeletal muscle.

The results in this study showed few significant outcomes, if the research was repeated and a working hypothesis established based on the resuts of the current study. An increase to the dosage of both CB_2 agonism and antagonism would be adviced. The exact concentration would be decided by a dose response protocol on a sample of animals. Futhermore as this research investigated the skeletal muscle response. It would be beneficial to have a group that was obese and underwent exercise during the treatment period. This would investigate if the increase in the expended energy required for execise training, results in an influence on the effects of CB_2 agonism and antagonism.

Further research into the mRNA expression of the whole muscle following CB_2 chronic antagonism is needed to fully understand if the increase in mitochondrial activity demonstrated by elevated citrate synthase activity results in increases in fatty acid oxidative gene expression. Regardless this is an exciting finding that further contributes to the understanding of CB_2 antagonist treatment in a DIO model.

6.10 Conclusion

This study is the first to confirm a relationship between *in vitro* CB_2 modulation and potentially positive skeletal muscle adaptions. In this study the null hypothesis has been neither accepted nor rejected as the results showed that the chronic activation of CB_2 receptors via agonist administration resulted in the increased detection of AdipoR1 mRNA expression and the detection of a decrease of AMPK mRNA expression in the EDL following acute adiponectin treatment. However, no changes were seen in the enzyme activity in the skeletal muscle of animals treated for six weeks with the CB_2 agonist, the null hypothesis has both been accepted and rejected in the case of CB_2 antagonism. Chronic blockage of the CB_2 receptors was shown to have no effect on the modulation of mRNA expression. However, in whole muscle CB_2

antagonist treatment resulted in an increase in citrate synthase in the highly oxidative red gastrocnemius skeletal muscle. Collectivley these data confirm a possible relationship between CB_2 modulation and skeletal muscle oxidative capacity the mechanisms that surround skeletal muscle adaptions during chronic CB_2 modulation in diet induced obesity is essential to fully understand the biologically processes involved.

Chapter 7 - Cannabinoid receptor antagonism mediates adiponectin receptor mRNA expression and adiponectin signalling in obese and obese diabetic derived human skeletal muscle myotubes.

7.0 Abstract

Adiponectin improves glucose tolerance, and increases fatty acid oxidation and mitochondrial biogenesis in skeletal muscle. Circulating levels of adiponectin however are decreased in obesity. Adiponectin once inside the cell binds with protein adapters APPL1 and APPL2 (Mao et al. 2006). It is unknown how blocking cannabinoid receptors alters the mRNA expression of key signalling pathways involved in adiponectin signalling. Human skeletal muscle samples were sourced from individuals undergoing routine lap band surgery, muscle was taken from the rectus abdominus muscle. Biopsies were obtained from obese and obese diabetic individuals (n=11 in each group). Human myotubes were grown and treated with either CB_1 antagonist, AM251, or CB_2 antagonist, AM630, in isolation or in combination with globular adiponectin (gAD). The AdipoR1 mRNA expression significantly increased in the myotubes derived from obese individuals when exposed to gAD. AdipoR1 mRNA expression increased in diabetic tissue when exposed to CB_1 antagonist treatment. AdipoR2 mRNA expression increased in myotubes derived from people with diabetes when exposed to the CB_2 antagonist AM630. This study provides further evidence to support the yet to be elucidated role of cannabinoid receptors in adiponectin signalling in skeletal muscle

7.1 Introduction

Adiponectin (also known as Acrp30, AdipoQ) is secreted predominately from adipocytes and plays a protective role against metabolic disorders such as obesity (Lihn et al. 2005; Bruce et al. 2005). Adiponectin induces beneficial effects in skeletal muscle by improving glucose tolerance, increasing fatty acid oxidation and mitochondrial biogenesis (Lihn et al. 2005; Yang et al. 2006; Mao et al. 2006). Circulating levels of adiponectin however are decreased in obese individuals (Kopp et al. 2005; Bruce et al. 2005; Yang et al. 2006). In skeletal muscle obesity may result in a change in fibre type; insulin resistance; increased intramyocellular triglycerides and the onset of adiponectin resistance (Yang et al. 2006).

Adiponectin is transported into the cell via two seven-transmembrane domain- containing proteins known as Adiponectin Receptor 1 (AdipoR1) and Adiponectin Receptor 2 (AdipoR2) (Tanabe et al. 2015). AdipoR1 is found primarily in skeletal muscle and binds principally to globular adiponectin (gAd) which makes up approximately 1% of circulating adiponectin (Fruebis et al. 2006). AdipoR2 although mainly expressed in the liver is also present in skeletal muscle, albeit at lower levels compared to AdipoR1 (Fruebis et al. 2006). AdipoR2 binds to full length adiponectin which makes approximately 99% of circulating adiponectin (Fruebis et al. 2006). Adiponectin signalling occurs via the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Rawlings et al. 2004, Kanatani et al. 2007). Adiponectin once inside the cell binds with protein adapters (Mao et al. 2006). It relies on this interaction to enable a cascade of signalling events involving multiple cell responses, for example intracellular adiponectin binds directly with APPL to initiate a downstream cascade of intramyocellular signalling (Mao et al. 2006, Wang et al. 2009). This multidomain adaptor protein is part of membrane trafficking involving a number of signal transduction pathways

(Zhu et al. 2007). APPL consists of two main isoforms known as APPL1 and APPL2 (Chial et al. 2008, Iwabu et al. 2010).

APPL1 is mediated by the N-terminal BAR domain (Zhu et al. 2007). APPL1 functions as a positive mediator in the adiponectin pathway (Mao et al. 2006). Studies have shown that APPL1 plays a crucial role in insulin stimulated glucose transporter signaling (Saito et al. 2007). Overexpression of APPL1 increases, and suppression of APPL1 level reduces, adiponectin signalling (Wang et al. 2009). APPL2 is an isoform of APPL1, and they share up to 54% identity in protein sequences (Mao et al. 2006; Wang et al. 2009). APPL2 has been found to play a vital role in cell proliferation and embryonic development (Miaczynska et al. 2004, Wang et al. 2009). APPL2 is thought to play an opposing role to APPL1, as APPL2 binds to AdipoR1 and AdipoR2, inhibiting the ability of APPL1 to initiate adiponectin mediated positive effects on fatty acid oxidation and insulin sensitivity (Wang et al. 2009).

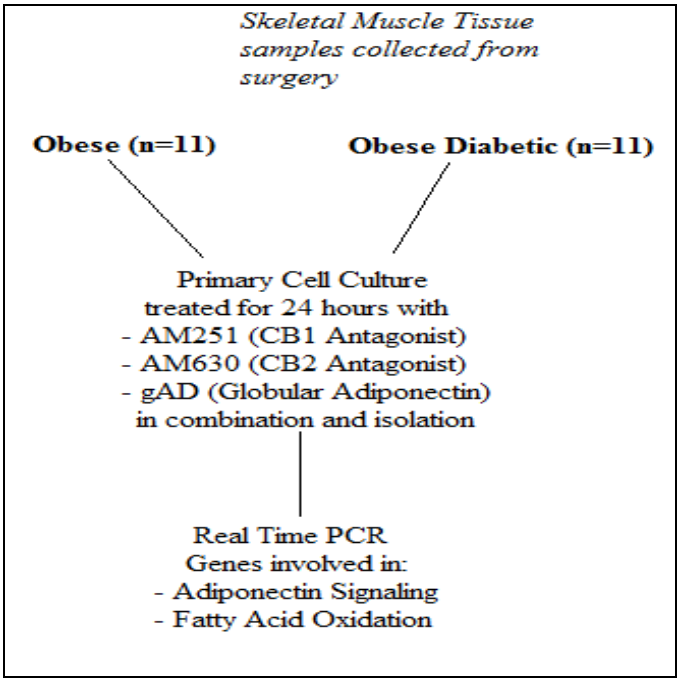
Once adiponectin has entered a skeletal muscle cell, intracellular proteins are modulated by downstream phosphorylation in a signalling cascade (Myeong et al. 2006). Peroxisome proliferator-activated receptors (PPAR) are a part of the nuclear receptor superfamily; they are involved in mitochondrial fatty acid catabolism and adipogenesis (Toshiya et al. 2003). Adiponectin increases PPARα activity and increases the expression of intramyocellular genes (ACO, CPT I, and FABP3) in C_2C_{12} myotubes (Myeong et al. 2006). Also involved in adipogenesis is the transcription factor Forkhead box protein O1 (FOXO1) (Wang and Tong, 2009). Adiponectin has been found to exert its modulation mechanisms through FOXO1 (Kajimura et al. 2009). Pyruvate dehydrogenase kinase 4 (PDK4) plays an important role in metabolism, through the regulation of mitochondrial fatty acid oxidation during times of metabolic stress for example: starvation and diabetes (Holness et al. 2000). Adiponectin has

been found decrease PDK4 in human myotubes from obese and diabetic tissue (McAinch and Cameron-Smith, 2009). Adiponectin works via PPAR gamma and FOXO1 to mediate its effects. PPAR gamma, FOXO1 and PDK4 are connected with adiponectin intracellular signalling via increases in fatty acid oxidation.

The endogenous cannabinoid system

The endogenous cannabinoid system is a lipid signalling system shown to play a role in the regulation of appetite and energy metabolism (Trillou et al. 2004; Bermudez-Silva et al. 2005; Cavuoto et al. 2007). Endocannabinoid ligands enter cells via cannabinoid receptors (Trillou et al. 2004). The two main recognized cannabinoid receptors are known as CB_1 and CB_2 which are both seven-transmembrane-domain G protein-coupled receptors (Carayon et al. 2009; Iwabu et al. 2010). The endocannabinoid receptor CB_1 has been found in skeletal muscle; liver and endocrine cells of the pancreas (Pagotto et al. 2007; Engali, 2008; Escartin-Perez et al. 2008). The endocannabinoid system is known to play an important role in body weight and metabolic regulation (Trillou et al. 2004; Despres et al. 2005; Bluher et al. 2006). In the obese state, circulating levels of endocannabinoids are increased, which results in over eating and a promotion of lipid storage (Trillou et al. 2004; Despres et al. 2005). However, in the presence of a CB_1 specific antagonist there is a suppression of appetite and progressive weight loss (Trillou et al. 2004; Despres et al. 2005; Bluher et al. 2006). CB_1 receptors are thought to be the most widely expressed and are directly related to metabolic pathways (Bensaid et al. 2002; Liu et al. 2004; Escartin-Perez et al. 2008). CB_2 mRNA has been detected in the marginal zones of the spleen; immune cells; brain microglia; intra-abdominal adipose tissue and skeletal muscle (Facci, 1995; Cavuoto et al. 2007; Bermudez-Silva et al. 2007; Staton et al. 2008).

Improvements in skeletal muscle fatty acid oxidation and insulin sensitivity in skeletal muscle have been seen when CB_1 signalling is blocked (Deveaux et al. 2009). Moreover, recent studies have also shown CB_2 knockout mice displayed the same positive effects (Deveaux et al. 2009; Agudo et al. 2010). The effect of CB_1 and CB_2 antagonism and the relationship between adiponectin receptor expression and resistance in skeletal muscle is not known. It is hypothesised that blockade of CB_1 and CB_2 receptors by an antagonist will result in increased adiponectin sensitivity. Therefore, the current study will determine the mRNA expression of AdipoR1, AdipoR2, APPL1 and APPL2 as well as PPAR, FOXO1, PDK4 in the presence of CB_1 and CB_2 antagonist following treatment with gAd to determine the relationship between adiponectin signalling and the cannabinoid receptors in human skeletal muscle myotubes. The hypothesis is that cannabinoid receptor antagonism will result in modulation of mRNA expression associated with improved adiponectin signalling in obese and obese diabetic derived human skeletal muscle myotubes.



