

ENDOCANNABINOIDS AND SKELETAL MUSCLE GLUCOSE UPTAKE

A Thesis Submitted by

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THESIS SUMMARY

Obesity is a risk factor for type 2 diabetes mellitus and cardiovascular disease. Obesity, in particular when the fat is predominantly visceral, is associated with insulin resistance and a reduced ability to increase the rate of fat oxidation in response to an increase in dietary fat intake. Skeletal muscle is a primary site for insulin-stimulated glucose uptake. Insulin responsiveness in skeletal muscle is regulated by a number of factors including growth hormone, cortisol, sex steroids, cytokines secreted by inflammatory cells and adipocytes, fatty acids, and fatty acid derivatives such as the endocannabinoids.

The most abundant endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are synthesised from arachidonic acid. They have autocrine or paracrine mechanisms of action which are rapidly terminated by cellular uptake and subsequent metabolism by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) which degrades AEA and 2-AG, respectively. AEA and 2-AG are ligands for the cannabinoid receptor type 1 (CB₁) and the cannabinoid receptor type 2 (CB₂); both are 7 transmembrane domain G-protein coupled receptors. AEA and 2-AG also bind to the transient receptor potential channel-vanilloid sub-family member 1 (TRPV1). TRPV1 is a putative six-transmembrane domain protein with a pore region between segments five and six

and cytoplasmic N and C termini. TRPV1 was initially discovered as a receptor for capsaicin, the main pungent component of hot chilli. Activation of TRPV1 leads to an increase in intracellular calcium either by entry through the plasma membrane or through calcium release of intracellular stores.

Endocannabinoids and their receptors form part of an endogenous system that regulates a number of homeostatic functions, including food intake (appetite and motivation to eat via effects in the hypothalamus and nucleus accumbens shell), the regulation of fat mass and intermediary metabolism. An overactivity of the endocannabinoid system in obesity may serve to maintain fat mass and may also underlie some of the associated metabolic consequences. Several studies have shown that inhibition of CB₁ in obese animal models improved the metabolic profile and reversed the deleterious effects of obesity on metabolism. The majority of this data was based on the effects of endocannabinoids on adipose tissue and liver. The studies that form the basis of this thesis examined the effect of endocannabinoids on glucose uptake and metabolism in skeletal muscle.

It was initially shown that CB₁ inhibition improves basal glucose uptake in primary cultures obtained from obese, but not lean humans. This is consistent with the notion of an “overactive endocannabinoid system” apparent even in the *ex-vivo* system of primary culture (**Chapter 3**). These data could not however all be

explained by the presence of a single type of endocannabinoid receptor in skeletal muscle. In a series of studies messenger RNA for CB₁, CB₂, TRPV1 and the enzyme FAAH was shown to be present in human and rat skeletal muscle biopsies, primary cultures of human skeletal muscle and a rat skeletal muscle cell line (L6) (**Chapter 4**).

Subsequent experiments to determine the effect of endocannabinoids on basal and insulin-stimulated glucose uptake and receptors mediating these effects were performed in L6 cells (**Chapter 5**). Chronic (24 h), but not acute (30 min) exposure to AEA and 2-AG increased insulin-stimulated glucose uptake and the effect of 2-AG was greater than that of AEA. 2-AG was used in subsequent studies. 2-AG-mediated glucose uptake was ameliorated by inhibition of CB₁ (SR141716), CB₂ (SR144528) or TRPV1 (SB366791) with no additional effect when more than one receptor was blocked concurrently. These studies are the first to demonstrate the presence of TRPV1 in skeletal muscle and that it has a role in glucose regulation.

To investigate a role for TRPV1 on glucose metabolism *in vivo*, targeted mutant mice with a deletion of the TRPV1 gene were utilised. The studies described in **Chapter 6** measured glucose tolerance in TRPV1^{-/-} mice in comparison to wild-type mice in response to a standard or high fat diet (HFD) via intraperitoneal glucose tolerance testing. At baseline the TRPV1^{-/-} mice were able to clear a glucose load

more efficiently than their wild-type counterparts. After 18 weeks of high fat feeding, body weight of the wild-type mice increased significantly and glucose tolerance was impaired. In contrast, the TRPV1^{-/-} mice were resistant to diet induced obesity, but their glucose tolerance was similar to that of the wild-type mice. The reason for the discrepancy between adiposity and glucose tolerance is unknown, however, *in vitro* studies describing an effect of endocannabinoids to increase insulin-stimulated glucose uptake via TRPV1 suggests a role for this receptor in the regulation of glucose utilisation. The novel observations relating to TRPV1 offer a new perspective on endocannabinoid mediated effects on peripheral metabolism with potential therapeutic implications. Further studies are required to determine the relationship between the effects of endocannabinoids on peripheral metabolism and the emerging role of TRPV1 in diabetes and obesity.

THESIS DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis when deposited in the University of Adelaide Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holders of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Thesis Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Paul Cavuoto

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PUBLICATIONS ARISING FROM THIS THESIS

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MANUSCRIPTS IN PREPARATION

Cavuto P, McAinch AJ, Janovská A, Hatzinikolas G, Lam YY, Cameron-Smith D, Wittert GA. *SR141716, a selective CB1 inverse agonist, increases basal glucose uptake in human skeletal muscle myotubes derived from obese patients.*

Cavuto P, Wittert GA, Janovska A, Lam YY, Hatzinikolas G, Blackshaw LA. *TRPV1 Mediates Discordant Effects on the Regulation of Fat Mass and Glucose Metabolism.*

OTHER MANUSCRIPTS IN PREPARATION

Lam YY, Janovská A, McAinch AJ, Hatzinikolas G, **Cavuto P**, Game P, Wittert GA. *Insulin-stimulated glucose uptake and pathways regulating energy metabolism in skeletal muscle cells: the effects of subcutaneous and visceral fat, and long-chain saturated, n-3 and n-6 polyunsaturated fatty acids.*

PUBLISHED ABSTRACTS

Cavuoto P, McAinch AJ, Hatzinikolas G, Wittert GA. *Effects of cannabinoid receptors on skeletal muscle oxidative pathways*. *Obesity Reviews*, 2006, **7**(supp.2): 130.

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CONFERENCE PRESENTATIONS

ORAL PRESENTATIONS

Cavuto P, McAinch AJ, Janovská A, Hatzinikolas G, Lam YY, Cameron-Smith D, Wittert GA. *The effect of the selective CB1 inverse agonist SR141716 on human skeletal muscle myotubes*. The 2009 Post Graduate Research Expo (Finalist), Faculty of Health Sciences, University of Adelaide, Adelaide, Australia, September 1, 2009.

Cavuto P, McAinch AJ, Janovská A, Hatzinikolas G, Lam YY, Cameron-Smith D, Wittert GA. *The effect of the first selective cb1 blocker rimonabant on human skeletal muscle myotube gene expression*. Centre of Clinical Research Excellence (CCRE) seminar, Discipline of Medicine, University of Adelaide, Adelaide, Australia, November 14, 2007.

Cavuto P, McAinch AJ, Janovská A, Hatzinikolas G, Lam YY, Cameron-Smith D, Wittert GA. *The effect of the first selective CB1 blocker rimonabant on human skeletal muscle myotube gene expression (Hot Topics)*. Australasian Society for the Study of Obesity 16th Annual Scientific Meeting, Canberra, Australia, August 31-September 2, 2007.

POSTER PRESENTATIONS

Wittert GA, **Cavuoto P**, Hatzinikolas G, Blackshaw LA. *TRPV1 mediates discordant effects on the regulation of fat mass and glucose metabolism*. The 70th Scientific Sessions, American Diabetes Association (ADA), Orlando, USA, 25-29 June 2010.

Cavuoto P, McAinch AJ, Janovská A, Hatzinikolas G, Lam YY, Cameron-Smith D, Wittert GA. *The effect of the selective CB1 inverse agonist SR141716 on human skeletal muscle myotubes*. The 2009 Australian Diabetes Society and Australian Diabetes Educators Association (ADS-ADEA) Annual Scientific Meeting, Adelaide, Australia, 26-28 August 2009.

Cavuoto P, Janovská A, Lam YY, Wittert GA. *Endocannabinoids and the regulation of insulin-stimulated glucose uptake in rodent skeletal muscle*. The 2008 Postgraduate Research Expo, Faculty of Health Sciences, University of Adelaide, Adelaide, Australia, 23 July 2008.

Lam YY, McAinch AJ, Janovská A, Hatzinikolas G, **Cavuoto P**, Wittert GA. *An adipose tissue-myotube co-culture system to study nutrient utilisation in skeletal muscle cells*. The 2007 Postgraduate Research Expo, Faculty of Health Sciences, University of Adelaide, Adelaide, Australia, 23 October 2007.

Cavuoto P, McAinch AJ, Janovská A, Hatzinikolas G, Lam YY, Cameron-Smith D, Wittert GA. *The effect of the selective CB1 blocker SR141716 on human skeletal muscle myotubes*. The 2007 Postgraduate Research Expo, Faculty of Health Sciences, University of Adelaide, Adelaide, Australia, 23 October 2007.

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McAinch AJ, **Cavuoto P**, Hatzinikolas G, Cameron-Smith D, Wittert GA. *The effect of the selective CB1 antagonist sr141716 on human skeletal muscle myotube gene*

expression. The 4th Asia-Oceania Conference on Obesity, Seoul, Korea, 9-11 February 2007.

LIST OF ABBREVIATIONS

Δ^9-THC	delta-9-tetrahydrocannabinol
2-AG	2-arachidonoylglycerol
ACC	acetyl coenzyme A carboxylase
ACEA	arachidonyl-2'-chloroethylamide hydrate
ADTP	Australasian Digital Thesis Program
AEA	anandamide
AMPK	AMP-activated protein kinase
AUC	area under the curve
BAT	brown adipose tissue
BDNF	brain derived neurotrophic factor
BMI	body mass index
BP	base-pairs
CaMKK	calmodulin-dependent protein kinase kinase
cAMP	cyclic AMP
CB₁	cannabinoid receptor type 1
CB₂	cannabinoid receptor type 2
CGRP	calcitonin-gene-related peptide
CoA	Coenzyme A
CPT-1	carnitine palmitoyltransferase-1
C_T	critical threshold

DAG	diacylglycerol
DOG	deoxy-D-glucose
DRG	dorsal root ganglia
ECM	extra cellular matrix
ECs	endocannabinoids
EDL	extensor digitorum longus
eNOS	endothelial nitric oxide synthase
FAAH	fatty acid amide hydrolase
FA	fatty acid
FAF	fatty acid free
FBS	fetal bovine serum
FFAs	free fatty acids
G-6-P	glucose-6-phosphate
GC-MS	gas chromatography coupled to mass spectrometry
HEK	human embryonic kidney
HFD	high fat diet
HPLC	high pressure liquid chromatography
HS	horse serum
IR	insulin receptor
K⁺_A	A-type potassium
K_{ir}	inwardly rectifying potassium
MAGL	monoacylglycerol lipase

MAPK	mitogen-activated protein kinase
MBH	mediobasal hypothalamus
MEF2C	myocyte enhancer factor 2C
NGF	nerve growth factor
NHS	National Health Survey
NRFs	nuclear respiratory factors
PDC	pyruvate dehydrogenase complex
PDKs	pyruvate dehydrogenase kinases
PDK4	pyruvate dehydrogenase kinase 4
PEA	<i>N</i> -palmitoylethanolamine
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1 α
PhD	Doctor of Philosophy
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B (also known as Akt)
PKC	protein kinase C
PPARγ	peroxisome proliferator-activated receptor γ
PVDF	polyvinylidene difluoride
RT-PCR	reverse transcription polymerase chain reaction
RTX	resiniferatoxin
SF1	steroidogenic factor 1
SOL	soleus

SP	substance P
SREBP-1c	sterol-regulatory element-binding protein-1c
T2DM	type 2 diabetes mellitus
TBS/T	tris-buffered saline/0.1% Tween 20
TG	triglycerides
TRPV1	transient receptor potential channel-vanilloid sub-family member 1
VMH	ventro medial hypothalamus
WHO	World Health Organisation

CHAPTER 1

BACKGROUND

1.1. INTRODUCTION

Skeletal muscle is the primary site of glucose and fatty acid (FA) oxidation accounting for up to 30% of basal energy requirements (Zurlo, Larson et al. 1990). Skeletal muscle rapidly adapts to changing energy needs such as following exercise or reduced energy intake by increasing FA oxidation (Henriksson 1995). Increased FA oxidation delays the consumption of glycogen stores within skeletal muscle and conserves circulating plasma glucose (Henriksson 1995). The capacity for skeletal muscle to adapt appropriately is dependent upon insulin sensitivity, leanness and aerobic fitness (Ukropcova, McNeil et al. 2005). Obesity, in contrast, is characterised by insulin resistance and impaired ability to oxidise dietary fats appropriately (Kelley 2005). However, up to 20% of the obese population maintain insulin sensitivity and a normal metabolic profile (Rasouli, Molavi et al. 2007). It has been suggested that fat distribution and skeletal muscle function are major determinates of insulin sensitivity and metabolic risk in obesity (Despres and Lemieux 2006).