7.2 Materials and methods

7.3 Skeletal Muscle samples

The human skeletal muscle samples were sourced from individuals undergoing routine lap band surgery at 'The Avenue Hospital, Melbourne, Australia'. Subjects were recruited to participate in this study by giving their written and informed consent. Individual subject characteristics were recorded. Following a fast (8-18h), general anaesthesia was induced with propofol (i.v.) and maintained by inhalation of a fentanyl and rocuronium and volatile anaesthesia mixture. Research was approved by Victoria University and The Avenue Hospital ethics committees (approval numbers 08/150 and trial 0100 respectively). A muscle biopsy was collected by the performing surgeon from the rectus abdominus muscle. Skeletal muscle biopsies were obtained from obese and obese diabetic (n=11) patients undergoing routine bariatric surgery. Human primary skeletal muscle myotubes were cultured according to the method previously described (McAinch et al. 2006). Briefly this involved cleaning the dissected muscle samples of visible adipose tissue and blood vessels. Then 3 ml 0.05% Trypsin-EDTA (Invitrogen® # 25300) was added and the tissue was minced with a sterile scalpel blade. After mincing, the samples were transferred along with the medium to a sterile conical flask with 12 ml of 0.05% Trypsin-EDTA (Invitrogen® # 25300) and placed on a shaker at low speed for 20 minutes at room temperature. Cells were grown on coated ECM and once deemed confluent at 70-80% the cells were split and cultivated, and then frozen (in LN_2 at -180^0C) for subsequent use. Once a cell line was thawed, it was again grown to 70-80% confluence before seeding onto 6 well plates. Once these cells were 70-80% confluent, the media was changed to induce differentiation (α-MEM, 2% horse serum (vol/vol), 0.5% penicillin (vol/vol), and 0.5% Fungizone (vol/vol) which continued for a period of four to five

days to enable cells to develop into mature myotubes. Following this period, the human myotubes were incubated for 2 hours in serum free α-MEM and 0.1% BSA treatment. Myotubes were treated with either a CB_1 or CB_2 antagonist in isolation or in combination with globular adiponectin for a 24 hour period; CB1 antagonist 5 µM - AM251 (Sigma® # 71670) and CB_2 antagonist 50 µM - AM630 (Enzo® # CR-107) both were dissolved in 100% Ethanol (EtOH). Globular Adiponectin was dissolved in EtOH and used at a concentration of 0.1 µg/ml. Total EtOH in each treatment was standardised to 0.2% (in alpha-MEM-BSA medium).

Table 7.1: Clinical Characteristics of skeletal muscle donors

	Obese (n=11)	**Obese Diabetic (n=11)**
Sex	6 Females 5 Males	6 Females 5 Males
Age	46.9 ± 3.76	47.4 ± 2.01
Weight (kg)	110 ± 5.58	126 ± 7.06
BMI (kg/m^2)	40.67 ± 1.54	42.68 ± 2.26
Fasting Plasma Glucose (mmol/L)	5.26 ± 0.09	10.91 ± 1.21*****
Plasma Insulin (mIU/ml)	5.51 ± 1.04	21.57 ± 5.30*****
HbA1c	5.51 ± 0.09	8.27 ± 0.64*****
Fasting Triglycerides (mmol/L)	1.53 ± 0.23	2.4 ± 0.44
Cholesterol (mmol/L)	5.16 ± 0.38	4.64 ± 0.37

7.4 RNA extraction and 'Real-Time' Polymerase chain reaction

Following hours of exposure to gAd, leptin, or control (α-MEM), the medium was removed and cells were rinsed with 1× phosphate-buffered saline. The homogenate was then stored at −80 °C before total cellular RNA extraction. First-strand cDNA was generated from 0.5 µg RNA using an AMV RT kit (Promega, Madison, WI). Real-time polymerase chain reaction was performed using the ABI PRISM 5700 sequence detection system (Applied Biosystems, Foster City, CA). Polymerase chain reactions were performed using SYBR Green I chemistry (Applied Biosystems). Oligonucleotide primers forward and reverse primers, respectively were designed using Primer Express software package, version 1.0 (Applied Biosystems) from GenBank sequences. Fluorescence emission data were captured, and gene expression was determined using the critical threshold (CT) values for each gene, with all data expressed relative to the housekeeping gene (cyclophilin) using the $2-\Delta CT$ equation, as arbitrary units (Schmittgen et al. 2000).

0.5 µg of RNA was added to the required amount of DEPC H_2O (Invitrogen® # 46-2224) to make a total solution volume of 5 µl. Using the iScript™ cDNA Synthesis Kit (Bio-Rad® # 170-8890) 2 µl of iScript reaction mix and 0.5 µl of iScript reverse transcriptase was added to each sample. This solution of RNA was subsequently incubated for a total of three reaction times being 5 minutes at 25 ˚C; 30 minutes at 42 ˚C; 5 minutes at 85 ˚C using the PCR Express Thermal Cycler. The 10 µl of cDNA was then diluted to a volume of 200 µl using nuclease free water and stored at -20 °C for subsequent analysis.

Real-Time RT-PCR was used to assess mRNA expression. Real-Time RT-PCR was performed using the BIO-RAD My iQ® single colour real-time PCR detection system (model no – My iQ® - optics model; serial number – 569BR/0611) and BIO-RAD iQ® SYBR® Green

Supermix. A master mix solution of 8 µl of 2 X SYBR Green; 2 µl of forward primers; 2 µl of reverse primers; 6 µl of sterile milli-Q water and 2 µl of sample cDNA was heated for 3 minute at 95 °C followed by 40 cycles of 10 seconds at 95 °C followed by 45 seconds at 60 °C, followed by 1 minute at 95 °C , followed by 1 minute at 55 °C, followed by the heat dissociation step utilising the BIO-RAD My iQ® sequence detection system. The heat dissociation step involved heating samples over 55 - 95 °C following the final cycle of the PCR. The increase in temperature causes PCR products to undergo denaturation that is accompanied by a decrease in fluorescence for solutions containing SYBR green chemistry.

Denaturation enables the molecules to be separated to form single strands. This step allows the specific annealing between the primers and the single strands of the target DNA. Analysis of PCR results were calculated using the ($2^{-\Delta\Delta CT}$) method normalized to the average of the house keeping genes cyclophilin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

7.5 Primers

Real-time PCR analysis primers were designed using Oligoperfect design (Invitrogen™) from gene sequences obtained from GenBank. Forward and reverse primer sequences are shown in table 7.2.

Table 7.2: Oligonucleotide primers for 'real-time' PCR primers

Genes	Accession number	Forward primer	Reverse primer
Cyclophilin	NM 021130.3	CATCTGCACTGGCAAGACTGA	TTCATGCCTTCTTTCACTTTGC
GAPDH	NM_002046.3	CAACGACCACTTTGTCAAGC	TTACTCCTTGGAGGCCATGT
AdipoR1	NM_001127687.1	CGCCATGGAGAAGATGGAA	TCATATGGGATGACCCTCCAA
AdipoR2	NC_000012.12	GGATCCCCGAACGCTTTTT	TGAGACACCATGGAAGTGAACAA
APPL1	NM_012096.2	TCACTCCTTCCCCATCTTTC	TAGAGAGAGGGCAGCCAAAT
APPL2	NM_018171.3	CACGCCCAATGGAAAATC	CGACTGCCTCAGGGTTGT
PPARα	NC_020173.2	ACGATGCTGTCCTCCTTGATG	GTGTGATAAAGCCATTGCCGT
PDK4	NM_053551.1	GGGATCTCGCCTGGCACTTT	CACACATTCACGAAGCAGCA
PGC-1α	NM_013261.3	ACCCACAGGATCAGAACAAACC	GACAAATGCTCTTTGCTTTATTGC
PDK4	NM_053551.1	GGGATCTCGCCTGGCACTTT	CACACATTCACGAAGCAGCA

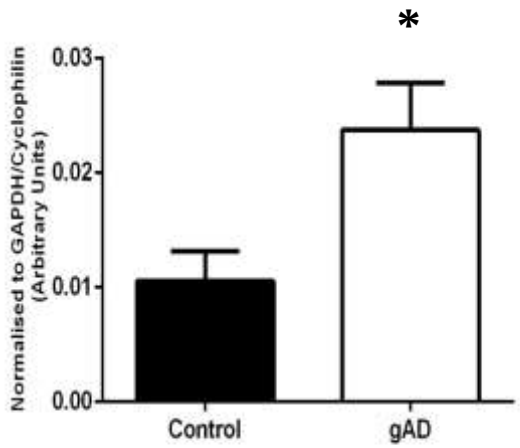
GAPDH: *glyceraldehyde-3-phosphate dehydrogenase;* ***AdipoR1:*** *Adiponectin receptor 1;* ***AdipoR2****: Adiponectin receptor 2;* ***APPL1*** *Adaptor protein containing PH domain, PTB domain and leucine zipper motif 1;* ***APPL2:*** *Adaptor protein containing PH domain, PTB domain and leucine zipper motif 2;* ***PPAR:*** *peroxisome proliferator-activated receptor gamma co-activator 1 alpha;* ***FOX01:*** *Forkhead box protein O1****; PDK4:*** *pyruvate dehydrogenase kinase 4.*

7.6 Statistical Analysis

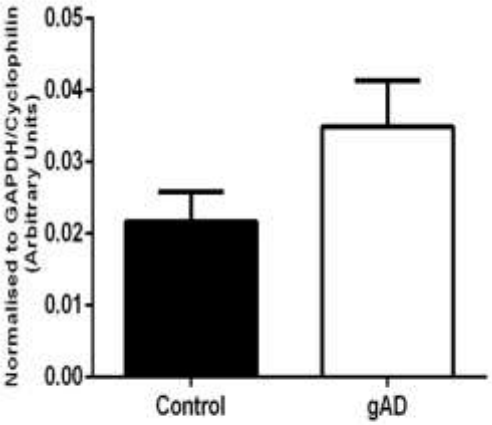
SPSS statistical package software (SPSS, Inc, Chicago, IL) was used for all statistical analysis. All data are presented as mean ± SEM. Analysis of groups was determined using an independent samples T-Test for two group direct analysis or mixed model ANOVA with Tukey's post-hoc tests. * Significance was accepted when $p < 0.05$.

7.7 Results

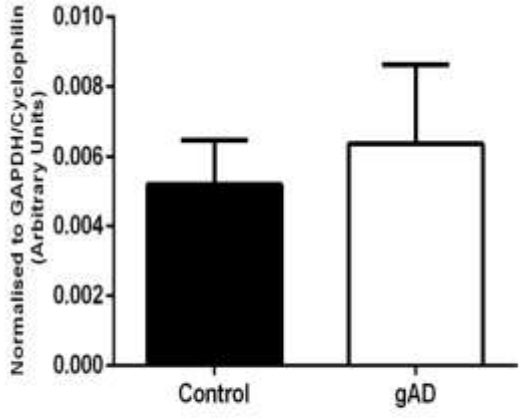
gAD treatment



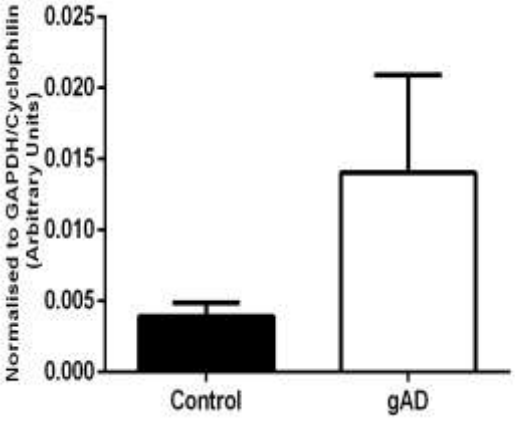
A; AdipoR1



B; AdipoR1

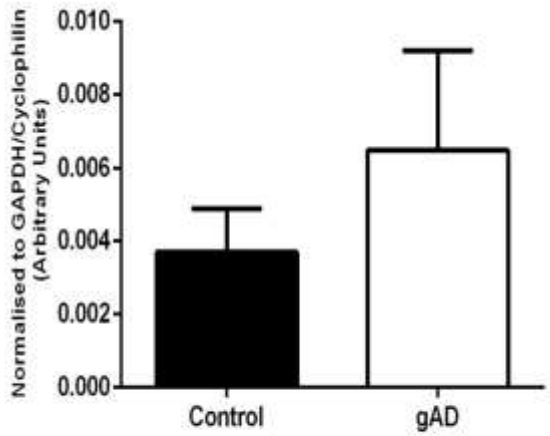


C; AdipoR2

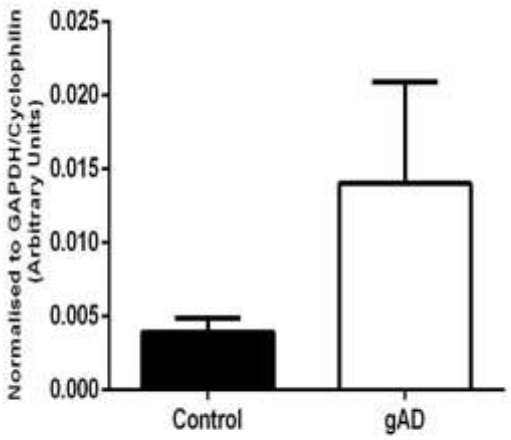


D; AdipoR2

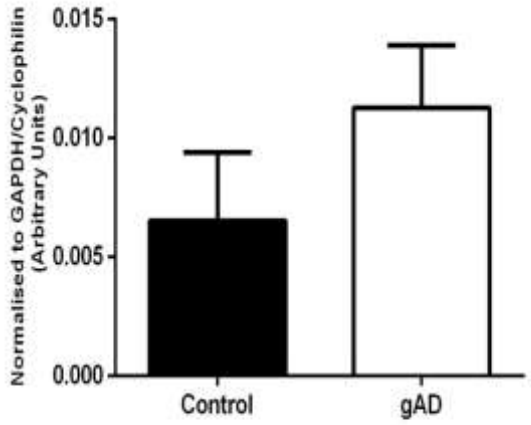
Figure 7.1.A *Human myotubes derived from skeletal muscle of obese individuals, treated with globular adiponectin and tested for mRNA levels of AdipoR1.* **B** *the mRNA gene expression of AdipoR1 in myotubes from diabetic human tissue treated with globular adiponectin.* **C** *Human myotubes derived from obese skeletal muscle and treated with globular adiponectin and tested for mRNA levels of AdipoR2.* **D** *the mRNA gene expression of AdipoR2 in myotubes from diabetic human tissue treated with globular adiponectin. Results shown in arbitrary units ± SEM. Significance is indicated by * compared to control ($p < 0.05$, $n = 11$).*



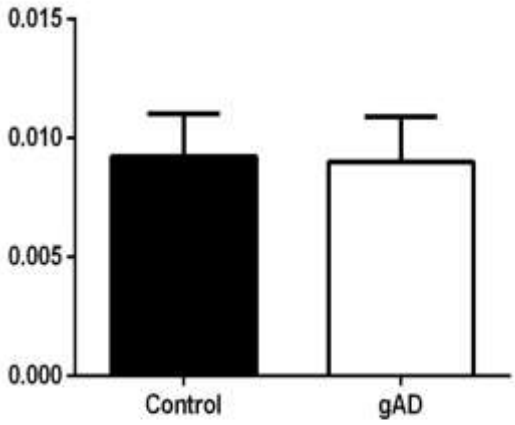
E; APPL1



F; APPL1



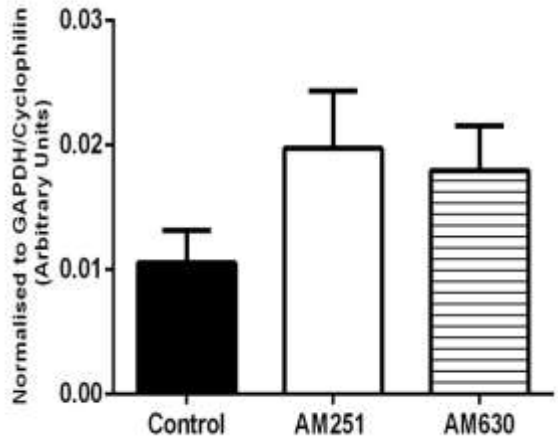
G; APPL2



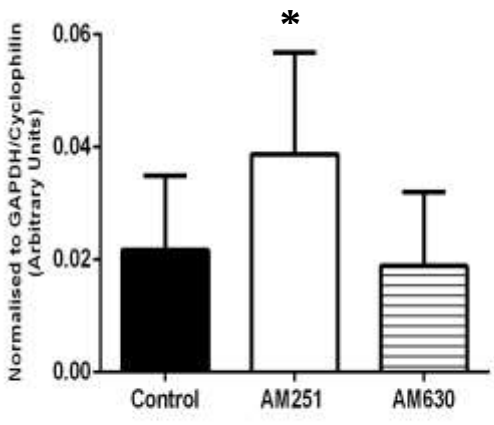
H; APPL2

Figure 7.1.E *Human myotubes derived from obese skeletal muscle, treated with globular adiponectin and tested for mRNA levels of APPL1.* **F** *the mRNA gene expression of APPL1 in myotubes from diabetic human tissue treated with globular adiponectin.* **G** *Human myotubes derived from obese skeletal muscle, treated with globular adiponectin and tested for mRNA levels of APPL2.* **H** *the mRNA gene expression of APPL2 in myotubes from diabetic human tissue treated with globular adiponectin. Results shown in arbitrary units ± SEM (n = 11).*

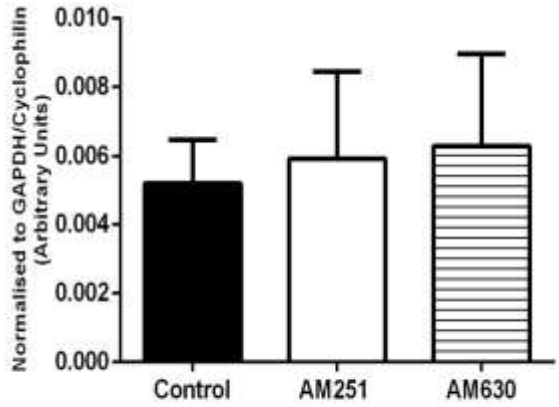
AM251, AM630 Treatment



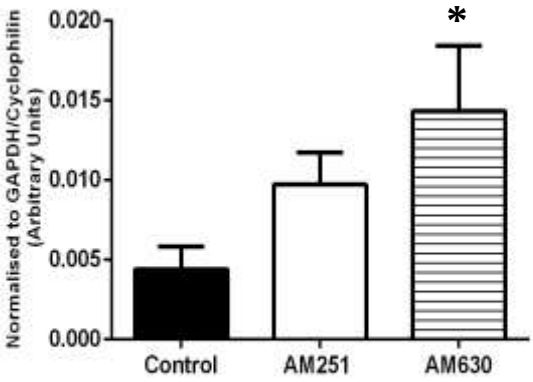
A; AdipoR1



B; AdipoR1

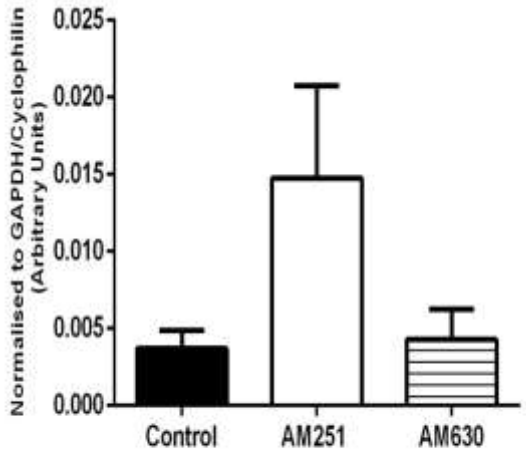


C; AdipoR2

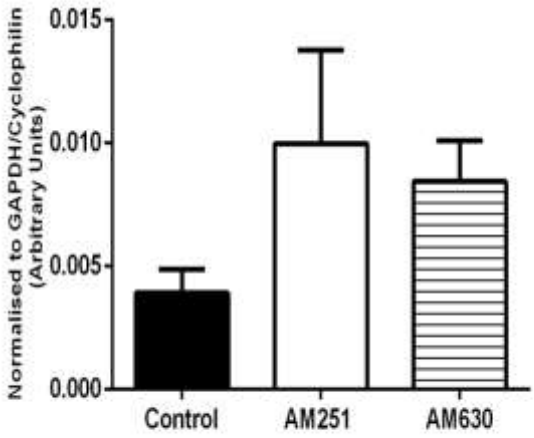


D; AdipoR2

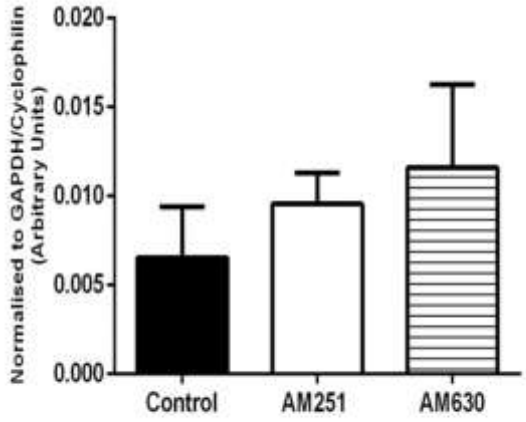
Figure 7.2.A *Human myotubes derived from obese skeletal muscle, treated with AM251 or AM630 and tested for mRNA levels of AdipoR1.* **B** *the mRNA gene expression of AdipoR1 in myotubes from diabetic human tissue treated with AM251 or AM630.* **C** *Human myotubes derived from obese skeletal muscle, treated with AM251 or AM630 and tested for mRNA levels of AdipoR2.* **D** *the mRNA gene expression of AdipoR2 in myotubes from diabetic human tissue treated with AM251 or AM630. Results shown in arbitrary units ± SEM. Significance is indicated by * compared to controls ($p < 0.05$, $n = 11$).*



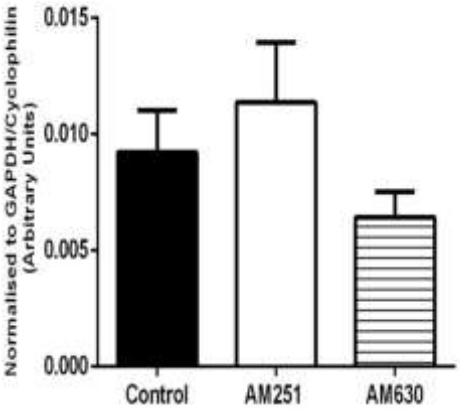
E; APPL1



F; APPL1



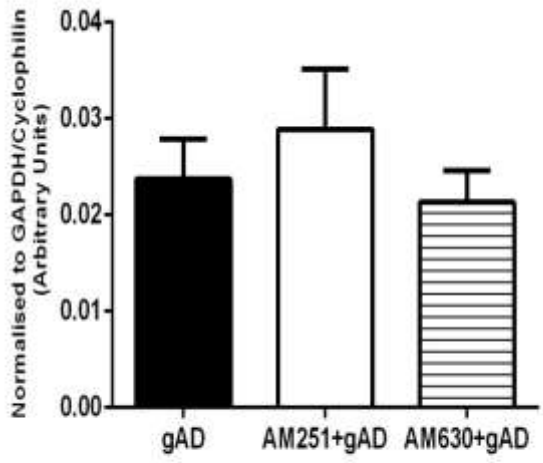
G; APPL2



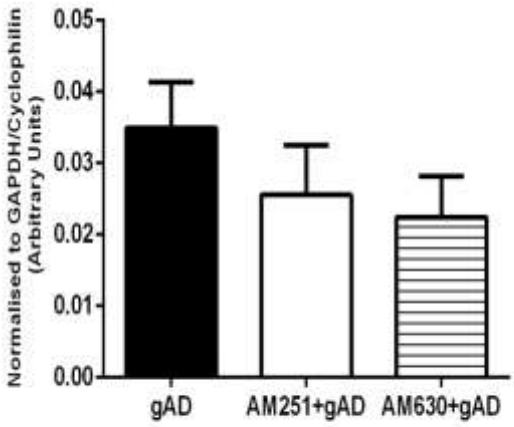
H; APPL2

Figure 7.2.E *Human myotubes derived from obese skeletal muscle, treated with AM251 or AM630 and tested for mRNA levels of APPL1.* **F** *the mRNA gene expression of APPL1 in myotubes from diabetic human tissue following antagonist treatment.* **G** *Human myotubes derived from obese skeletal muscle, treated with either CB_1 or CB_2 antagonist and tested for mRNA levels of APPL2.* **H** *the mRNA gene expression of APPL2 in myotubes from diabetic human tissue ($p < 0.05$, $n = 11$).*