The endocannabinoids, which form part of a lipid derived endogenous signalling system are over-expressed in obesity (Engeli, Bohnke et al. 2005; Cote, Matias et al.

2007) and present a possible mechanism for the obesity related impairment of skeletal muscle energy utilisation. Endocannabinoids are derived from arachidonic acid and have an autocrine or paracrine mechanism of action (Deutsch and Chin 1993). The most abundant endocannabinoids are anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Devane, Hanus et al. 1992; Sugiura, Kondo et al. 1995). AEA and 2-AG are endogenous ligands for cannabinoid receptors type 1 (CB₁) and type 2 (CB₂) (Matsuda, Lolait et al. 1990; Munro, Thomas et al. 1993). The transient receptor potential vanilloid sub-family member 1 (TRPV1), a neuronally expressed receptor, is also a receptor for AEA and possibly 2-AG (Smart and Jerman 2000; McVey, Schmid et al. 2003; Golech, McCarron et al. 2004).

Endocannabinoids mediate energy intake centrally by promoting hunger and motivation to eat via activation of CB₁ in the hypothalamus and nucleus accumbens shell, respectively (Williams and Kirkham 1999; Duarte, Alonso et al. 2004). Targeted inhibition of CB₁ in obese humans and animals decreases body weight, improves insulin sensitivity and promotes oxidation of glucose and lipids over storage (Pagotto, Marsicano et al. 2006). The effects of endocannabinoids and their receptors on skeletal muscle glucose utilisation, particularly in obesity, remain to be fully elucidated and are the subject of this thesis. This chapter reviews the current data on skeletal muscle energy metabolism, the impact of obesity on insulin sensitivity and glucose homeostasis and the effects of the endocannabinoid system on peripheral metabolism.

1.2. SKELETAL MUSCLE

1.2.1. Skeletal Muscle Glucose Metabolism

Glucose is the principle source of cellular energy and substrate storage (Bouche, Serdy et al. 2004). In response to a hyperglycaemic and hyperinsulinemic challenge, skeletal muscle accounts for up to 95% of whole body glucose uptake (Baron, Brechtel et al. 1988). Therefore, skeletal muscle is a key site in maintaining post-prandial glycaemia. The physiology of skeletal muscle glucose transport and disposal is discussed below.

1.2.1.1. Glucose Transport

The hydrophilic nature of glucose and the observation that glucose uptake may be inhibited by cytochalasin B in human erythrocytes (Bloch 1973) provided the early evidence of a glucose transporter. The first glucose transporter was purified in 1978 in human erythrocytes by anion-exchange chromatography (Zoccoli, Baldwin et al. 1978). Subsequent studies utilising rodent adipocytes reported that glucose transporters are pooled in sub-cellular vesicles and are recruited to the cell surface in response to insulin (Wardzala, Cushman et al. 1978; Cushman and Wardzala 1980; Suzuki and Kono 1980; Kono, Suzuki et al. 1981). The discovery of a unique facilitated glucose transporter in adipose tissue and skeletal muscle (James, Brown et al. 1988) provided the first evidence of a glucose transporter family. Glucose transporters are referred to as GLUTs, with each unique GLUT numbered in the order in which it was isolated. Currently, there are 14 known GLUTs. In skeletal

muscle, the dominant glucose transporters are GLUT1 and GLUT4 (Flier, Mueckler et al. 1987; Fukumoto, Kayano et al. 1989). GLUT1 is predominately membrane bound, transporting glucose under basal conditions (Olson and Pessin 1996). GLUT4 is located in intracellular storage sites and is translocated to the cell surface in response to insulin (Goodyear and Kahn 1998). A schematic of cellular glucose transport is shown in Figure 1.1.

1.2.1.2. Basal Glucose Uptake

Basal glucose uptake sustains respiration in many cell types and is increased in response to metabolic stresses such as hypoxemia, inhibition of oxidative phosphorylation or osmotic stresses (Bouche, Serdy et al. 2004). Studies utilising Clone 9 (rat epithelial) cells have suggested that AMP-activated protein kinase (AMPK), a fuel-sensing enzyme that is present in all mammalian cells, may mediate these stress responses (Barnes, Ingram et al. 2002; Richter and Ruderman 2009).

The majority of basal glucose uptake occurs in non-insulin responsive tissues such as the brain and gut (Baron, Brechtel et al. 1988). The large proportion of total body mass represented by skeletal muscle and the observation that basal glucose uptake is decreased in patients with type 2 diabetes mellitus (T2DM) (Ciaraldi, Mudaliar et al. 2005) suggests that this tissue is also a significant contributor to non-insulin mediated glucose utilisation in health and disease.

1.2.1.3. Insulin-Stimulated Glucose Uptake

Under basal conditions, GLUT4 is localised in intracellular storage sites in adipose tissue, cardiac and skeletal muscle (Khan and Pessin 2002). Insulin signalling via the insulin receptor in skeletal muscle promotes GLUT4 translocation to the cell surface increasing glucose uptake as well as promoting synthesis of glycogen, triglyceride and protein and the up-regulation of genes involved in anabolic respiration and cell growth (Heesom, Harbeck et al. 1997). Translocation of GLUT4 to the plasma membrane is the rate-limiting step in insulin-stimulated glucose uptake in adipose tissue and skeletal muscle (Bouche, Serdy et al. 2004).

GLUT4 translocation is also induced by muscle contraction independently of insulin (Wallberg-Henriksson and Holloszy 1985; Holloszy 2003). There is a large body of evidence linking increases in GLUT4 as a result of muscle contraction to AMPK activity and increased myocyttoplasmic Ca^{2+} (Klip 2009; Richter and Ruderman 2009). The mechanisms of action of AMPK and Ca^{2+} on GLUT4 translocation are currently unknown.

Figure 1.1

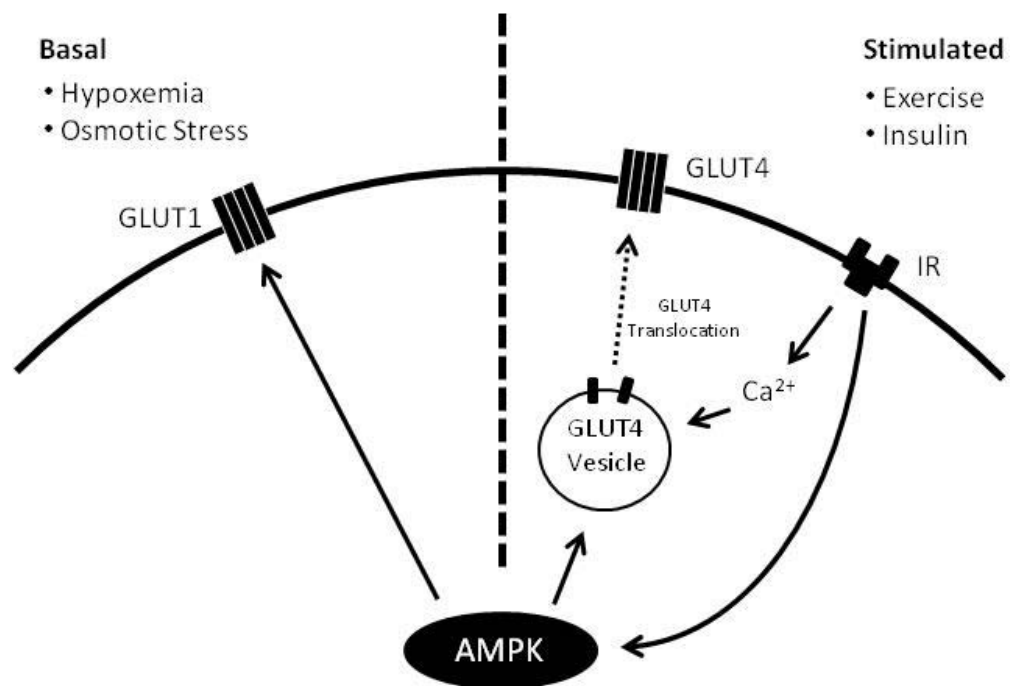


Figure 1.1: Schematic of cellular glucose transport in skeletal muscle. Basal glucose uptake occurs predominately via GLUT1. Insulin-stimulated glucose uptake occurs predominately via GLUT4 which is translocated to cell surface in response to insulin. Both basal and insulin-stimulated glucose uptake may be mediated by AMPK. AMPK, AMP-activated protein kinase; IR, insulin receptor.

1.2.2. Glucose Disposal in Skeletal Muscle

The metabolism of glucose in skeletal muscle is summarised in Figure 1.2. Once in the cell, glucose is rapidly phosphorylated and converted to glucose-6-phosphate (G-6-P) by the enzyme hexokinase II (Printz, Koch et al. 1993). Skeletal muscle does not possess the enzyme glucose-6-phosphatase and as a consequence, is unable to release glucose back into circulation. Therefore, the conversion of glucose into G-6-P is a terminal step. There are three major pathways for G-6-P disposal: 1) glycolysis 2) glycogenesis or 3) the hexosamine biosynthetic pathway (Bouche, Serdy et al. 2004).

Glycolysis is the primary pathway in the oxidation of glucose, forming 2 molecules of pyruvate. Under aerobic conditions, pyruvate may enter the mitochondria where it undergoes conversion into acetyl-coenzyme A (CoA) by the pyruvate dehydrogenase complex (PDC). Acetyl-CoA is then oxidised via the citric acid cycle releasing CO₂ and energy (Bouche, Serdy et al. 2004). The PDC is a major determinant of glucose oxidation in skeletal muscle and is inactivated by high stores of energy (in the form of ATP) and inhibitory phosphorylation by the pyruvate dehydrogenase kinases (PDKs) (Sugden and Holness 2003). PDK4 is the predominant PDK isoenzyme in skeletal muscle (Rowles, Scherer et al. 1996) and expression of PDK4 is rapidly up-regulated during starvation to conserve glucose (Sugden, Kraus et al. 2000).

Another pathway mediating glucose disposal is glycogenesis which results in the storage of glucose as glycogen. Most of the glucose from insulin-stimulated glucose uptake in skeletal muscle is stored as glycogen which is used as an energy source for muscle contraction (Bouche, Serdy et al. 2004). The rate limiting step in glycogen synthesis is the regulation of the enzyme glycogen synthase. Glycogen synthase is activated in response to two known mechanisms: 1) insulin-stimulation and 2) glycogen depletion. AMPK may inhibit glycogen synthase in favour of glucose oxidation, both *in vivo* and *in vitro* (Halse, Fryer et al. 2003; Jorgensen, Nielsen et al. 2004).