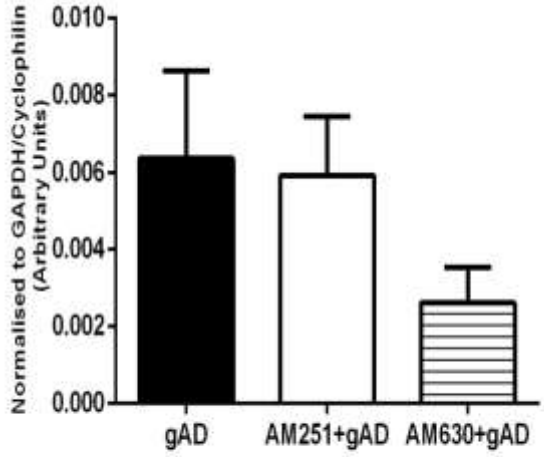
gAD, AM251 + gAD, AM630 + gAD Treatment



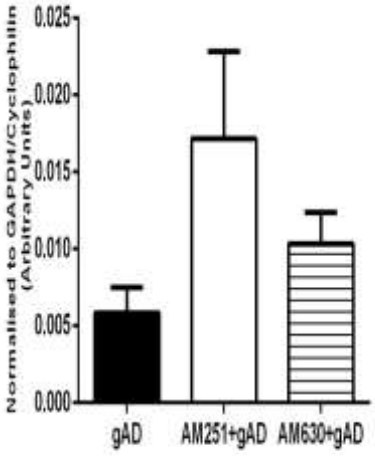
A; **AdipoR1**



B; **AdipoR1**

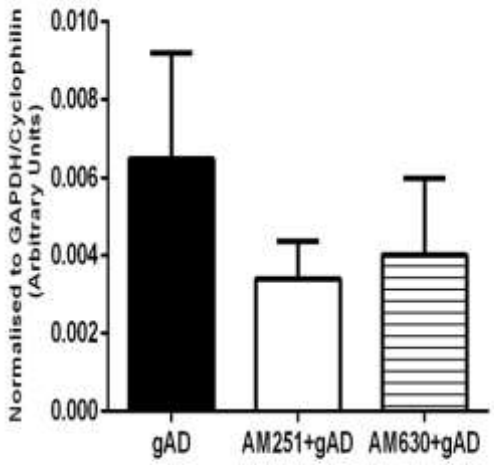


C; **AdipoR2**

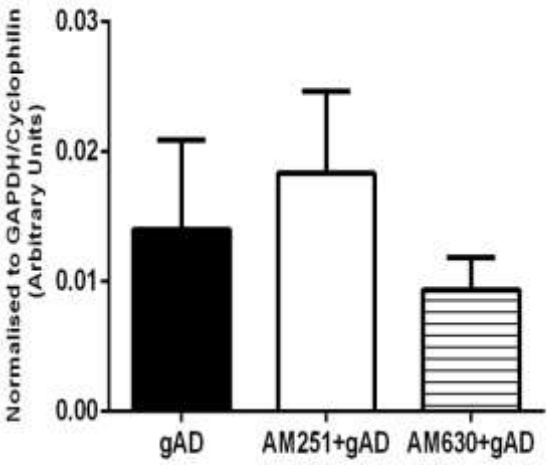


D; **AdipoR2**

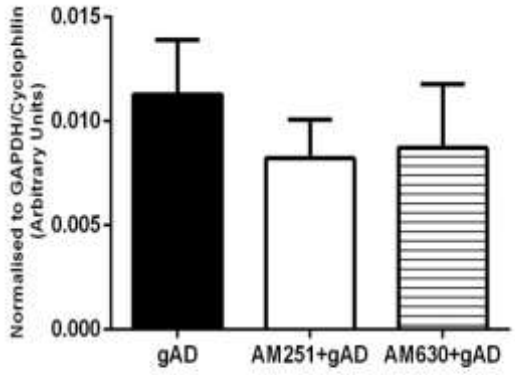
Figure 7.3.A *Human myotubes derived from obese skeletal muscle, treated with either globular adiponectin in isolation or in combination with CB_1 antagonist AM251 or CB_2 antagonist AM630 and tested for mRNA levels of AdipoR1.* **B** *the mRNA gene expression of AdipoR1in myotubes derived from diabetic tissue.* **C** *Human myotubes derived from obese skeletal muscle and treated with globular adiponectin or CB_1 or CB_2 antagonist, and tested for mRNA levels of AdipoR2.* **D** *the mRNA gene expression of AdipoR2 in myotubes from Diabetic tissue ($p < 0.05$, $n = 11$).*



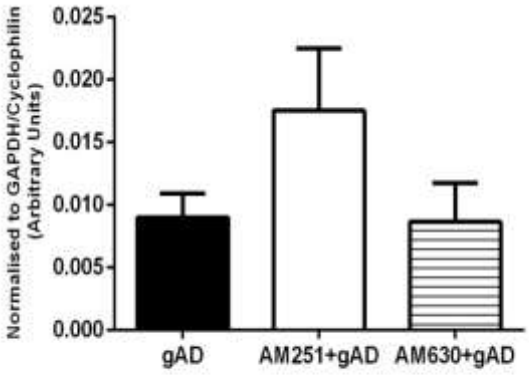
E; APPL1



F; APPL1



G; APPL2

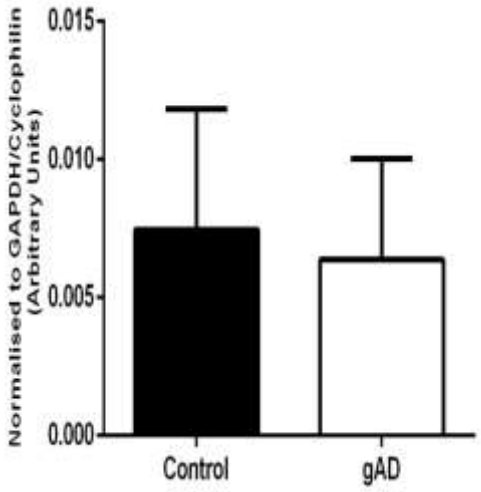


H; APPL2

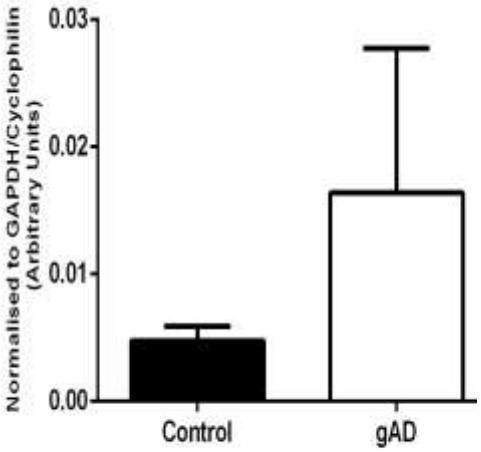
Figure 7.3.E *Human myotubes derived from obese skeletal muscle, treated in isolation or combination with CB_2 antagonist with gAD and tested for mRA levels of APPL1.* **F** *the mRNA gene expression of APPL1 in myotubes from diabetic human tissue.* **G** *Human myotubes derived from obese skeletal muscle, treated in combination with CB_1 and CB_2 antagonist with gAD and tested for mRNA levels of APPL2.* **H** *the mRNA gene expression of APPL2 in myotubes from diabetic human's results shown in arbitrary units ($p < 0.05$, $n = 11$).*

Exposure to gAD resulted in an increase in AdipoR1 mRNA expression in the obese derived human primary skeletal muscle myotubes (p=0.01) *(Figure 7.3.A).* Following gAd exposure AdipoR1 in the diabetic derived myotubes failed to reach significance at (p=0.09), and no other changes were following this treatment. The next section of results looked at whether treatment with the CB_1 antagonist, AM251, or CB_2 antagonist, AM630, altered gene expression following 24 hours of exposure. There was a significant increase in the mRNA expression of AdipoR1 in the diabetic derived myotubes (P=0.02) following treatment with AM251 *(Figure 7.3.B).* Similarly, in the diabetic derived myotubes there was a significant increase in AdipoR2 mRNA expression when exposed to CB_2 antagonist treatment for 24 hours *(Figure 7.3.D).* APPL1 in the obese tissue failed to reach significance (p=0.07) in either AM251 or AM630 gene expression in these groups did not alter compared to gAD alone, or when antagonist treatment was combined with gAD.

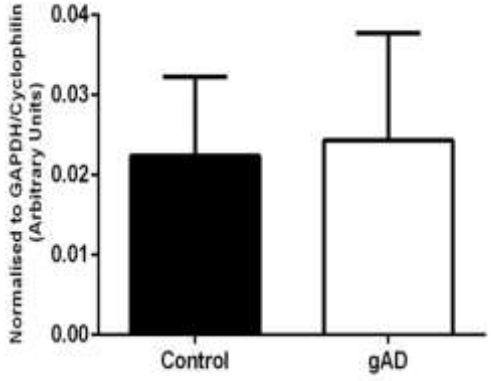
Intramyocellular proteins known to be modulated by adiponectin



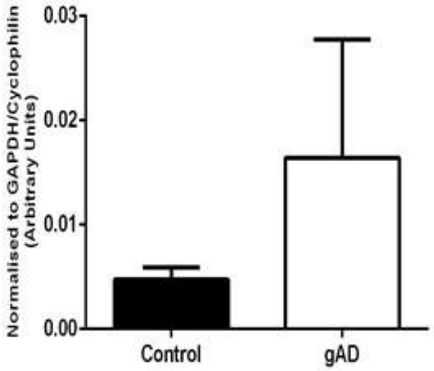
A; PPAR



B; PPAR

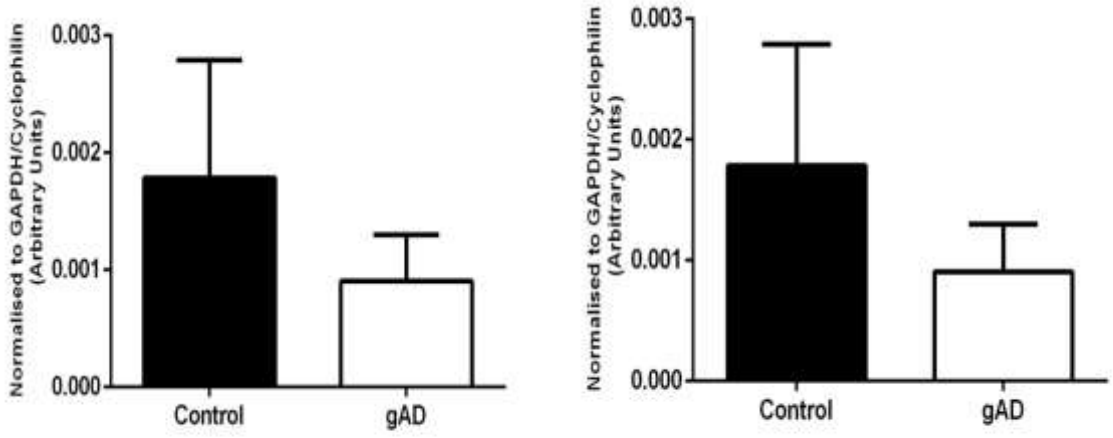


C; PDK4



D; PDK4

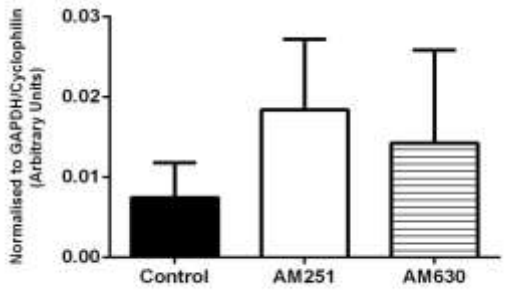
Figure 7.4.A *Human myotubes derived from skeletal muscle of obese individuals, treated with globular adiponectin (n=6) and tested for mRNA levels of PPAR against control (n=8).* **B** *the mRNA gene expression of PPAR in myotubes from diabetic human tissue treated with globular adiponectin (n=6) compared to control (n=6).* **C** *Human myotubes derived from obese skeletal muscle and treated with globular adiponectin (n=6) and tested for mRNA levels of PDK4 compared to control (n=7).* **D** *the mRNA gene expression of PDK4 in myotubes from Diabetic human's following acute exposure to globular adiponectin (n=4) and compared to control (n=5).*



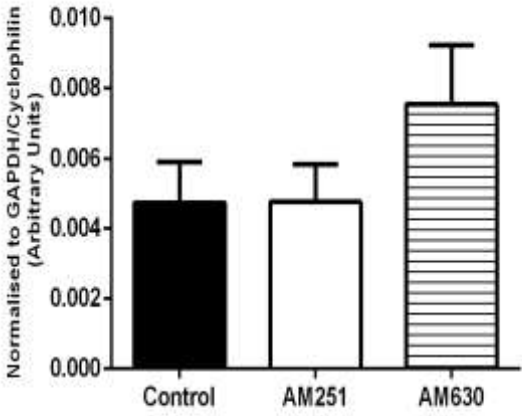
E; FOX01 **F; FOX01**

Figure 7.4.E *Human myotubes derived from obese skeletal muscle treated with globular adiponectin (n=6) and tested for mRNA levels of FOX01 against control (n=6).* **F** *the mRNA gene expression of FOX01 in myotubes from diabetic human's following acute globular adiponectin exposure (n=4) and compared to control (n=4).*

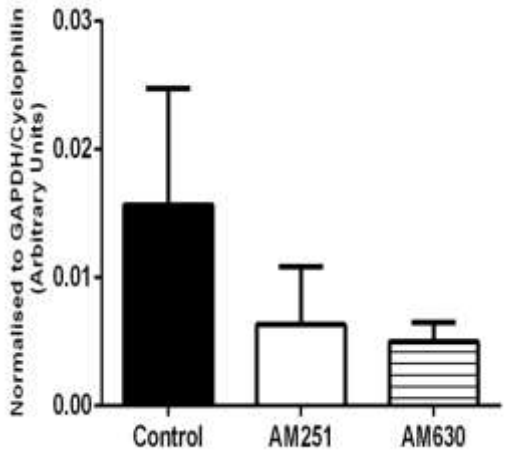
AM251 + AM630



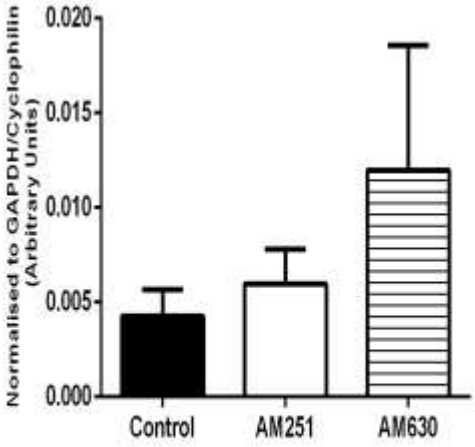
A; PPAR



B; PPAR

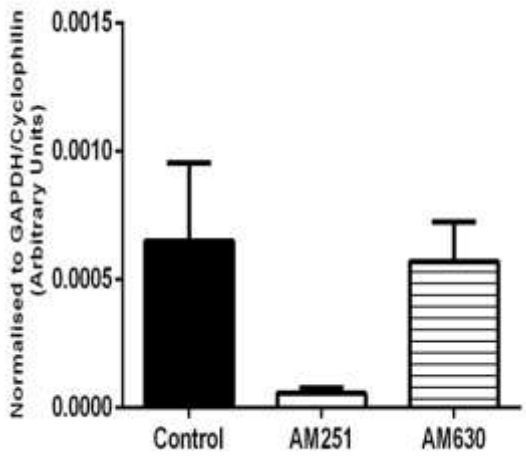


C; PDK

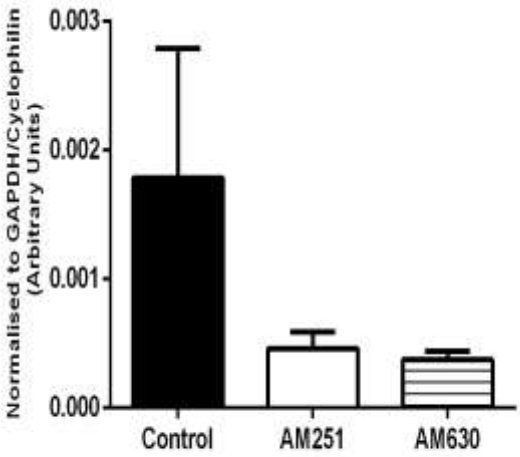


D; PDK4

Figure 7.5.A *Human myotubes derived from obese skeletal muscle, treated with AM251 (n=6) or AM630 (n=6) and tested for mRNA levels of PPAR compared to control (n=8).* **B***the mRNA gene expression of PPAR in myotubes from diabetic human tissue following treatment with AM251 (n=8) or AM630 (n=7) and compared to control (n=8).* **C** *Human myotubes derived from obese skeletal muscle, treated with either CB_1 (n=6) or CB_2 (n=6) antagonist and tested for mRNA levels of PDK4 compared to control (n=7).* **D** *the mRNA gene expression of PDK4 in myotubes from diabetic human tissue following treatment with either AM251 (n=7) or AM630 (n=7) and compared to control (n=7).*



E; FOX01



F; FOX01

Figure 7.5.E *Human myotubes derived from obese skeletal muscle, treated with CB_1 (n= 3) or CB_2 (n=7) antagonist and acutely exposed to globular adiponectin and tested for mRNA levels of FOXO1compared to control (n=7).* **F** *the mRNA gene expression of FOXO1 in myotubes from diabetic human's following treatment with AM251 (n=7) or AM630 (n=7) and compared to control (n=6) results shown in arbitrary units.*

gAd, AM251+gAD and AM630+gAD

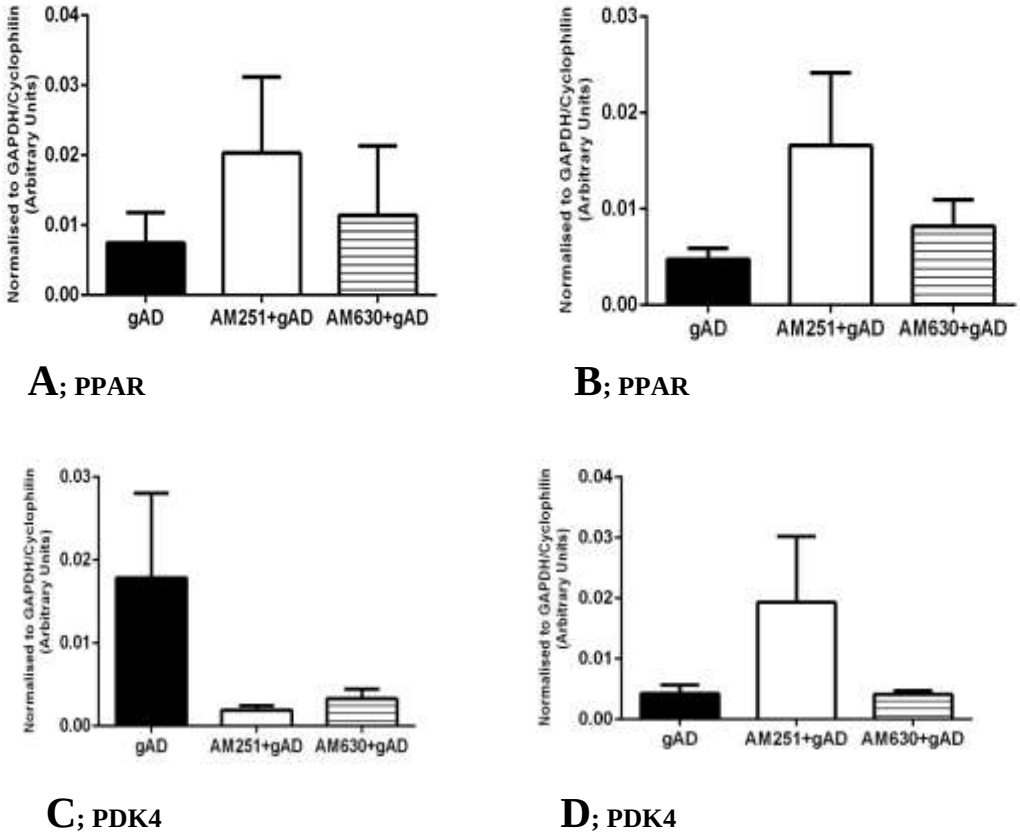
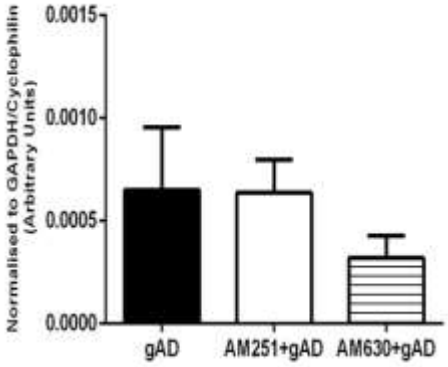
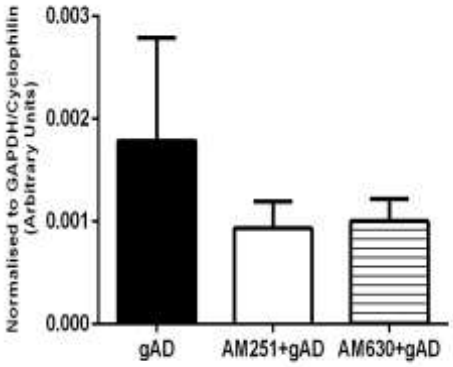


Figure 7.6.A *Human myotubes derived from obese skeletal muscle, treated with either globular adiponectin (n=8), or globular adiponectin combined with CB_1 antagonist AM251 (n=4) or CB_2 antagonist AM630 (n=7) and tested for mRNA levels of PPAR.* **B** *PPAR in myotubes from diabetic human tissue acutely exposed to globular adiponectin (n=7) in isolation or combined with CB_1 antagonist AM251 (n=4) or CB_2 antagonist AM630 (n=6).* **C** *Human myotubes derived from obese skeletal muscle, and treated iwith globular adiponectin in isolation or in combination with CB_1 AM251 (n=6) or CB_2 AM630 (n=6) mRNA levels of PDK4.* **D** *the mRNA gene expression of PDK4 in myotubes from diabetic globular adiponectin (n=4, AM251 (n=6), AM630 (n=8).*



E; FOX01



F; FOX01

Figure 7.6.E *Human myotubes derived from obese skeletal muscle, treated with globular adiponectin (n=4) or in combination with CB_1 antagonist AM251 (n=6) CB_2 antagonist (n=6) and tested for mRNA levels of FOX01.* **F** *the mRNA gene expression of FOX01 in myotubes from diabetic humans acutely exposed to globular adiponectin (n=4), AM251 (n=6), AM630 (n=8).*

Treatment with globular adiponectin did not alter PPAR, PDK4 or FOXO1 mRNA expression in either the obese (PPAR (*Figure 7.4.A;* P=0.85) PDK4 (*Figure 7.4.C;* P=0.16) or FOX01 (*Figure 7.4.E;* P=0.74)) or obese + diabetic (PPAR (*Figure 7.4.B;* P=0.32) PDK4 (*Figure 7.4.D;* P=0.32) or FOX01 (*Figure 7.4.F;* P=0.44) derived human skeletal muscle myotubes. The next section of results looked at CB_1 antagonist treatment with AM251, and CB_2 antagonist treatment with AM630 on gene expression following 24 hours of exposure. There was no change in the mRNA expression of PPAR (*Figure 7.4.G;* P=0.61), PDK4 (*Figure 7.4.I;* P=0.89) or FOX01 (*Figure 7.4.K;* P=0.25) in the obese derived myotubes or PPAR (*Figure 7.4.H;* P=0.30), PDK4 (*Figure 7.4.J;* P=0.47) or FOX01 (*Figure 7.4.L;* P=0.07) mRNA expression in the obese + diabetic derived myotubes. Similarly no change was seen in the mRNA expression of PPAR (*Figure 7.4.M; P=0.54*), PDK4 (*Figure 7.4.O; P=0.42*) or FOX01 (*Figure 7.4.Q; P=0.46*) in the obese derived myotubes, or PPAR (*Figure 7.4.N; P=0.26*), PDK4 (*Figure 7.4.P; P=0.16*) or FOX01 (*Figure 7.4.R; P=0.42*) in the obese + diabetic derived myotubes following treatment with CB_1 antagonist, AM251, combined with globular adiponectin or CB_2 antagonist, AM630, combined with globular adiponectin compared to globular adiponectin treatment alone. Difficulties in the measurement of PPAR, PDK4 and FOXO1 mRNA expression were encountered in these experiments due to low expression levels of these genes and despite repeating the 'Real-Time' RT-PCR analysis a number of the samples failed to yield a result.