A small amount of glucose (approximately 1-3%) is disposed via the hexosamine biosynthetic pathway, creating substrates for the synthesis of glycoproteins (Hawkins, Barzilai et al. 1997).

Figure 1.2

NOTE:
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of the print copy of the thesis held in
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Figure 1.2: Simplified schematic of glucose utilisation in skeletal muscle. Upon entry into the cell, glucose is rapidly phosphorylated and converted to glucose-6-phosphate and may undergo one of three major pathways for disposal: 1) glycolysis for oxidation via the Citric Acid Cycle 2) glycogenesis for synthesis of glycogen 3) the hexosamine biosynthetic pathway (Bouche, Serdy et al. 2004).

1.2.3. Intracellular Mechanisms Mediating Skeletal Muscle Glucose Utilisation

Several intracellular factors may regulate glucose utilisation in skeletal muscle. The studies in this thesis will analyse three key components of glucose metabolism in skeletal muscle in response to alterations in endocannabinoid signalling: AMPK, peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) and PDK4.

1.2.3.1. AMP-Activated Protein Kinase (AMPK)

AMPK, a ubiquitous serine/threonine protein kinase, is a heterotrimeric complex containing a catalytic alpha subunit of which there are 2 isoforms and 2 regulatory subunits beta and gamma of which there are 2 and 3 isoforms, respectively (Hardie, Carling et al. 1998).

AMPK is allosterically activated by AMP in response to a rise in the AMP/ATP ratio. The resultant increase in ATP inhibits AMPK activity (Hardie and Hawley 2001). Hypoxia, hypoglycaemia, heat shock and prolonged exercise (Winder and Hardie 1999), which deplete cellular ATP, induce an increase in AMPK activity. In most cell types, AMPK is activated by the tumour suppressor kinase LKB1 to protect against apoptosis from rising levels of AMP (Shaw, Kosmatka et al. 2004). Inhibition of AMPK may occur via Cidea, a regulator of energy expenditure in brown adipose tissue (BAT) in mice (Qi, Gong et al. 2008), although it is unclear whether this mechanism exists in skeletal muscle.

The endocannabinoids, in particular AEA, stimulate AMPK activity in the hypothalamus and cardiac muscle while inhibiting AMPK in liver and adipose tissue (Kola, Hubina et al. 2005). In skeletal muscle, AM251, a specific CB₁ inverse agonist, increases AMPK α 1 mRNA content in primary cultures from obese individuals (Cavuoto, McAinch et al. 2007).

AMPK performs several roles in the regulation of skeletal muscle energy utilisation; a visual representation of these functions is shown in Figure 1.3. AMPK is activated by the hormones leptin, adiponectin and ghrelin to promote FA oxidation (Minokoshi, Kim et al. 2002; Yamauchi, Kamon et al. 2002; Barazzoni, Bosutti et al. 2005). Activation of AMPK leads to phosphorylation of acetyl-CoA carboxylase (ACC) (Abu-Elheiga, Matzuk et al. 2001) thereby inhibiting production of malonyl-CoA, an enzyme responsible for the inhibition of carnitine palmitoyltransferase-1 (CPT-1), a rate limiting step for the entry of FAs for oxidation in the mitochondria (Winder 2001).

In addition to promoting basal and insulin-stimulated glucose uptake, AMPK inhibits synthesis of glycogen by phosphorylating glycogen synthase (Halse, Fryer et al. 2003; Jorgensen, Nielsen et al. 2004). The drug metformin, which lowers blood sugar levels in Type 2 diabetics, stimulates AMPK activity in skeletal muscle *in vitro*. Whether or not this is a significant mechanism of effect *in vivo* is uncertain as

circulating concentrations of the drug are decreased between the gut and the periphery (Towler and Hardie 2007).

AMPK has also been shown to increase expression and activity of PGC-1 α , a transcription factor which induces mitochondrial biogenesis and enhances oxidative capacity of skeletal muscle (Jager, Handschin et al. 2007).

Figure 1.3

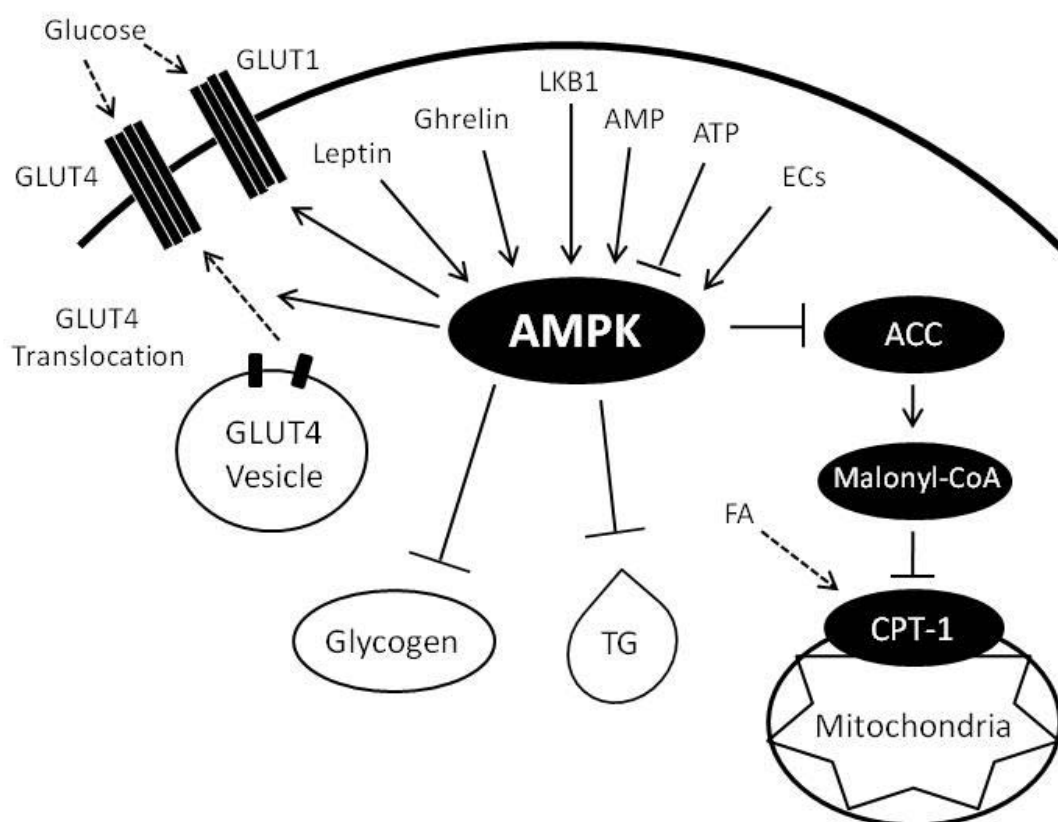


Figure 1.3: Regulation and function of AMPK in skeletal muscle metabolism. AMPK is activated by LKB1, leptin, ghrelin, increasing levels of AMP and possibly the endocannabinoids. Increasing levels of ATP inhibit AMPK activity. AMPK promotes fatty acid oxidation by inhibition of ACC, allowing entry of FAs into the mitochondria via CPT-1. AMPK also promotes glucose uptake via GLUT4 translocation and GLUT1 and inhibits glycogen and TG synthesis. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CPT-1, carnitine palmitoyltransferase-1; ECs, endocannabinoids; FA, fatty acids; TG, triglycerides.

1.2.3.2. Peroxisome Proliferator-Activated Receptor γ Co-Activator 1 α (PGC-1 α)

PGC-1 α is a transcriptional co-activator of several genes involved in energy metabolism (Houten and Auwerx 2004) and its mRNA content in skeletal muscle may be regulated by endocannabinoids (Cavuoto, McAinch et al. 2007). PGC-1 α was first described as a cold-induced activator of the transcription factor peroxisome proliferator-activated receptor γ (PPAR γ), suggesting a role in adaptive thermogenesis (Puigserver, Wu et al. 1998). PGC-1 α stimulates mitochondrial biogenesis by modulating regulators of mitochondrial transcription/replication such as the nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (Wu, Puigserver et al. 1999). PGC-1 α via co-activation with the muscle selective transcription factor myocyte enhancer factor 2C (MEF2C) induces increases in GLUT4 mRNA content in skeletal muscle in an AMPK-dependent manner (Michael, Wu et al. 2001; Oliveira, Ueno et al. 2004).

PGC-1 α also promotes oxidative phosphorylation of mitochondria by up-regulating several components of the respiratory chain such as cytochrome c oxidase subunits II and IV, cytochrome c, and β -ATP synthetase (Wu, Puigserver et al. 1999). In addition, PGC-1 α mediates fibre-type switching from Type II to Type I fibres in transgenic mice over-expressing PGC-1 α by increasing mitochondrial density (Lin, Wu et al. 2002). Mitochondrial density is a major contributor to cold-induced adaptive thermogenesis particularly in lean healthy humans as skeletal

muscle accounts for approximately 40-50% of body weight and levels of BAT are relatively low (Puigserver and Spiegelman 2003). Taken together, these data suggest that PGC-1 α is a key regulator of skeletal muscle energy utilisation.

1.2.3.3. Pyruvate Dehydrogenase Kinase 4 (PDK4)

The PDK isoenzymes regulate glucose oxidation by phosphorylation and subsequent inactivation of PDC, a rate limiting step in the conversion of pyruvate into acetyl-CoA (Sugden and Holness 2003). A schematic of PDK4 regulation of skeletal muscle glucose utilisation is shown in Figure 1.4. PDK4 is a downstream target of PGC-1 α (Ma, Zhang et al. 2005) and may be regulated at the mRNA level in skeletal muscle in response to endocannabinoids (Cavuoto, McAinch et al. 2007).

PDK4 expression is unchanged in skeletal muscle from lean healthy humans after 90 minutes of exercise (Stephens, Norton et al. In Press). However, PDK4 mRNA is increased after prolonged exercise (Pilegaard and Neufer 2004), possibly to restore depleted glycogen stores. PDK4 expression is also increased in skeletal muscle during starvation which may be fibre type dependent (Rodriguez, Sanchez et al. 2010). Time-course effects of starvation in rats resulted in larger increases in PDK4 mRNA content in gastronemius (glycolytic) than soleus (oxidative) muscle which may act as a mechanism to conserve glucose (Rodriguez, Sanchez et al. 2010).

In the same study, PDK4 expression increased in response to free fatty acids (FFAs) in C2C12 muscle cell culture (Rodriguez, Sanchez et al. 2010). In cultured human skeletal muscle cells, the effects of FFAs to up-regulate PDK4 are ameliorated by insulin (Abbot, McCormack et al. 2005). Insulin suppression of PDK4 is impaired in the insulin resistant state in rats (Kim, Lee et al. 2006). Taken together, these data present a possible mechanism for impaired PDK4 activity and therefore glucose utilisation in obesity.

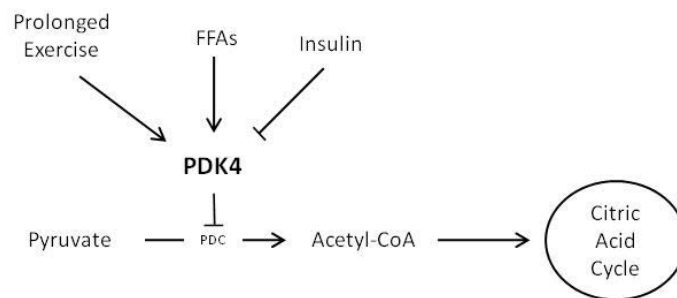
Figure 1.4

Figure 1.4: Regulation and function of PDK4 on skeletal muscle glucose utilisation. PDK4 expression is up-regulated in response to prolonged exercise and FFAs and inhibited by insulin. PDK4 inhibits PDC, a rate limiting step in the conversion of pyruvate to acetyl-CoA for entry into the Citric Acid Cycle. FFAs, free fatty acids; PDC, pyruvate dehydrogenase complex; PDK4, pyruvate dehydrogenase kinase 4.

1.3. OBESITY AND INSULIN RESISTANCE

1.3.1. Obesity

Obesity is the result of an imbalance between energy intake and energy expenditure, leading to accumulation of adipose tissue (James 2004). Factors that are linked to obesity include increased availability of energy dense foods, a lack of physical activity and genetic predisposition (Astrup, Buemann et al. 1994; Astrup, O'Hill et al. 2004). The prevalence of obesity in Australia is increasing. The most recent surveys on obesity were performed by the Australian Bureau of Statistics in the 2007-2008 National Health Survey (NHS) which reported that 25% of adults were obese (with a body mass index [BMI] ≥ 30) as compared to 7.1% in 1980 and 18.4% in 2000 (NHS 2007-2008).

The World Health Organisation (WHO) classifies Caucasians with a BMI ≥ 30 kg/m² as obese (James 2004). BMI as a measure of obesity is limited as it does not take into consideration differences in body fat distribution as a result of sex, ethnicity or age (Baumgartner, Heymsfield et al. 1995). Therefore, classifications of obesity by BMI may differ between population groups. For example, adult Asians with a BMI ≥ 25 are classified as stage I obese (James 2004). Such differences are also evident in the Australian population as Australians of European descent have markedly different body fat distribution and fat mass for a given BMI than Indigenous Australians (Piers, Rowley et al. 2003).

Individuals with central or visceral obesity have increased risk of chronic conditions such as T2DM, stroke and heart disease (Allison, Zannolli et al. 1999; Adams, Schatzkin et al. 2006). In addition, visceral obesity is associated with insulin resistance (Despres and Lemieux 2006). In contrast, obese individuals with low levels of visceral adiposity may maintain a normal metabolic profile (i.e. high sensitivity to insulin, high high-density lipoproteins and low triglycerides) (Ferrannini, Natali et al. 1997).

Thus an alternative to BMI is to measure waist circumference (van der Kooy, Leenen et al. 1993). A waist circumference of > 102 cm for non-Asian men and >88 cm for non-Asian women correlates with a BMI > 30 and suggests a high risk to health (Lean, Han et al. 1995). Waist circumference thresholds are set lower for individuals of Asian descent (Huxley, Barzi et al. 2007). A waist circumference of > 90 for Asian men and > 80 for Asian women is indicative of abdominal obesity and increased risk to health, although these numbers may slightly vary for Koreans and Asian Indians (Vikram, Pandey et al. 2003; Baik 2009).

1.3.2. Insulin Resistance

Insulin resistance is a condition in which insulin sensitive cells have an impaired ability to produce a normal insulin response. In skeletal muscle insulin resistance is characterised by an impaired ability of the tissue to take up glucose in response to insulin (Corcoran, Lamon-Fava et al. 2007). Individuals with insulin resistance

typically have elevated levels of circulating insulin and impaired control of blood glucose.

Multiple mechanisms have been identified as mediators of insulin resistance. This includes, but is not limited to, excess accumulation of visceral adipose tissue (Pan, Lillioja et al. 1997), increased triglyceride content ectopically in skeletal muscle, liver, pancreas and cardiac tissue (Despres and Lemieux 2006) and increased secretion of inflammatory adipokines, such as IL-6 and TNF- α , from adipose tissue into circulation (Cote, Mauriege et al. 2005). In addition, there is emerging evidence suggesting that endocannabinoids derived from adipose tissue may also mediate skeletal muscle insulin resistance (Watt 2009). Furthermore, increased expression of TNF- α is associated with elevated activity of the endocannabinoid system (Kempf, Hector et al. 2007). This was demonstrated via *in vitro* stimulation of human visceral adipose tissue by TNF- α which decreased expression of the endocannabinoid metabolising enzyme fatty acid amide hydrolase (FAAH), and adiponectin (Kempf, Hector et al. 2007).

Expression of endocannabinoids is increased in viscerally obese humans (Engeli, Bohnke et al. 2005; Cote, Matias et al. 2007). Inhibition of CB₁ in obese rats decreases triglyceride content and increase glucose uptake and insulin sensitivity in skeletal muscle (Herling, Kilp et al. 2008; Nogueiras, Veyrat-Durebex et al. 2008; Cota, Sandoval et al. 2009). Taken together, these studies suggest a role for the

endocannabinoid system in mediating insulin sensitivity in skeletal muscle and will be investigated in this thesis.

1.4. THE ENDOCANNABINOID SYSTEM

1.4.1. Isolation of Delta-9-Tetrahydrocannabinol (Δ^9 -THC) and the receptors for Endocannabinoids

In 1964, Gaoni and Mechoulam first characterised delta-9-tetrahydrocannabinol (Δ^9 -THC) the main psychoactive constituent of marijuana (Gaoni and Mechoulam 1964). Studies into the cellular effects of Δ^9 -THC found that it inhibited adenylyl cyclase (Howlett and Fleming 1984). Inhibition of adenylate cyclase reduces the conversion of ATP to cyclic AMP (cAMP), a second messenger in cell signalling. The inhibitory effect of Δ^9 -THC could be blocked via pertussis toxin (Howlett, Qualy et al. 1986) suggesting the presence of a $G_{i/o}$ protein. These findings eventually led to the cloning of CB_1 , a specific receptor for cannabinoids (Matsuda, Lolait et al. 1990). Shortly after, a subsequent peripheral cannabinoid receptor subtype, CB_2 , was cloned (Munro, Thomas et al. 1993).

1.4.2. The Cannabinoid Receptors

Cannabinoid receptors belong to the 7-transmembrane region receptor family and are $G_{i/o}$ protein coupled (Matsuda, Lolait et al. 1990; Munro, Thomas et al. 1993). The signal transduction pathways have been extensively described in many reviews and are shown in Figure 1.5 (Piomelli 2003; De Petrocellis, Cascio et al. 2004; Howlett 2005; Demuth and Molleman 2006). In brief, activation of CB_1 induces a signalling cascade which begins with the stimulation of $G_{i/o}$ proteins that,

in turn, inhibit the adenylate cyclase mediated conversion of ATP to cAMP. The action of cAMP involves the activation of protein kinase A (PKA), resulting in the phosphorylation of A-type potassium (K^+_A) channels. Therefore, the inhibition of adenylate cyclase by CB_1 action results in the activation of K^+_A channels. CB_1 activation can also stimulate several intracellular kinases, which include the p38 mitogen-activated protein kinase (MAPK), MEK/ERK MAPK, and phosphoinositide 3-kinase (PI3K) pathways (Pagotto, Marsicano et al. 2006). MAPK is a key signalling mechanism that regulates critical cellular functions such as growth, transformation and apoptosis. Stimulation of intracellular kinases can lead to the induced expression of transcription factors such as c-fos, c-jun and zif268 and the brain derived neurotrophic factor (BDNF). Ion channels can also be modulated by CB_1 activation through the $G_{i/o}$ protein, which can directly inhibit N- or P/Q-type Ca^{2+} channels and activate inwardly rectifying potassium (K_{ir}) channels. These two channels are regulated by protein kinase C (PKC) which, once activated, can phosphorylate CB_1 resulting in an uncoupling of the receptor from the ion channels. The signal transduction pathways of CB_2 are similar to that of CB_1 with the exception of ion channel regulation. It should be noted, however, that numerous studies have identified diverse pathways of cannabinoid signal transduction that are not always mediated by the known cannabinoid receptors (see review by Demuth and Molleman (2006)).

Figure 1.5

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of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.5: Main effects of CB₁ on intracellular signalling cascades (Pagotto, Marsicano et al. 2006). Activation of CB₁ leads to stimulation of G_{i/o} proteins which inhibit adenylate cyclase mediated conversion of ATP to cAMP, regulate potassium and calcium channels and stimulate several intracellular kinases. BDNF, brain derived neurotrophic factor; CB₁, cannabinoid receptor type 1; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C

CB₁ is expressed centrally in areas of the brain associated with higher cognitive function, control of movement, and control of motor and sensory functions consistent with the effects of cannabinoids such as Δ^9 -THC from marijuana use (Glass, Dragunow et al. 1997). Furthermore, CB₁ is found in abundance in the hypothalamus and nucleus accumbens shell where it may mediate effects on appetite and motivation to eat (Harrold and Williams 2003). Peripherally, prolonged CB₁ activity may impair energy disposal in several metabolically active tissues such as skeletal muscle, adipose tissue, liver, pancreas and the gastrointestinal tract (Pagotto, Marsicano et al. 2006; Cavauto, McAinch et al. 2007). A detailed synopsis of endocannabinoid mediated effects on peripheral tissues which predominately occur via CB₁, is presented in section 1.4.7.

CB₂ was initially thought to be expressed in some cells of the immune system and some peripheral tissues such as the spleen (Munro, Thomas et al. 1993; Schatz, Lee et al. 1997). Recent studies have characterised glial and neuronal CB₂ expression in the brain and central nervous system, suggesting a potential role for CB₂ in neurodegenerative and neuropsychiatric disorders (Nunez, Benito et al. 2004; Benito, Tolon et al. 2008; Nunez, Benito et al. 2008). The current understanding of the peripheral effects of CB₂ remain limited, however, the observations that CB₂ is expressed in insulin-secreting pancreatic beta cells (Starowicz, Cristino et al. 2008)

and skeletal muscle (Cavuto, McAinch et al. 2007) suggests that this receptor may have a role in energy metabolism and warrants further investigation.

1.4.3. Other Receptors for Cannabinoids

GPR55, a G-protein coupled receptor has been identified as a possible third cannabinoid receptor as it binds to synthetic cannabinoid ligand CP55940 (Ryberg, Larsson et al. 2007). Whether GPR55 is a true cannabinoid receptor remains controversial as it has been reported by some that AEA and 2-AG bind to GPR55 (Ryberg, Larsson et al. 2007), but not by others (Oka, Nakajima et al. 2007). In addition, GPR55 shares only ~13.5% sequence homology with CB₁ and ~14.4% homology with CB₂ (Ross 2009).

TRPV1, an ion channel, is known to bind to some endocannabinoids (Di Marzo 2008) and is discussed in detail in section 1.5.

1.4.4. Cannabinoid Receptor Agonists and Inverse Agonists

1.4.4.1. Agonists

The endogenous cannabinoid AEA was isolated shortly after the initial cloning of CB₁ (Devane, Hanus et al. 1992). This was quickly followed by the isolation of 2-AG (Sugiura, Kondo et al. 1995). AEA and 2-AG are the most abundant endocannabinoids and are both synthesised in cellular membranes from

arachidonic acid (Fowler and Jacobsson 2002). Arachidonic acid is an omega-6 essential FA that is cleaved from phospholipids or synthesised from diacylglycerol (DAG) (Baynes and Dominiczak 2005). AEA has the highest affinity for the cannabinoid receptors whereas 2-AG has the highest binding efficiency (Di Marzo 2008). To date, numerous cannabinoids have been identified, and can be categorised into three groups: herbal, endogenous and synthetic (Di Marzo and Petrocellis 2006).

The actions of AEA and 2-AG are brief as they are rapidly metabolised by FAAH and monoacylglycerol lipase (MAGL), respectively (Figure 1.6) (Deutsch and Chin 1993; Goparaju, Ueda et al. 1999). The mechanisms by which endocannabinoids are taken up into the cell remain speculative; it is unknown whether a transporter for AEA and 2-AG exists or if these endocannabinoids enter the cell via simple diffusion (Glaser, Kaczocha et al. 2005; Hermann, Kaczocha et al. 2006).