7.8 Discussion

Adiponectin has a role in exerting anti-inflammatory, anti-diabetic and strong insulin sensitizing mechanisms (Waki et al. 2004). There are two recognized cannabinoid receptors known as CB_1 and CB_2 (Carayon et al. 1998. CB_1 has subsequently been shown to play a significant role in energy metabolism, appetite control, endocrine and metabolic regulation (Pagutto et al. 2006). Although there is limited research that has been conducted on CB_2 it appears both CB_1 and CB_2 are involved in the regulation of fatty acid oxidative pathways in skeletal muscle (Zhao et al. 2010). In the obese state skeletal muscle becomes resistant to the biologically decreased amounts of circulating adiponectin (Mullen et al. 2008).

This thesis has presented results in previous studies (Chapter 3, 4, 5, 6) which investigated the effects of chronic CB_1 antagonism with (AM1241) or CB_2 antagonist (AM630) treatment. In the DIO animals CB_1 antagonist treatment resulted in weight loss and reduced fat pad stores. An increased reliance on fatty acids was supported by a reduction in plasma ghrelin and leptin levels with an increase in glucagon and reduction in fat pad weight despite the recovery in daily food intake. When the skeletal muscle of these animals was acutely exposed to adiponectin it resulted in a decrease in the mRNA expression of AdipoR1 in the soleus muscle, AdipoR2 in the EDL and a decrease in PGC1-α gene expression. In the DIO animals model CB_2 antagonist treatment for six weeks resulted in decreased brown fat pad mass and decreased metabolic hormones, ghrelin and leptin.

Acute adiponectin exposure in the rat skeletal muscle following chronic AM630 treatment in DIO model, however, resulted in increased levels of citrate synthase expression in the highly oxidative red gastrocnemius skeletal muscle, which could not be explained by any alterations in mRNA expression of key markers of oxidative metabolism.

When considering these results of the skeletal muscle upon acute adiponectin exposure, it is possible that the results may have been influenced by the external influences of chronic treatment of CB_1 and CB_2 antagonism in-vivo. Plasma from animals in both treatments was found to have significantly decreased levels of ghrelin and leptin (Chapter 3 and Chapter 5). Altered circulating levels in ghrelin and leptin have been found to result in adaptive changes in skeletal muscle tissues (Jennings et al. 2011). Chronic CB antagonist treatment resulted in adaptive changes in the DIO rats in both vivo and ex-vivo studies within this thesis (Chapter 3, Chapter 4, Chapter 5, Chapter 6). To further elucidate if CB antagonism has an effect on adiponectin signalling pathways in skeletal muscle, a cell culture model reflecting mRNA adaption without circulating influences was tested. Moreover to investigate if the changes identified within the DIO model also occur in human skeletal muscle when exposed to adiponectin, primary human myotubes (collected from obese and diabetic humans) investigated the effects of CB_1 and CB_2 specific antagonism in the presence of gAD in human skeletal muscle myotubes.

In the human primary muscle myotubes AdipoR1 gene expression significantly increased in the myotubes derived from obese individuals when exposed to gAD. AdipoR1 gene expression increased in myotubes derived from diabetic tissue. AdipoR2 mRNA expression also increased in myotubes derived from diabetic tissue when exposed to CB_2 antagonist treatment. This data

provides further evidence to support the yet to be elucidated role of endocannabinoids and adiponectin signaling in the skeletal muscle adaptions of obesity and diabetes.

The physiological adaptation to obesity by skeletal muscle in a cell culture model has found that adiponectin resistance and the down regulation of AdipoR1 are associated with inflammation (Sente et al. 2016). The low grade inflammation that occurs in obesity induces changes in skeletal muscle by reducing fatty acid oxidation and glucose clearance (Liu et al. 2010). The increase in adipose tissue storage in obesity has been shown to have a direct effect on the down regulation of AdipoR1 expression (Cho et al. 2015, Ramos-Ramírez et al. 2016). In similar research to the current study using a similar cell culture model, AdipoR1 gene expression was found to increase in lean myotubes, whereas in obese and diabetic tissue, gene expression did not alter (McAinch et al. 2006). The study by McAinch et al (2006) exposed the skeletal muscle mytoubes to gAD treatment for 6 hours, whereas the current study treated the samples for 24 hours. It is therefore possible that the longer exposure time may have resulted in an up regulation of AdipoR1 in the obese skeletal muscle myotubes.

The downstream activation of genes such as AMPK by gAD in either obese or obese type II diabetic myotubes, has been found to be regulated by mechanistic changes as opposed to alterations in the number or concentration of adiponectin receptors (Chen et al. 2009). Studies in humans however have found that AdipoR1 down regulation is more closely related to a family history of diabetes rather than obesity parameters such as the reduced plasma levels of adiponectin (Civitarese et al. 2004). Research findings suggest that both the levels of circulating adiponectin and the concentration of adiponectin receptors are equally responsible for the changes that occur in skeletal muscle in the obese state (Blüher et al. 2006). There is a significant increase in AdipoR1 gene expression in the obese tissue when exposed to gAD.

This contrasts with the DIO rats chronically treated with CB_1 antagonist AM251 where acute treatment of the soleus muscle with adiponectin caused a decrease in AdipoR1 gene expression soleus muscle (Chapter 4).

It is difficult to hypothesise on the exact CB mechanisms following six weeks treatment with AM251 in a DIO animal model. This group displayed evidence of lipolysis and inflammation post treatment (Chapter 3). The gene expression of AdipoR1 in their highly oxidative skeletal muscles decreased once acutely exposed to adiponectin. Interestingly AdipoR1 gene expression in human skeletal muscle obese myotubes responded to exposure to globular adiponectin by increasing AdipoR1 gene expression. The decrease in AdipoR1 in the DIO rat muscle may have decreased due to a negative feedback loop which is a possible explanation for the mechanistic changes in skeletal muscle in obesity, when uptake of adiponectin into a cell decreases (Chapter 4).

This provides evidence to support further research into adaptive changes of AdipoR1 in obesity and CB_1 antagonist treatment, increasing our understanding of receptor adaption and expression changes in a variety of biological settings. Regardless of the tissue distribution of adiponectin receptors, further research is required to investigate the downstream genes that are involved in the fatty acid oxidative pathway. The blocking of AdipoR1 results in decreased mitochondrial activity, a reduction in highly oxidative muscle fiber types and an increase in insulin resistance (Iwabu et al. 2010). In agreement with these recent pharmacological advances have found that adipoR agonism (bind to both AdipoR1 and AdipoR2 in vitro causes a similar biological effect as adiponectin in muscle. This includes increasing fatty acid oxidative pathways and the amelioration of insulin resistance in mice fed a high-fat diet (Okada-Iwabu et al. 2010).

The results in this study found an increase in AdipoR1 gene expression in diabetic tissue when exposed to CB_1 antagonist treatment. It is intriguing that this occurred in only in diabetic tissue. Genetic variations in adiponectin concentrations, but not receptors have been implicated in insulin resistance and metabolic disturbances in diabetic human populations (Peters et al. 2013). CB_1 antagonism in skeletal muscle has been demonstrated to increase glucose uptake in oxidative muscle and the expression of genes involved in oxidative metabolism (Cavuoto et al. 2007, Pagotto et al. 2006, Miller et al. 2011, Osei-Hyiaman et al. 2005). Endocannabinoid levels have been found to be increased in both high and low oxidative fibre types, in a high fat fed animal model.

Exercise training induced improvements in skeletal muscle were coupled with a reduction in the level of endocannabinoids in the low oxidative fibre type tissue but not in the high oxidative fibre type (Gamelin et al. 2016). Evidence suggests that there is an increase in low oxidative fibre type and reduction in muscle function in obese mice fed a high fat diet (Ciapaite et al. 2015). This suggests that low oxidative fibre type skeletal muscle may be more sensitive to changes when CB_1 signaling is blocked. This is in support of why the diabetic myotubes changed and not the obese. The diabetic myotubes may have higher percentage of low oxidative fibre type or lower oxidative capacity than obese-derived myotubes.

An increase in the activity of circulating levels of endocannabinoid ligands increases food intake, weight gain and increases the risk of metabolic disorders (Engeli et al. 2005). In the current study exposing cultured myotubes to cannabinoid receptor antagonists will enable a possible snapshot of what may occur in vivo if there was an increase in circulating endocannbinoids and if this inturn alters the mechanisms of CB receptors, and therefore effecting adiponectin signalling,

Exposing cells to an antagonist in a cell culture model has an isolative effect; the blocking of receptors occurs and effects downstream signalling without impacting on the circulating levels of endocannabinoids or adiponectin. This enables the establishment of changes in function of cannabinoid receptors. AM251 (known to closely resemble Rimonabant©) is a specific cannabinoid CB_1 receptor antagonist that is also an inverse agonist, with 306-fold selectivity over CB_2 receptors (McAllister et al. 2003).

The increase in AdipoR1 in the diabetic tissue when exposed to CB_1 antagonist is a significant funding in this study. Activation of CB_1 decreases adiponectin and this effect is reversed by CB_1 blockade (Ge et al. 2013). Increasing adiponectin and AdipoR1 in diabetic tissue is a viable target in the treatment of obesity induced diabetes. This study is this the first to show that in the presence of a CB_1 antagonist, AdipoR1 gene expression is up regulated in diabetic tissue.

The gene expression of AdipoR2 in this study was also significantly increased in diabetic derived skeletal muscle myotubes when treated with a CB_2 antagonist. AdipoR2 has been shown to modulate regulation of downstream adiponectin signaling via activation of the nuclear receptors PPARγ and PPARα (Tan, et al. 2014, Tomita et al. 2008). In skeletal muscle the upregulation of PPAR is associated with increased fatty acid oxidation (Yoon et al. 2006). Similarly CB agonism increases PPAR activation (Kaczocha et al., 2012, Yu et al., 2014). Whether the increase in AdipoR2 gene expression in the diabetic tissue in this study had an impact on PPAR by adiponectin initiated by AdipoR2 has yet to be determined.

This study is the first to discover a relationship between AdipoR2 and CB_2 antagonism in human diabetic skeletal muscle. The results of the current study found that APPL1 and APPL2 did not alter, suggesting that the up-regulation of AdipoR1 occurred at a cellular level. Recent advances have revealed the structure of AdipoR1 and AdipoR2 and have concluded that

adiponectin receptors are a novel class of receptors that may interact with the extracellular face, rather than the C-terminal flexible tail, of the receptors (Tanabe et al. 2015). Whether adaptations in obese and diabetic skeletal muscle occur as a result of adiponectin resistance at the receptor or intracellular level has yet to be determined. There were no changes in this study in the gene expression of APPL1 or APPL2 when AM251 or AM630 were treated in combination with gAD. In this study CB antagonism did not alter gene expression in obese or diabetic tissue when combined with gAD, suggesting that the relationship between adiponectin resistance and cannabinoid signaling blockade is a complicated one.

The effect of cannabinoid receptor modulation and adiponectin exposure was investigated further in this study by looking at intramyocellular genes that have been shown to be modulated through adiponectin exposure. The results showed that mRNA expression of PPAR, PDK4 and FOX01 did not alter in either the obese or diabetic groups. The samples testing these genes had low numbers in the groups to compare (n=3-8). Despite repeated testing some gene expression was too low to give accurate results. This is a limitation of the current study and there is a need to generate more concentrated samples to accurately determine any change. Due to the candidature timeline and sample depletion, this was beyond the scope of this thesis. Further research is required to understand the mechanisms behind the endocannabinoid – adiponectin modulation in skeletal muscle that occurs in obesity and diabetes.