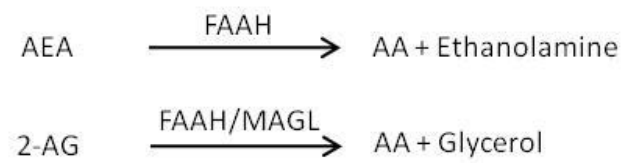
Figure 1.6

Figure 1.6: Metabolism of AEA and 2-AG by FAAH and MAGL (Mechoulam, Fride et al. 1998). Once in the cell AEA and 2-AG are rapidly metabolised by FAAH and MAGL, respectively. 2-AG, 2-arachidonoylglycerol, AA, arachidonic acid, AEA, anandamide, FAAH, fatty acid amide hydrolase, MAGL, monoacylglycerol lipase.

1.4.4.2. Inverse Agonists

Many studies investigating the potential role of the endocannabinoid system in appetite regulation and energy balance have primarily utilised the selective CB₁ inverse agonist SR141716 (also known as rimonabant) (Cota, Marsicano et al. 2003; Ravinet Trillou, Arnone et al. 2003). An inverse agonist binds to the same receptor binding site as an agonist and reduces the constitutive activity of the receptor as opposed to an antagonist which binds to the receptor without activating it (Kenakin 2004). SR141716 was the first selective CB₁ inverse agonist to be synthesised (Rinaldi-Carmona, Barth et al. 1994). SR141716 had, until recently, been viewed as a therapeutic agent for weight loss (Gary-Bobo, Elachouri et al. 2007). However, the central side effects of SR141716 have precluded its use as a therapeutic agent (Christensen, Kristensen et al. 2007).

SR144528, a selective CB₂ inverse agonist of similar structure to SR141716, was also the first of its type to be synthesised (Rinaldi-Carmona, Barth et al. 1998). SR144528 has been used in several studies investigating immune activation and suppression of inflammation (Di Marzo 2008). However, the effects of SR144528 on energy utilisation have not hitherto been reported. Although SR141716 and SR144528 are described as being selective, there is some evidence that these compounds may partially bind to and act as a partial agonists at the opposing cannabinoid receptor (Krylatov, Maslov et al. 2005). These data have not limited the

use of SR141716 and SR144528 as these agents remain the most utilised cannabinoid receptor blockers in the research community despite a large catalogue of similar compounds (Muccioli and Lambert 2005).

1.4.5. Levels of Endocannabinoids in Unstimulated Tissues and Cells

Several analytical methods, mostly based on high pressure liquid chromatography (HPLC) or gas chromatography coupled to mass spectrometry (GC-MS) have been utilised for the quantification of endocannabinoids in tissues and biological fluids (Mechoulam, Fride et al. 1998). Both AEA and 2-AG have been detected in several tissues including the brain, skeletal muscle, adipose tissue, liver, pancreas and gut leading to the suggestion that they may induce a wide range of effects via cannabinoid receptors on energy utilisation in these tissues. In rat brain, AEA was found in concentrations ranging from 'not detectable' (Kempe, Hsu et al. 1996) to 29 pmol/g tissue whereas higher levels (up to 148 pmol/g tissue) were found in the human brain (Felder, Nielsen et al. 1996). In contrast, levels of 2-AG in the rat brain were found to be up to 170 times higher than those of AEA (Sugiura, Kondo et al. 1995; Stella, Schweitzer et al. 1997). Peripherally, 2-AG levels appear to be significantly higher than that of AEA in most tissues (Mechoulam, Fride et al. 1998). Specifically, in mouse soleus muscle, AEA levels were ~65-70 pmol/g tissue whereas 2-AG levels were much higher ~2-4 nmol/g tissue (Matias, Petrosino et al. 2008). Whilst circulating levels of 2-AG are increased in obesity in humans (Cote, Matias et al. 2007), endocannabinoid content in human skeletal muscle is unknown.

1.4.6. Central Effects of Endocannabinoids on Energy Metabolism

Several studies have reported a major role of the endocannabinoid system in the central regulation of food intake (see review by Di Marzo and Matias (2005)). Endocannabinoid mediated effects on energy metabolism, particularly in the hypothalamus, is associated with leptin. Leptin is a hormone that is secreted by adipocytes into the peripheral circulation in proportion to the amount of fat stored and regulates not only food intake, but also the central regulation of energy expenditure (Friedman and Halaas 1998). Di Marzo *et al.* (2001) showed that defective leptin signalling in the hypothalamus of obese *db/db* and *ob/ob* mice and Zucker rats was associated with an increase in endocannabinoid content. Furthermore, acute leptin treatment in normal rats and *ob/ob* mice reduced AEA and 2-AG levels in the hypothalamus suggesting a role for endocannabinoids in the neural circuitry regulated by leptin (Di Marzo, Goparaju *et al.* 2001). For example, cannabinoid and leptin signals are integrated in lateral hypothalamic neurons (Jo, Chen *et al.* 2005).

Leptin is also involved in the regulation of neural networks via neurons in the arcuate and ventro medial hypothalamus (VMH). The VMH is involved in the regulation of thermogenesis as well as appetite and overall energy balance. Selective loss of leptin receptors in the VMH results in increased adiposity and the metabolic syndrome (Preston, Triandafillou *et al.* 1989; Bingham, Anderson *et al.* 2008). CB₁ is selectively expressed in steroidogenic factor 1 (SF1) expressing VMH

neurons. Absence of these neurons leads to weight gain, and their excitability is decreased in the presence of CB₁ agonists and increased by leptin (Dhillon, Zigman et al. 2006).

In addition to leptin, there is indirect evidence that ghrelin, a peptide hormone that is secreted by P/D1 cells in the stomach, may increase appetite via the endocannabinoid system in the hypothalamus. Studies have reported that the orexigenic effect of ghrelin in the hypothalamus is inhibited by SR141716 (Tucci, Rogers et al. 2004) and does not appear in CB₁^{-/-} mice (Kola, Farkas et al. 2008).

While these data do not prove a role for the endocannabinoid system in the regulation of energy expenditure, or more specifically thermogenesis at the level of the hypothalamus (as opposed to the clear effects on food intake), it is at least plausible, given the actions of leptin and possibly ghrelin, that this is the case.

1.4.7. Peripheral Effects of Endocannabinoids on Energy Metabolism

1.4.7.1. Skeletal Muscle

The first evidence for a role of the endocannabinoid system in skeletal muscle was provided by Liu *et al.* (2005) who demonstrated that SR141716 increased glucose uptake and oxygen consumption in isolated soleus muscle preparations of genetically obese *Lep^{ob}/Lep^{ob}* mice. Pagotto *et al.* (2006) then reported that the expression of CB₁ in skeletal muscle is up regulated in mice fed a high fat diet (HFD).

More recent *in vivo* studies have demonstrated that SR141716 decreases triglyceride content and increases glucose uptake and insulin sensitivity in diet induced obese rats (Herling, Kilp et al. 2008; Nogueiras, Veyrat-Durebex et al. 2008; Cota, Sandoval et al. 2009).

In vitro, inhibition of CB₁ with SR141716 mediates beneficial effects on the insulin signalling pathway in skeletal muscle cultures. In L6 culture, 100 nM SR141716 stimulated basal 2-deoxy glucose uptake via regulation of PI3K (Esposito, Proto et al. 2008). In another study, also using L6 culture, chronic (24 hour) exposure to 100 nM of the synthetic cannabinoid receptor agonist WIN55,212-2 inhibited insulin-mediated activation of ERK, but not protein kinase B (PKB; also known as Akt) (Lipina, Stretton et al. 2010). In the same study, similar effects were observed using the selective CB₁ agonist arachidonyl-2'-chloroethylamide hydrate (ACEA). Conversely, 24 hour exposure to 100 nM SR141716 enhanced the insulin-mediated activation of ERK and PKB (Lipina, Stretton et al. 2010). Endocannabinoids from adipose tissue may also impair skeletal muscle insulin sensitivity (Eckardt, Sell et al. 2009). Adipocyte-conditioned medium from overweight and obese subjects contained naturally occurring levels of AEA and 2-AG and induced insulin resistance in cultures of human skeletal muscle; an effect which was inhibited by SR141716 (Eckardt, Sell et al. 2009).

Additionally, *in vitro* studies have implicated CB₁ in the regulation of AMPK mRNA. The selective CB₁ inverse agonist AM251 increased AMPK α 1 mRNA expression in human primary skeletal muscle myotubes derived from obese, but not lean humans (Cavuoto, McAinch et al. 2007). It is not known whether changes in AMPK α 1 mRNA expression in this model is associated with an increase in glucose uptake and warrants further investigation.

In contrast to the effects of CB₁ blockers, no effect of cannabinoids has been observed on AMPK mRNA expression in skeletal muscle of either lean rats (Kola, Hubina et al. 2005), or lean and obese humans (Cavuoto, McAinch et al. 2007). In cardiac myocytes, which depend on FA oxidation for energy under basal conditions, expression and functional activity of AMPK is increased by endocannabinoids (Kola, Hubina et al. 2005).

1.4.7.2. Adipose Tissue

In mice, cannabinoids promote lipogenesis and the storage of adipose tissue via CB₁ (Cota, Marsicano et al. 2003). The expression of CB₁ in adipose tissue is up-regulated in rodent models of obesity (Bensaid, Gary-Bobo et al. 2003; Yan, Liu et al. 2007; Starowicz, Cristino et al. 2008). In contrast, inhibitors of CB₁ promote lipolysis *in vivo* and inhibit pre-adipocyte proliferation *in vitro* (Jbilo, Ravinet-Trillou et al. 2005; Gary-Bobo, Elachouri et al. 2006; Herling, Kilp et al. 2008). It has been

suggested that this may, at least in part, be due to the up-regulation of adiponectin gene expression in adipocytes, secretion of protein, and consequently increased circulating adiponectin levels (Bensaid, Gary-Bobo et al. 2003). Secretion of adiponectin in human adipose tissue *in vitro* is up-regulated by AMPK which also stimulates lipid oxidation and inhibits lipogenesis (Lihn, Jessen et al. 2004). In addition, in adipose tissue from male Lister hooded rats, cannabinoids have an inhibitory effect on AMPK, thereby increasing lipid stores (Kola, Hubina et al. 2005). Another mechanism by which cannabinoids promote lipogenesis and the storage of fat was demonstrated in 3T3-L1 adipocyte culture via a CB₁ dependent increase in insulin sensitivity (Motaghedi and McGraw 2008).

There is evidence to suggest the endocannabinoid system might regulate energy expenditure is by the induction of mitochondrial biogenesis. In mouse primary white adipocytes, SR141716 induced mitochondrial biogenesis and increased AMPK activity via an endothelial nitric oxide synthase (eNOS) dependent mechanism (Tedesco, Valerio et al. 2008). Obesity as a result of a HFD is associated with a reduction in mitochondrial biogenesis that is, together with fat accumulation, reversed by inhibition of CB₁ with SR141716 (Tedesco, Valerio et al. 2008).

Voluntary exercise augments the effect of CB₁ inverse agonists to induce lipolysis in rodents (Zhou and Shearman 2004), the mechanism of this effect is not known.

Human adipocytes express functional CB₁ and CB₂ receptors. Activation of CB₁ receptors increases intracytoplasmic cAMP, whereas activation of CB₂ decreases intracytoplasmic cAMP (Roche, Hoareau et al. 2006). The significance of this in the regulation of energy balance remains unclear.

1.4.7.3. Liver

Endocannabinoids in the liver, for example AEA, increase in response to a HFD and promote hepatic lipogenesis (Osei-Hyiaman, DePetrillo et al. 2005). The increase in liver fat induced by CB₁ agonists is mediated by increased sterol-regulatory element-binding protein-1c (SREBP-1c) and its targets acetyl-CoA carboxylase-1 and fatty acid synthase in wild type mice; an effect that does not occur in CB₁^{-/-} mice (Osei-Hyiaman, DePetrillo et al. 2005). These CB₁ deficient mice do not develop fatty livers in response to a HFD. Blockade of the CB₁ by SR141716 in obese Zucker (*fa/fa*) rats led to resolution of the liver fat (Gary-Bobo, Elachouri et al. 2007). This was associated with an increase in adiponectin and decrease in TNF- α , promoting an increase in the oxidation of fat in the liver. In addition, levels of plasma FFAs, triglycerides and total cholesterol decreased (Gary-Bobo, Elachouri et al. 2007).

1.4.7.4. Pancreas

The effects of cannabinoids on insulin secretion by pancreatic beta cells remain unresolved. Inhibition of insulin secretion in isolated islet cells from young mice (Nakata and Yada 2007) and stimulation in RIN-m5F beta-cells under high glucose conditions have both been observed (Matias, Gonthier et al. 2006). There is also conflicting data as to the distribution of CB₁ and CB₂ in the pancreas although both have been reported to be present (Starowicz, Cristino et al. 2008). Inhibition of insulin secretion has variably been reported to be mediated by CB₁ (Nakata and Yada 2007) and CB₂ (Juan-Pico, Fuentes et al. 2006). More recent studies reveal the presence of CB₁, CB₂ and FAAH, the enzyme that catabolises endocannabinoids, in human islets although data relating to the patterns of expression on different cell types within the islets are conflicting (Bermudez-Silva, Sanchez-Vera et al. 2007; Tharp, Lee et al. 2008). One group reported that CB₁ is predominantly found in glucagon-secreting alpha cells and less so in insulin-secreting beta cells and CB₂ is limited to somatostatin-secreting delta cells (Bermudez-Silva, Sanchez-Vera et al. 2007) whereas others, while confirming the presence of CB₁ in beta cells found specific expression in somatostatin-secreting delta cells (Tharp, Lee et al. 2008). Activation of CB₁ induces insulin and glucagon secretion, whereas CB₂ mediates a decrease in glucose-dependent insulin secretion (Bermudez-Silva, Sanchez-Vera et al. 2007).

1.4.7.5. Gastrointestinal Tract

The digestion and absorption of nutrients are pivotal to the regulation of energy balance. The rate of gastric emptying and proximal intestinal motility modify the rate at which nutrients appear in the peripheral circulation. Cannabinoids inhibit gastrointestinal motility in both rodents and humans via CB₁ (Coutts and Pertwee 1998; Izzo, Mascolo et al. 1999; Esfandyari, Camilleri et al. 2006). Mice fed a HFD display slower rates of gastric emptying which is reversed by CB₁ inhibition (Di Marzo, Capasso et al. 2008). Food deprivation increases small intestinal AEA content and slows the rate of gastric emptying, an effect reversed by re-feeding or administration of SR141716 (Gomez, Navarro et al. 2002). In the intestine the CB₁ mediated effect appears to attenuate intestinal motor responses in the myenteric components of the peristaltic reflex (Yuece, Sibaev et al. 2007). CB₁ in the gastrointestinal tract may directly modulate food intake. These receptors are located on capsaicin sensitive neurons suggesting a role for the TRPV1, which is a receptor for endocannabinoids as well as capsaicin (Gomez, Navarro et al. 2002). Endocannabinoid levels and expression of CB₁ on vagal afferents vary according to states of satiety. Vagal CB₁ is also regulated by gut peptides such as cholecystokinin and ghrelin. In the brainstem CB₁, CB₂ and TRPV1 all appear to mediate the effects of vagally mediated alterations in gut function (Gomez, Navarro et al. 2002).

1.4.8. Effects of Endocannabinoids on Energy Metabolism During Onset of Obesity

In humans, circulating levels of 2-AG correlate with increased levels of visceral adipose tissue, BMI and waist circumference (Cote, Matias et al. 2007). In mice, plasma endocannabinoid levels increased in those which consumed a HFD for 8 weeks as compared to mice fed standard chow (Starowicz, Cristino et al. 2008), but were indistinguishable between the dietary groups by 14 weeks, suggesting that endocannabinoid activity increases during the onset of obesity rather than at a later stage (Matias, Petrosino et al. 2008). The decrease in FAAH expression that is associated with obesity reduces the rate at which endocannabinoids are metabolised resulting in prolonged endocannabinoid effects regardless of plasma levels (Engeli, Bohnke et al. 2005; Starowicz, Cristino et al. 2008).

Insulin and leptin resistance and decreased FA oxidation are characteristics of obesity (Evans, Murray et al. 1984; Lonngqvist, Nordfors et al. 1997; Kelley, Goodpaster et al. 1999) and effect the regulation of, or are themselves subject to regulation by, the endocannabinoid system. Insulin induces an increase in the activity of the endocannabinoid degrading enzymes FAAH and MAGL in 3T3-L1 adipocytes (D'Eon, Pierce et al. 2008). Accordingly, the decrease in FAAH activity and resultant increase in endocannabinoid levels in obesity may at least partly contribute to the development of insulin resistance.

The infusion of leptin into the mediobasal hypothalamus (MBH) of rats increased FAAH expression and activity in white adipose tissue thereby decreasing intracellular endocannabinoids and suppressing lipogenesis. This effect of leptin can be inhibited by peripheral infusion of a CB₁ agonist (Buettner, Muse et al. 2008). These data suggest that one of the central effects of leptin is to decrease peripheral endocannabinoid tone; the failure of this to occur may be of pathophysiological importance in the maintenance of the obese state in the presence of central leptin resistance (Buettner, Muse et al. 2008).

The production of adiponectin is lowered in response to high fat feeding in mice, an effect which is not seen in mice deficient in CB₁ (Osei-Hyiaman, DePetrillo et al. 2005). Therefore, increases in endocannabinoid tone in response to high fat feeding reduce circulating adiponectin thereby promoting not only the maintenance of the obese state but also impairments in glucose metabolism.

Summary tables listing the actions of AEA and SR141716 in different tissues (Table 1.1) and *in vivo* (Table 1.2) in obesity are presented below.

Table 1.1: Tissue-Specific Effects of AEA and SR141716 in Obesity

Tissue	Mechanism	AEA	SR141716	Species
Hypothalamus	Appetite	↑	↓	Humans, Rats, Mice
Nucleus Accumbens	Motivation to Eat	↑	↓	Humans, Rats, Mice
Skeletal Muscle	Glucose Uptake	↓	↑	Rats, Mice
Adipose Tissue	Lipogenesis	↑	↓	Humans, Rats, Mice
Liver	Lipogenesis	↑	↓	Mice, Rats
Pancreas	Insulin Secretion	↑	↓	Mice
GI Tract	Gastric Emptying	↓	↑	Humans, Rats, Mice

Table 1.2: *In vivo* Effects of AEA and SR141716 in Obesity

Mechanism	AEA	SR141716	Species
Body Weight	↑	↓	Humans, Rats, Mice
Blood Glucose	↑	↓	Humans, Rats, Mice
Insulin Sensitivity	↓	↑	Humans, Rats, Mice
Leptin	↑	↓	Rats
Adiponectin	↓	↑	Humans, Mice
Plasma Free Fatty Acids	↑	↓	Humans, Rats, Mice
Triglycerides	↑	↓	Humans, Rats, Mice
Total Cholesterol	↑	↓	Humans, Rats, Mice

There is emerging data suggesting that endocannabinoids may mediate effects on energy balance and metabolism via TRPV1 (Suri and Szallasi 2008) which is discussed below.

1.5. TRANSIENT RECEPTOR POTENTIAL CHANNEL-VANILLOID

SUB-FAMILY MEMBER 1 (TRPV1)

1.5.1. Isolation and Roles of TRPV1

TRPV1 was first cloned in 1997 and described as a heat-activated receptor for capsaicin, the pungent component of hot peppers (Caterina, Schumacher et al. 1997). TRPV1 is a putative six-transmembrane domain protein with a pore region between segments five and six, and cytoplasmic N and C termini (Caterina, Schumacher et al. 1997). TRPV1 is expressed on primary sensory neurons and acts as a polymodal nociceptor with a dynamic threshold of activation that may be lowered under inflammatory conditions (Numazaki and Tominaga 2004). In addition to capsaicin, TRPV1 may be activated or sensitised by several agonists including: nerve growth factor (NGF) (Chuang, Prescott et al. 2001), endocannabinoids (Zygmunt, Petersson et al. 1999; McVey, Schmid et al. 2003), TNF- α (Nicol, Lopshire et al. 1997) and ATP (Tominaga, Wada et al. 2001). Activation of TRPV1 leads to increases in intracellular Ca^{2+} either by entry through the plasma membrane or through Ca^{2+} release of intracellular stores (Figure 1.7) (Olah, Szabo et al. 2001; Liu, Liu et al. 2003; Karai, Russell et al. 2004).

TRPV1 is believed to play a major role in the regulation of inflammatory mediated hyperalgesia (Krause, Chenard et al. 2005). Capsaicin-sensitive neurons in the dorsal root ganglia (DRG) are generally bipolar neurons with unmyelinated

axons (C-fibres) or thinly myelinated axons (A δ fibres) (Holzer 1988). These neurons are known to release pro-inflammatory neuropeptides such as substance P (SP) and calcitonin-gene-related peptide (CGRP), which are key contributors to neurogenic inflammation (Lundberg, Franco-Cereceda et al. 1985). TRPV1 is highly expressed in the DRG and the majority of TRPV1 positive neurons also express SP and CGRP (Planells-Cases, Garcia-Sanz et al. 2005). In addition, TRPV1 expression is increased during nerve-injury-induced thermal hyperalgesia and diabetic neuropathy making TRPV1 a therapeutic target for pain relief (Rashid, Inoue et al. 2003).

Figure 1.7

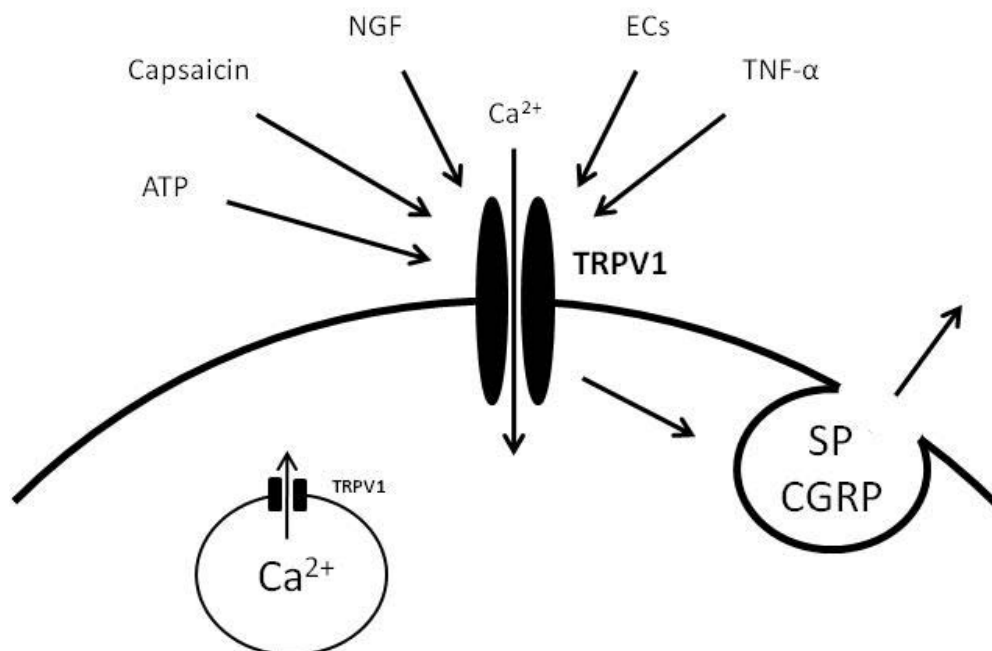


Figure 1.7: TRPV1 signalling increases intracellular calcium and release of pro-inflammatory neuropeptides. TRPV1 is activated by many different agonists including: ATP, capsaicin, NGF, TNF- α and some endocannabinoids. TRPV1 may be membrane bound or bound to the membranes of intracellular stores. Activation of membrane-bound TRPV1 allows for entry of calcium into the cytoplasm and release of pro-inflammatory neuropeptides CGRP and SP. Calcium may also be released from intracellular storage sites via TRPV1 activation. CGRP, calcitonin-gene-related peptide; ECs, endocannabinoids; NGF, nerve growth factor; SP, substance P; TRPV1, transient receptor potential channel-vanilloid sub-family member 1.