7.9 Conclusion

In summary AdipoR1 gene expression significantly increased in the obese tissue when exposed to gAD. AdipoR1 gene expression increased in diabetic tissue when exposed to CB_1 antagonist treatment. AdipoR2 gene expression increased in diabetic tissue when exposed to CB_2 antagonist treatment. Little is known about CB_2 function. Despite these increases in receptor

expression there was no additive effect of CB_1 or CB_2 antagonist treatment when combined with exposure to gAd. Therefore, the null hypotheses is accepted, despite minor changes cannabinoid receptor antagonism did not result in improved adiponectin signalling via modulation of mRNA expression in obese and obese diabetic derived human skeletal muscle myotubes. These data, provide further evidence to support the yet to be elucidated role of CB in adiponectin signaling in skeletal muscle.

Chapter 8 - General discussion

8.0 General discussion

This thesis presents *in vivo, in vitro* and *ex vivo* studies investigating the effects of chronic endocannabinoid receptor modulation on skeletal muscle adiponectin sensitivity in diet induced obesity and diabetes. This consisted of complex data collected following chronic and acute treatments, different cohorts undergoing specific pharmacological treatments, specific skeletal muscle types and both rat and human samples. This discussion will firstly discuss the thesis aims and the overall key findings of the rat studies for each specific drug (CB_1 antagonist AM251 treatment – *chapter 3 and chapter 5*; CB_2 agonist AM1241 treatment – *chapter 4 and chapter 6*; CB_2 antagonist AM630 treatment *chapter 4 and chapter 6).* The key findings of the human cell culture model (*chapter 7*) will then be discussed. This thesis chapter will then conclude with limitations, future directions for investigating cannabinoid receptors in the treatment of obesity and diabetes and finally, a summary and conclusion.

8.1 Animal studies (*in vivo* & *ex vivo*)

CB_1 antagonism

The first two aims of this study investigated the effects of chronic CB_1 antagonism AM251 in a DIO model. ***Aim 1:*** *'Investigate the role of Chronic CB_1 antagonism on plasma hormone and cytokine markers'****Aim 2:*** *Investigate the effects of chronic CB_1 antagonism and adiponectin resistance on fatty acid oxidative pathways in skeletal muscle.* The *in vivo* effects of chronic CB_1 antagonism with AM251 resulted in significant weight loss and a reduction in fat pad stores. This result suggests that the animals in this study had an increased reliance on fatty acids as a metabolic fuel. In support of these findings, plasma leptin levels increased and glucagon

levels decreased. Although weight loss and a reduction in fat pad mass has been seen in previous studies investigating the effects of CB_1 antagonism (Bensaid et al. 2003), the presence of increased inflammatory makers in this cohort is a novel finding and considering previous research which described negative psychoactive effects in humans treated with a CB_1 antagonist, this result warrants further investigation.

CB_2 agonist

This thesis further investigated the novel findings of chronic CB_2 modulation. ***Aim 3:*** *Investigate the role of chronic CB_2 agonism and antagonism on obesity plasma hormone and cytokine markers and* ***Aim 4:*** *Investigate the effects of chronic CB_2 agonism and CB_2 antagonism on adiponectin resistance on fatty acid oxidative pathways in skeletal muscle.*

The effects of chronic CB_2 agonism on a DIO model showed no change in weight, body fat percentage or food intake. It did however result in the novel finding of an increase in circulating levels of IL12p70 and a decrease in fat pad mass of epididymal, peri-renal and brown fat pads following chronic CB_2 agonism. Research suggests that the natural CB agonist anandamide (AEA) decreases expression of IL-12p70 (Correa et al. 2011). This is in contrast to the chronic administration of AM1241 in a DIO model in the current studies as the animals showed no other inflammation other than IL-12, which is interesting considering it has been found to be up regulated in autoimmune disorders and that levels increase in line with worsening disorder progression (Comabella et al, 1998, Hart et al. 2005). Moreover, the decrease in fat pad mass despite no decrease in body fat percentage should be further investigated to determine what mechanisms involved in the activation of CB_2 could result in a decrease in fat pad mass.

In the skeletal muscle of the DIO animals chronically treated with the CB_2 agonist and acutely exposed to adiponectin, treatment caused a significant increase in both AdipoR1 and PGC-1α and a decrease in AMPK mRNA expression in the EDL muscle. These data provide a rationale for further investigation into the intracellular mechanisms responsible for and elicited by these changes. PGC-1α is strongly associated with adaptations of fibre types, mitochondrial biogenesis and function (Iwabu et al. 2010). The significant decrease found in mRNA expression of AMPK upon acute adiponectin exposure is intriguing as AMPK is known to play a key role in fatty acid oxidation. The modulation of AMPK has been found to induce the activation of AMPK by adiponectin (Yamauchi et al. 2007). The increase in both AdipoR1 and PGC-1α and the down regulation in AMPK following acute adiponectin exposure and considering the increase in IL-12p70 and decreases in the fat pad mass of this group, there may be other underlying mechanisms occurring in chronic CB_2 agonism treatment in a DIO model. Further investigation is required to elucidate the results of this study and investigate the chronic effect of CB_2 agonism on acute adiponectin exposure.

CB_2 antagonist

The effects of chronic CB_2 antagonism on a DIO model showed no change in weight, body fat percentage or food intake. It did however result in a decrease in the brown fat pad mass and a decrease in leptin and ghrelin levels. Previous research has found a relationship between CB_2 and leptin (Lopez-Rodriguez et al. 2016). There is limited research into the effects of chronic CB_2 antagonism and its potential relationship with ghrelin. In humans, obesity negatively correlates with low levels of circulating ghrelin (Tschop et al. 2001). The DIO control group in this study failed to show changes in the circulating ghrelin levels. Therefore, obesity alone did not cause the down regulation of circulating levels of leptin and ghrelin seen in this study.

This supports CB_2 antagonism as playing a yet to be determined role in influencing the circulating levels of metabolic hormones leptin and ghrelin. Further research is warranted to determine the mechanisms involved.

Chronic CB_2 antagonism via AM630 on a DIO model resulted in increased levels of citrate synthase in the highly oxidative red gastrocnemius in whole muscle sample, despite no alterations observed in mRNA expression of key markers of oxidative metabolism and adiponectin treatment. Citrate synthase is used as a quantitative marker of enzyme content of intact mitochondria (Holloszy et al 1970). Further work is warranted given that in CB_2 knockout mice (-/-) fed a high fat diet there were improvements in insulin sensitivity (Agudo et al. 2010). Citrate synthase has previously been found to be down regulated in red muscle fibre types in diet induced diabetes (Simoneau et al. 1985). This highlights the use of CB_2 antagonism as a potential target for the treatment of diabetes and further research should be undertaken to fully elucidate its therapeutic action.

8.2 Human study (*in vitro*)

Chapter 7 of this thesis had the following aim: ***Aim: 5:*** *Investigate the effects of cannabinoid receptor antagonism on adiponectin receptor mRNA expression and adiponectin signalling in obese and obese diabetic derived human skeletal muscle myotubes.*

The results of this study showed that in human muscle myotubes, AdipoR1 gene expression significantly increased in the obese tissue when exposed to globular adiponectin. This is despite no changes detected in the gene expression of APPL proteins or intramyocellular genes shown to be modulated by adiponectin. To date the relationship between cannabinoids and adiponectin on skeletal muscle, obesity and diabetes are limited. Further research is required to understand

the mechanistic changes occurring when obese derived myotubes are acutely exposed to adiponectin treatment. Moreover, in diabetic derived myotubes, the mRNA expression of AdipoR1 was significantly increased following exposure to the CB_1 antagonist, AM251. In contrast, the decrease in plasma adiponectin in obesity has been found to be reversed by CB_1 blockade (Ge et al. 2013). Interestingly AdipoR2 gene expression also increased in the diabetic tissue when exposed to CB_2 antagonist treatment. Due to the lack of clarity in the results with no change seen in the downstream genes associated with adiponectin, the increases seen in AdipoR1 and AdipoR2 in diabetic tissue requires further investigation to determine if CB modulation is a viable target in the treatment of obesity induced diabetes.

8.3 Limitations

To date there has been very little research conducted on the effects of the endocannabinoid system and skeletal muscle. There are also many gaps in the literature that contribute to the lack of knowledge associated with the effects of obesity and diabetes on skeletal muscle. This is intriguing considering that skeletal muscle is responsible for more than half of both glucose and fatty acid clearance in the body.

In the studies in this thesis there was a lack of clarity with measurements of glucose and insulin in the DIO animals. There were no alterations in the circulating levels of either glucose or insulin. Thus despite the increased weight and body fat percentage in this DIO model, the skeletal muscle was still sensitive to insulin. This was despite the diet being utilized by our lab previously (which resulted in both glucose and insulin resistance in DIO animals). These animals failed to display any evidence of glucose or insulin resistance notwithstanding the significant increase in body fat (%). To avoid variability the animals were the same strain, sex, aged matched, weight matched and were all purchased from the same animal supplier, and they

were purchased in intervals and were staggered in batches for their introduction into the study. The animals were measured at the same time daily and all testing was performed at the same time of day (Bowe et al. 2014).

A limitation to the measure of glucose regulation and insulin resistance in the rats was the capacity to use what is considered as the 'gold standard' for measuring whole body insulin responsiveness. The procedure, known as hyperinsulinaemic-euglycaemic clamp (Defenzo et al. 1979) requires surgical incision of a small tube that is connected to the rat's circulation. Insulin is infused into the fasted animal, through an intravenous catheter to maintain hyperinsulinaemia. Glucose is concomitantly infused to maintain euglycemia (Bowe et al. 2014). The data collected from this procedure is considered high quality giving the most accurate depiction of glucose tolerance. It was not used in this study as repeated surgical cannulation and recovery would have impacted too much on the rats and compromised other parameters. Moreover, the nature of the research posed difficulties when the research design was being established. The animals became obese within weeks of consuming the high fat diet, the animals were already undergoing multiple testing. As the animals were mature in age (for rodents) when the treatments began, the risk of complications for the animals increased when administering anaesthesia for surgery. Lack of disposable funds associated with a PhD scholarship grant meant that animals that did not complete the study were not able to be replaced. As the research design called for in vivo and ex vivo-in vitro testing, it was considered paramount that all animals complete the 15 weeks of the study.

The animals (as discussed in Chapter 2) were chosen to mimic what occurs in a group of humans when they consume a high fat diet. Some animals will exhibit an obese phenotype and will result in the excess storage of adipose when the animals consume a high fat diet. Similarly,

some animals will display the lean phenotype which will result in the animal not exhibiting a weight increase (Levin, Dunn-Meyell et al. 1997, Farley, Cook et al. 2003). It is possible that the animals although staggered were generated from the same genetic background and all exhibited a phenotype which prohibited glucose resistance.

The animals did not have any form of activity or exercise and alterations in movement were not recorded. To fully elucidate the hypothesis and aims of this thesis and to provide clarity to the results, the use of animals that are both glucose and insulin resistant would be beneficial.

The animals did not have any form of activity or exercise and alterations in movement were not recorded. To fully elucidate the hypothesis and aims of this thesis and to provide clarity to the results, the use of animals that are both glucose and insulin resistant would be beneficial.

8.4 Future Directions

In order to fully investigate and understand if the endocannabinoid system modulation effects adiponectin receptors and/or adiponectin resistance in skeletal muscle in the presence of either obesity, diabetes or a high fat diet, a future study should be repeated with adiponectin knock-out animals. This would establish if the effects of increased adipose tissue storage, increased glucose resistance or increased circulating levels of free fatty acids, results in changes to the capacity of skeletal muscle to adapt without the influence of changes in adiponectin. The use of adiponectin knockout mice (adipo -/-) has previously been used in research to ascertain the effects of adiponectin, for example; to investigate the effects of adiponectin and supplementation (Resveratrol) on colorectal tumours (Boddicker et al. 2011). Investigating the role of adiponectin on cardiac hypertrophy and contractile dysfunction in adipo -/- mice on a high fat diet (Guo et al. 2013), and to investigate the effects of CB_1 antagonism would be of

value. Rimonabant (10 mg/kg) was injected via IP injection to both adipo -/- mice and wild type controls (C57B1/6J) which were placed on a high fat diet for seven days (Tam et al. 2014). Although the gastrocnemius was collected, the study tested for glucose uptake and glycogen synthesis alone in mixed fibre skeletal muscle, which found no change despite the administration of Rimonabant. Thus the results of this study lack clarity and require further investigation to determine the effects of chronic CB_1 antagonism on skeletal muscle in a DIO model and the effects that occur in adiponectin intramyocellular signalling. This thesis presents a rationale for the use of Adipo -/- animals to further investigate the effects on skeletal muscle of chronic CB receptor modulation, adiponectin, fatty acid and glucose clearance in obesity and diabetes.