1.5.2. TRPV1, Obesity and Insulin Resistance

Capsaicin, a TRPV1 agonist, protects against obesity both *in vitro* in 3T3-L1 pre-adipocyte cell culture (Zhang, Yan Liu et al. 2007) and *in vivo* in human subjects (Westerterp-Plantenga, Smeets et al. 2005). In 3T3-L1 culture, capsaicin increased intracellular Ca^{2+} and prevented adipogenesis; an effect that was attenuated by gene silencing of TRPV1 with RNAi (Zhang, Yan Liu et al. 2007). Capsaicin may potentially act in a TRPV1-independent manner. Other studies also using 3T3-L1 culture have reported that adipogenesis is inhibited via direct activation of caspase-3 (Hsu and Yen 2007). In human subjects, studies utilising capsaicin as a dietary supplement reported increased satiety and decreased energy intake (Westerterp-Plantenga, Smeets et al. 2005). It is not known whether these observations are specifically due to TRPV1 mediated effects.

A critical component of the beneficial effects of TRPV1 agonists may be the desensitisation of TRPV1 in primary sensory neurons. Subcutaneous injection of the ultra potent capsaicin analogue and TRPV1 agonist resiniferatoxin (RTX) in obese and diabetic Zucker rats improves glucose tolerance (Gram, Hansen et al. 2005). Again, it is not known whether these effects were specifically due to TRPV1 desensitisation; however there is some speculative evidence suggesting that this is the case (Gram, Hansen et al. 2005). As mentioned above, TRPV1-positive neurons are a major source of CGRP. In rodent skeletal muscle (L6) cells and isolated soleus

muscle, CGRP induced insulin resistance (Kreutter, Orena et al. 1993). It has been proposed that CGRP is associated with age-related insulin resistance and obesity (Melnyk and Himms-Hagen 1995) presenting a possible mechanism of action for the effects of TRPV1 in obesity.

There is evidence suggesting that CGRP may act as a negative regulator for TRPV1 signalling in the pancreas. TRPV1 is expressed in pancreatic beta cells and is sensitised by insulin (Van Buren, Bhat et al. 2005). CGRP released from TRPV1-positive neurons in the pancreas inhibit insulin secretion from beta cells (Pettersson, Ahren et al. 1986). It has been proposed that decreased CGRP content in mice deficient in TRPV1 leads to improved glucose tolerance and this is supported by preliminary data from TRPV1^{-/-} mice (Razavi, Chan et al. 2006). These mice are protected against diet induced obesity (Motter and Ahern 2008), however, whether glucose tolerance is also protected is unknown and will be investigated.

1.5.3. TRPV1 and the Endocannabinoid System

There is a growing body of evidence suggesting that TRPV1 is a functional component of the endocannabinoid system. TRPV1 and the cannabinoid receptors are often expressed in the same tissues (Smart and Jerman 2000; McVey, Schmid et al. 2003; Golech, McCarron et al. 2004). AEA has similar affinity for TRPV1 as

capsaicin, however, whether 2-AG binds to TRPV1 remains speculative (Ross 2003; Di Marzo 2008).

TRPV1 has been associated with the endocannabinoid system in several physiological pathways, both *in vitro* and *in vivo*. Studies utilising goldfish retina amacrine cells have demonstrated that TRPV1 and FAAH may co-localise, possibly acting as a regulator of AEA content (Zimov and Yazulla 2007). In addition, in cultured DRG neurons from neonatal rats, chronic exposure to NGF increased receptor crosstalk between TRPV1 and CB₁, an effect that was measured using voltage-clamp electrophysiology and Fura-2 Ca²⁺ imaging (Evans, Scott et al. 2007). In rats, stress-induced hyperalgesia increased expression of TRPV1 and AEA and decreased expression of CB₁ in DRG neurons (Hong, Fan et al. 2009); in ferrets, the synthetic cannabinoid Arvanil (*N*-[3-methoxy-4-hydroxy-benzyl]-arachidonamide) and endocannabinoids AEA and *N*-arachidonoyl-dopamine inhibited emesis via CB₁ and TRPV1 (Sharkey, Cristino et al. 2007). Despite the emerging data, little is known on the combined effects of TRPV1 and the cannabinoid receptors with respect to obesity and glucose metabolism and will be investigated in this thesis.

1.6. AIMS AND HYPOTHESES

The overall aim of the studies described in this thesis was to investigate the role of endocannabinoids on skeletal muscle glucose uptake and pathways regulating glucose metabolism. Preliminary studies suggested that the effect of endocannabinoids on glucose uptake in skeletal muscle may be mediated via receptors other than CB₁ (Cavuto, McAinch et al. 2007). The studies described within this thesis tested the following hypotheses: (i) CB₁ inhibition will increase basal glucose uptake and regulate key genes and proteins involved in glucose metabolism in human primary skeletal muscle cell culture, (ii) myotubes derived from obese donors will be more responsive to CB₁ inhibition than those from lean, (iii) The three main endocannabinoid receptors (CB₁, CB₂ and TRPV1) and the enzyme responsible for the degradation of AEA (FAAH) are expressed in human and rodent skeletal muscle, (iv) chronic exposure to endocannabinoids will have a deleterious effect on basal and insulin-stimulated glucose uptake in rodent skeletal muscle (L6) cell culture, (v) CB₁, CB₂ and TRPV1 will have differential effects on skeletal muscle glucose uptake and (vi) mice that do not express TRPV1 will be resistant to diet induced obesity but will have poor glucose tolerance.

CHAPTER 2

MATERIALS AND METHODS

2.1. CELL CULTURE

2.1.1. Collection of Human Tissue

Human tissue was collected from lean and obese patients following written informed consent. External abdominal oblique muscle and subcutaneous adipose tissue was obtained from lean patients undergoing inguinal hernia surgery. Rectus abdominus muscle, omental (intra-abdominal) and subcutaneous adipose tissue was obtained from obese patients undergoing gastric by-pass surgery. External abdominal oblique and rectus abdominus muscle are directly comparable as both are mixed muscles and contain similar distribution of fibre types (~55-58% Type I, 15-23% Type IIA, 21-28% Type IIX) (Haggmark and Thorstensson 1979). Whole tissue samples were immediately dissected into two portions with one portion placed in α MEM (Invitrogen, Mount Waverly, Australia) for cell culture and the second portion frozen in liquid nitrogen and stored at -80°C . Collection of tissue was approved by the Human Ethics Research committees of the Royal Adelaide Hospital, Calvary Hospital and Burnside Hospital (South Australia, Australia).

2.1.2. Primary Human Skeletal Muscle Cell Culture

Human primary skeletal muscle myotubes were cultured according to the method described by Blau and Webster (1981) and modified by Gaster *et al.* (2001). Muscle samples, approximately 50-100 mg, were washed three times in α MEM. Samples were then transferred to a 10 cm Petri dish and enzymatically disassociated in 3 ml 0.05% Trypsin/EDTA (Sigma–Aldrich, St. Louis, MO, USA) while being minced with a size 22 scalpel. Minced tissue was then transferred to a sterile 50 ml conical flask. 15 ml 0.05% Trypsin/EDTA was added to the flask which was then gently shaken for 20 min. The supernatant (~12-15 ml) was collected and transferred to a 50 ml conical tube containing 5 ml fetal bovine serum (FBS, Sigma–Aldrich) and placed on ice. This process was repeated in triplicate such that the 50 ml conical tube contained approximately 45 ml supernatant. The supernatant was then filtered through a 100 μ m cell strainer. Cells were collected using centrifugation (1600 rpm, 7 min) and re-suspended in 5 ml primary growth media containing α MEM with 10% FBS, 0.5% penicillin–streptomycin (Sigma) and 0.5% amphotericin B (Sigma). Cells were cultured onto an uncoated 25 cm² flask and incubated for 20 min at 37°C in 5% CO₂. Following incubation, the cells were transferred to an extra cellular matrix (ECM) coated 25 cm² flask and grown to confluence at 37°C in 5% CO₂ with primary growth media being changed every second day.

Cells were passaged at approximately 70% confluence, using 1.5 ml 0.05% Trypsin/EDTA to disassociate the cells for 3 min at 37°C in 5% CO₂. Cells were then re-suspended onto eight ECM coated 75 cm² flasks at a 1:10 dilution. At confluence, cells were disassociated with 3 ml 0.05% Trypsin/EDTA and collected in two 50 ml conical tubes and centrifuged at 1600 rpm for 5 min, then re-suspended in 2 ml growth media and 2 ml FBS per tube. 4 ml sterile filtered freezing media containing growth media with 10% DMSO (Sigma) was then added to each tube and immediately placed in -20°C in 1.5 ml aliquots for 30 min. Cells were then stored overnight at -80°C and then in liquid nitrogen.

In preparation for experimental use, cells were thawed and added to 15 ml conical tubes containing 9 ml growth media and centrifuged at 1600 rpm for 5 min. Cells were then plated onto an ECM coated 75 cm² flask and grown to confluence. At confluence, cells were passaged in a 1:10 dilution onto either ECM coated glass slides for immunostaining, 6-well plates for mRNA analysis, 12-well plates for glucose uptake studies or 10 cm Petri dishes for protein analysis using western blot. At confluence, cells underwent 3 days of differentiation using differentiation media containing α MEM with 2% horse serum (HS, Sigma), 0.5% penicillin–streptomycin and 0.5% amphotericin B. Experiments were conducted on the fourth day.

2.1.3. Collection of Rodent Skeletal Muscle

Following approval from the Animal Ethics committees of the University of Adelaide and Institute of Medical and Veterinary Science and the Royal Adelaide Hospital, rodent skeletal muscle and adipose tissue were obtained from male Wistar rats aged 20 weeks being fed a standard laboratory diet or high-fat diet (Janovska, Hatzinikolas et al. 2008). Immediately after sacrifice, skeletal muscle was obtained from the hind limb and adipose tissue from the mesenteric fat depot, immediately frozen in liquid nitrogen, and stored at -80°C.

2.1.4. Rodent Myogenic Cell Culture (L6)

L6 cells were kindly donated by Dr. David Cameron-Smith (Deakin University, Melbourne, Australia). In preparation for experimental use, L6 cells were thawed and added to 15 ml conical tubes containing 9 ml growth media containing α MEM with 10% FBS and 1% penicillin–streptomycin and centrifuged at 1600 rpm for 5 min. Cells were then plated onto a 75 cm² flask and grown to confluence. At confluence, cells were passaged in a 1:10 dilution onto either 6-well plates for mRNA analysis, 12-well plates for glucose uptake studies or 10 cm Petri dishes for protein analysis using western blot. At confluence, cells underwent 3 days of differentiation using differentiation media containing α MEM with 2% HS and 1% penicillin–streptomycin. Experiments were conducted on the fourth day.