The implementation of further treatment groups would assist in the determination of the aims in this thesis. For example the induction of a CB_1 agonist group may assist in determining the effects of activating CB_1. CB_1 activation studies have traditionally investigated the effects of the agonist in neuronal tissue (Katona et al. 1999, Gerdeman + Lovinger, 2001, Aso et al. 2012). The introduction and legalisation of marijuana for medical purposes (Cerdá et al. 2012) has seen new studies investigating the effects of specific CB_1 activation in other tissues. CB_1 agonism treatment using the specific agonist ACPA was used in an *ex vivo* model using the lung tissue of male Sprague-Dawley rats (Chou-Ming et al. 2016). The study found that CB_1 activation attenuated the induction of reactive oxygen species (ROS) (Chou-Ming et al. 2016). Considering the CB_1 antagonist AM251 group in this thesis (*chapter 3*) displayed the expected reduction in weight and body fat percentage, but also showed an unanticipated increase in circulating inflammation markers, investigation of the effects of chronic CB_1 agonism in the

skeletal of obese and diabetic DIO model would be advantageous for clarification of the results in this thesis.

Introducing both an exercise and a weight loss group would also be beneficial in determining if CB modulation has an effect on adiponectin sensitivity in skeletal muscle. When considering the biological mechanisms of an organ such as skeletal muscle and adiponectin there are many variables to test. This is evident when the effect on both dietary intake and exercise in isolation or combination were tested in seventy-nine obese males and females (Christiansen et al. 2010). The mRNA expression of AdipoR1 in skeletal muscle was significantly increased by exercise only, however the combination of both decreased energy intake and exercise resulted in an increase in both AdipoR1 and AdipoR2 mRNA expression (Christiansen et al. 2010).

The introduction of an exercise group and a weight loss group would assist in determining the best possible functional adaptions of adiponectin receptors in skeletal muscle. This data would be beneficial in understanding the therapeutic treatments best recommended for those with obesity or diabetes. This thesis investigated what occurs in human myotubes when treated in isolation and in combination with a CB antagonist and globular adiponectin in tissue collected from both obese and diabetic humans. In considering the findings from this thesis, the next step to fully elucidate the relationship between CB modulations in obesity and diabetes is to test the aims of this thesis in a DIO adipo -/- model. This model would ensure that the animals in futures studies are resistant to glucose uptake and reduced in fatty acid oxidation.

'Future Aims: Determining the effect of ex-vivo acute adiponectin exposure on the skeletal muscle of chronic CB modulation in adiponectin knockout model of obesity'

A kinetic model PET/CT (FDG and position emission tomography/computed tomography) imaging has recently been used to explain glucose uptake by apoliproteins in a diabetic mouse model (db/db) (Cochran et al. 2016). The fasted animals were injected via IP injection with apoA-1. After two hours the animals were anesthetised using isoflurane. Insulin was injected and during the following 30 minutes, images were collected of the radiotracer uptake into the tissues (Cochran et al. 2016). This model was very effective with a small sample size (n=4 per group) and determined that the apoA-1 treatment increased the rate of insulin-dependent glucose phosphorylation (Cochran et al. 2016). Moreover the same research group investigated *ex-vivo* skeletal muscle glucose uptake in gastrocnemius muscle, following removal and incubation for 60 minutes in an insulin bath at 37^{o}C (Cochran et al. 2016). Then the muscle was exposed to either glucose or insulin 10 minutes, glucose uptake was measured using liquid scintillation counting following exposure to ice cold buffer containing cytochalasin (Cochran et al. 2016). Future recommendations would be the use of this method on a DIO adipo -/- model. In vivo treatment with CB agonists and antagonists and then the use of PET/CT scan would assist in understanding the effect on various organs including skeletal muscle. Following this Soleus and EDL should be exposed to an organ bath treatment similar to the organ bath method used in this thesis; following the technique of liquid scintillation should then be used. This will provide valuable information on the influence of endocannabinoids in skeletal muscle adiponectin sensitivity in DIO model. It will assist in enhancing our knowledge of the role CB modulation and adiponectin in skeletal muscle. The *ex-vivo* CB_2 agonist treatment in a DIO model in this thesis (Chapter 6) noted an increase in the gene expression of both AdipoR1 and PGC-1α in the EDL muscle when acutely exposed to adiponectin for 30 minutes. Due to candidature restraints CB_2 agonism was not looked at in this DIO model.

'Future Aims: Determining the effects of contraction on acute adiponectin exposure on skeletal muscle signalling pathways of glucose uptake and fatty acid oxidation by chronic CB modulation in an adiponectin knockout model of obesity'

Muscle contraction *ex vivo* has been shown to stimulate phosphorylation of kinase 1 and 2 (ERK1/2), and p38MAPK (Ryder et al. 2000). There were a number of genes tested in this study shown to be modulated by adiponectin (Chapter 4 and chapter 6). Little is known about the effects of CB modulation on adiponectin downstream signalling pathways in skeletal muscle, notwithstanding the alterations that may occur between the downstream signalling in an active muscle and the downstream signalling within a sedentary muscle. Thus the effects of chronic CB modulation in muscle at rest and muscle under contraction would help to establish acute alterations in pathway responses. The model of sedentary muscle presented in this thesis (Chapter 6) resulted in an increase in AdipoR1 and PGC-1α in the EDL of DIO rats chronically treated with AM1241 and acutely exposed to adiponectin. If these observed changes also occur when muscles are actively contracting, this will show that the observed changes in gene expression occurred because of receptor adaption from chronic CB modulation when acutely exposed to adiponectin. In the obese human myotubes (Chapter 7) treated with globular adiponectin for 24 hours the gene expression of AdipoR1 also increased as this occurred in non-contracting skeletal muscle myotubes. Investigating if the mechanisms involved alter in contraction may contribute to targeting for therapeutic treatments.

In contrast, CB_1 antagonist treatment via AM251, caused an increase in AdipoR1 gene expression in diabetic tissue. AdipoR1 appears to have the strongest reaction in both human obese and diabetic skeletal muscle and resulted in adaptions in inflammation and hormone levels and an increase in weight loss when CB modulation occurs. Therefore, focusing on the

changes that occur in AdipoR1 in a range of presented models would be a future recommendation. Intriguingly AdipoR2 gene expression increased in the human diabetic myotubes when exposed to CB_2 antagonist, AM630. Moreover the detected levels of citrate synthase (Chapter 6) was significantly increased the soleus muscle. These data offer, further insight into a complex system and it is recommended that AdipoR2 be investigated for possible alterations in gene expression when investigating adiponectin in a DIO model.

To fully elucidate what is occurring and the mechanistic changes that occur at a molecular level, the introduction of a human lean cell line should be established. Research showing changes differ between lean and obese/diabetic tissue (McAinch et al. 2006) to understand if the results in this thesis differ from the results that would occur in human lean tissue under the same treatments is not yet known. Thus investigations into human lean tissue and adiponectin uptake in skeletal muscle is warranted.

Due to the normal restraints of candidature, the collection of data was limited to time, tissue abundance and accessibility to both funds and equipment. Recent advances has seen glucose uptake imaging successfully depicting insulin sensitivity and intracellular glucose phosphorylation in both in vivo and in vitro models (Cochran et al. 2016). Chapter one of this thesis discusses ceramide and its unknown role in the inflammation and adiponectin resistance in skeletal muscle. It has an association with cannabinoid receptors and there is sufficient rationale to support further research into the role of ceramide and adiponectin resistance (Herrera et al. 2006). In conclusion, determining if chronic CB1 and CB2 modulation altered circulating levels of endocannabinoid ligands in the presence of obesity or diabetes would be advantageous in elucidating the changes that occurred. Thus, testing the circulating plasma levels using high-performance liquid chromatography (HPLC) is recommended. This

procedure has been successful in determining the different ligands and concentrations by others (Sergi et al. 2013).

8.5 Cannabinoid receptors in the treatment of obesity and diabetes

Obesity and its comorbidities is a universal problem that put us on a path where our future generations will have an increased morbidity rate as our species struggle to adapt to a lifestyle change of sedentary behaviour and a high fat diet. Modern pharmaceuticals continue to be developed and target new treatments to assist in the fight against obesity. The increase in lipid storage associated with obesity results in the dysfunction and stress in biological tissue. Research thus far has delivered sufficient evidence in both *in vitro* and *in vivo* studies suggesting the involvement of the endocannabinoid system in a variety of pathological conditions. The pharmaceutical blockade of the CB_1 pathway in metabolic peripheral tissues has resulted in positive effects in weight control and metabolic profile. However, in humans this resulted in negative psychoactive effects. New advances in pharmaceuticals (Alonso et al, 2012; Tam et al. 2010) (*discussed in detail in chapter 1*) may potentially see the specific targeting of CB_1 (and given the results of CB_2 modulation discovered in this thesis) and CB_2 as viable targets in the future treatment of obesityy and its associated co- morbidities.

8.6 Summary of Results

The results of this thesis found that in an animal DIO model chronic CB_1 antagonist treatment resulted in weight loss and a reduction in fat pad mass. Potential effects on metabolism were evidenced by an increase in the circulating plasma levels of leptin and a decrease in glucagon. Collectively these results are indicative of lipolysis.Chronically blocking CB_1 signalling also caused an increase in circulating inflammatory cytokines. In human skeletal muscle myotubes

from diabetic individuals, CB_1 antagonist treatment for 24 hours resulted in an increase in AdipoR1 gene expression. It is not known if these adaptions were a result of inflammation caused *in vivo* or *in vitro* as a result of damage to the skeletal muscle myotubes caused from modulation due to the diabetic traits of the donors. It does however offer further evidence to support the relationship between adiponectin signalling and CB_1 modulation and mechanistic changes that occur to promote weight loss in a DIO model.

Chronic CB_2 agonist treatment resulted in changes *in vivo* by increasing circulating levels of IL12p70 and decreases in the fat pad mass of epididymal, peri-renal and brown fat pads of the DIO animals. The acute *ex-vivo* exposure of their skeletal muscle to adiponectin resulted in an increase in AdipoR1 and PGC-1α gene expression; moreover, the gene expression of AMPK was significantly reduced in the EDL muscle. This emphasises the modulating effects of CB modulation on adiponectin signalling. It provides evidence to support the relationship between adiponectin signalling and CB_2 agonism and the mechanistic changes that occur to promote a decrease fat mass in a DIO model.

Chronic CB_2 antagonism in an *in vivo* model resulted in a reduction in leptin and ghrelin and a decrease in brown fat pad mass. In *ex-vivo* whole muscle, blockade of CB_2 signalling increased levels of citrate synthase in the highly oxidative red gastrocnemius skeletal muscle. In human myotubes CB_2 antagonism resulted in an increase in AdipoR2 signalling in diabetic tissue. These data combined demonstrate that blocking CB_2 signalling results in changes to metabolism, brown fat reduction, increased fatty acid oxidation and an increase in AdipoR2 in a DIO model receptor tissues.

The modulation of each cannabinoid receptor in the DIO model treatment group resulted in decreases to at least one fat pad mass and adaptions in adiponectin signalling gene expression,

metabolic hormone changes and adaptions to whole muscle. In human primary skeletal muscle there were adaptions in gene expression in CB antagonism.

8.7 Final Conclusion

Chronic CB_1 and CB_2 antagonism and CB_2 agonism *in vivo* and *ex-vivo* in a DIO animal model results in changes to metabolic markers via circulating hormone, skeletal muscle adaptions and gene adaptions following acute adiponectin exposure. In human primary skeletal muscle, AdipoR1 gene expression increased in obese myotubes following CB_1 antagonist treatment. Moreover, CB_2 antagonism increases AdipoR2 gene expression in diabetic myotubes. In conclusion, this thesis is the first to establish a relationship between cannabinoid receptor modulations, adiponectin signalling and skeletal muscle changes in a diet induced obese model. In the fight against obesity and diet induced obesity, this thesis has provided evidence to support further investigation into the pharmacological targeting of the endocannabinoid system for potential therapeutic use for inducing weight loss, adiponectin modulation and increases in fatty acid oxidation in obesity and diabetes.

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