2.2. mRNA ANALYSIS

2.2.1. RNA Extraction

Human primary skeletal muscle myotubes or L6 cells were cultured onto 6-well plates and prepared for experimental use as described in sections 2.1.2. and 2.1.4., respectively. Following 24 h exposure to the experimental conditions, human primary skeletal muscle myotubes and L6 myocytes were washed twice with 1x PBS, then lysed with 800 μ l TRIzol reagent (Invitrogen) and transferred to 1.5 ml eppendorf tubes (Eppendorf, North Ryde, Australia). For whole tissue samples, portions of 10-20 mg of skeletal muscle or 60-80 mg of adipose tissue were placed in 800 μ l TRIzol reagent in 1.5 ml eppendorf tubes containing one 5 mm stainless steel bead (Qiagen, Melbourne, Australia), then homogenized using the Qiagen TissueLyser (Qiagen) for 1.5 min at 30 Hz.

RNA was extracted according to the method described by Chomczynski and Sacchi (1987). 200 μ l chloroform (Sigma) was added to the homogenates, inverted to mix, placed on ice for 5 min and then centrifuged at 13000 rpm at 4°C for 15 min. The upper clear phase was then moved to a fresh 1.5 ml eppendorf tube containing an equal volume (approximately 600 μ l) 2-propanol (Sigma) and 10 μ l of 5 M NaCl gently mixed and then placed on ice for 2 h. Samples were centrifuged at 13000 rpm at 4°C for 20 min forming a small white RNA pellet. Supernatant was removed and 400 μ l 75% ethanol (containing DEPC treated H₂O) added to the RNA pellet. Samples were centrifuged at 9000 rpm at 4°C for 8 min. Ethanol was aspirated off

and the RNA pellet was left to briefly air dry at room temperature for 5 min. The RNA pellet was dissolved in 5 μ l heated (60°C) nuclease-free H₂O (Ambion, Austin, TX, USA). To ensure purity for mRNA sequences containing a single exon, the RNA was treated with the TURBO DNA-free™ kit (Ambion). Total RNA concentration was quantified using nanodrop spectrometry at 260 nm.

2.2.2. Reverse Transcription of RNA

Two unique protocols were used for reverse transcription. The first used the AMV Reverse Transcription Kit (Promega, Madison, WI, USA) and the second used the QuantiTect Reverse Transcription Kit (Qiagen).

The AMV Reverse Transcription Kit utilized 0.5 μ g RNA which was added to nuclease-free H₂O to a volume of 4.9 μ l. Samples were then heated to 65°C for 10 min and cooled to 4°C for 5 min using the Eppendorf Mastercycler® Gradient (Eppendorf). Reverse transcription samples were prepared to a final volume of 10 μ l containing: sample RNA (4.9 μ l), 25 mM MgCl₂ (2 μ l), reverse transcription 10x buffer (1 μ l), 10 mM dNTP mix (1 μ l), recombinant RNasin® ribonuclease inhibitor (0.25 μ l), AMV reverse transcriptase (0.35 μ l) and oligo(dT)₁₅ primer (0.5 μ l). Reverse transcription was completed using the following profile: 42°C for 60 min, 99°C for 5 min and 4°C for 5 min. Generated cDNA was then diluted 1:20 stored at -20°C.

The QuantiTect Reverse Transcription Kit also utilized 0.5 µg RNA which was added to nuclease-free H₂O to a volume of 6 µl. 1 µl genomic DNA wipeout buffer was added to the samples which were heated to 42°C for 2 min using the Eppendorf Mastercycler® Gradient and then immediately placed on ice. Reverse transcription samples were prepared to a final volume of 10 µl containing: sample RNA containing genomic DNA wipeout buffer (7 µl), Quantiscript RT 5x buffer (2 µl), Quantiscript reverse transcriptase (0.5 µl) and RT primer mix (0.5 µl). Reverse transcription was completed using the following profile: 42°C for 30 min, 95°C for 3 min and 4°C for 5 min. Generated cDNA was then diluted 1:3 or 1:6 and stored at -20°C.

2.2.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed using the Eppendorf Mastercycler® Gradient with HotStarTaq Plus Master Mix (Qiagen). The following cycle profile was used: 95°C for 5 min, then 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s and finishing with 72°C for 10 min. Primers were designed using the Primer Express software package Version 3.0. Gene sequences were obtained from GenBank. BLAST searches for all primers confirmed homologous binding to desired mRNA of human or rodent samples for all chosen genes. Primer sequences are described in section 2.2.5.

RT-PCR products were analysed using gel electrophoresis on a 2.5% agarose gel (Sigma) containing 0.5x TBE buffer (Brody and Kern 2004) and stained with ethidium bromide (Sigma). Gels were placed on top of an ultraviolet transilluminator and visualized with the Kodak EDAS 290 digital camera.

2.2.4. 'Real Time' RT-PCR

'Real Time' RT-PCR was performed using the RotorgeneTM 3000 real time thermal cycler (Corbett Research, Sydney, Australia) with either SYBR green master mix (Applied Biosystems) or the QuantiTect SYBR green PCR kit (Qiagen). The RotorgeneTM 3000 was programmed with the following cycle profile: 95°C for 15 min, then 50 cycles of 95°C for 30 s and 60°C for 60 s followed by 72°C for 4 min, then a melt sequence starting at 60°C and incrementally increasing 1°C every 30 s until 95°C. Primers sequences are described in section 2.2.5.

Expression of mRNA was analysed using RotorgeneTM 6 software (Corbett Research). A candidate housekeeping gene was used to compensate for variations of input RNA and efficiency of reverse transcription with fluorescence being analysed for critical threshold (C_T) values. The relative quantification was calculated from a standard curve and expressed as a ratio of target to housekeeping gene.

2.2.5. Primer Sequences

Primers were designed using the Primer Express software package Version 3.0. Gene sequences were obtained from GenBank. BLAST searches for all primers confirmed homologous binding to desired mRNA of human or rodent samples for all chosen genes. Primers designed 'in-house' and genotyping primers for TRPV1^{-/-} mice were purchased from Geneworks. Primers not designed 'in-house' were purchased from Qiagen. PCR reactions were designed uniquely for Geneworks primers (10 µl SYBR green, 4 µl nuclease-free H₂O, 2 µl forward primer, 2 µl reverse primer and 2 µl sample cDNA) or Qiagen primers (10 µl SYBR green, 6 µl nuclease-free H₂O, 2 µl QuantiTect primer assay and 2 µl sample cDNA). Human primer sequences are described in Table 2.1, rodent primer sequences are described in Table 2.2 and genotyping primers for TRPV1^{-/-} mice are described in Table 2.3.

Table 2.1: Human Primer Sequences

Gene	Primer	Product Size (bp)	Accession Number
AMPKα1	F- GACT GCT ACT CCA CAG AGA TCG	72	NM_006251
	R- TCA GCA TCT GAA TCA CTC CTTT		
AMPKα2	F- AAC TGC AGA GAG CCA TTC ACT TT	75	NM_006252
	R- GGT GAA ACT GAA GAC AAT GTG CTT		
CB₁	F- CGC TTT CCG GAG CAT GTT	66	NM_016083
	R- TCC CCC ATG CTG TTA TCC A		
CB₂	F- TAT GGG CAT GTT CTC TGG AA	141	NM_001841
	R- GAG GAG CAC AGC CAA CAC TA		
FAAH	F- AGG CCC AGA TGG AAC ATT ACA G	112	NM_001441
	R- GGC AGC CCC ACA CTC TTC T		
PDK4	F- ATG GAT AAT TCC CGG AAT GCT	73	NM_002612
	R- ACT TGG CAT ACA GAC GAG AAA TTG		
PGC-1α	F- CAA GCC AAA CCA ACA ACT TTA TCT CT	105	NM_013261
	R- CAC ACT TAA GGT GCG TTC AAT AGT C		
TRPV1	F- TTC ACC ATG GCT GCC TAC TAC A	111	NM_018727
	R- TCC TCC TAA CAC AGA CAG GAT CTC T		
Cyclophilin A	F- CAT CTG CAC TGC CAA GAC TGA	72	NM_021130
	R- TTC ATG CCT TCT TTC ACT TTG C		

Table 2.1: Human primer sequences used for 'Real Time' and end-point RT-PCR. AMPK α 1 and α 2, AMP-activated protein kinase alpha 1 and 2; CB₁ and CB₂, cannabinoid receptor type 1 and type 2; FAAH, fatty acid amide hydrolase; PDK4, Pyruvate dehydrogenase kinase 4; PGC-1 α , Peroxisome proliferator-activated receptor γ co-activator-1 α ; TRPV1, transient receptor potential channel-vanilloid sub-family member 1. Numbers (NM_) of the genes are NCBI accession numbers obtained from the NIH Database.

Table 2.2: Rodent Primer Sequences

Gene	Primer	Product Size (bp)	Accession Number
CB₁	F- CAA GCA CGC CAA CAA CAC A	68	NM_012784
	R- TCT TAA CGG TGC TCT TGA TGC A		
CB₂	F- TTC CCC CTG ATC CCC AAC GAC TA	369	NM_020543
	R- CTC TCC ACT CCG CAG GGC ATA AAT		
FAAH	F- CTG CCC AGC TGG TTT AAA AGA	87	NM_024132
	R- AGG ACG CAT ACT GTT GAG AAA GG		
TRPV1	F- GGA CGT TGC CCG GAA GAC AGA CA	228	NM_031982
	R- GAC AGG GGC AGC TCA CCA AAG TAG		

Table 2.2: Rodent primer sequences used for end-point RT-PCR. CB₁, cannabinoid receptor type 1; CB₂, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; TRPV1, transient receptor potential channel-vanilloid sub-family member 1. Numbers (NM_) of the genes are NCBI accession numbers obtained from the NIH Database.

Table 2.3: Genotyping Primer Sequences for TRPV1^{-/-} Mice

TRPV1	Primer	Product Size (bp)	JAX Code
Mutant	CAC GAG ACT AGT GAG ACG TG	600	oIMR0297
Wild-Type	CCT GCT CAA CAT GCT CAT TG	984	oIMR1561
Common	TCC TCA TGC ACT TCA GGA AA	-	oIMR1562

Table 2.3: Mouse primer sequences used for genotyping of TRPV1^{-/-} mice. All 3 primers were used in the same PCR reaction to determine genotype. TRPV1, transient receptor potential channel-vanilloid sub-family member 1. JAX Code numbers for the primers are sku numbers obtained from Jackson Laboratories (Bar Harbour, ME, USA).

2.3. GLUCOSE UPTAKE

2.3.1. Human Primary Skeletal Muscle 2-[³H]deoxy-D-glucose Uptake

Human primary skeletal muscle myotubes were cultured in ECM coated 12-well plates and prepared for experimental use as described in section 2.1.2. On the day of the experiment, myotubes were washed 3 times with uptake buffer at pH 7.4 containing: 150 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM NaH₂PO₄, 1.2 mM CaCl₂, 10 mM HEPES and 0.1% BSA (Ciaraldi, Abrams et al. 1995). Myotubes were then exposed to the experimental conditions (i.e. a compound of interest), diluted in 1 ml uptake buffer and incubated for 30 min at 37°C in 5% CO₂. Exposure to the experimental conditions was conducted in triplicate for each experiment.

The glucose uptake reaction was initiated with the addition of 2-[³H]deoxy-D-glucose (DOG; 1 µCi/ml) (Perkin Elmer, Boston, MA) and 2-DOG (10 µmol/ml) (Sigma) to the wells. After 15 min of incubation at 37°C in 5% CO₂, the uptake buffer was rapidly aspirated and the myotubes were rinsed 4 times in ice-cold 1x PBS. Myotubes were then lysed with 0.1 M NaOH for 30 min at room temperature and lysates were divided into two aliquots, either for the determination of 2-[³H]DOG content using Ultima Gold™ scintillation fluid (Perkin Elmer), or for the quantification of protein content with the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA). After subtraction of non-specific radioactivity, values for glucose uptake in experimental samples and controls were corrected for quantity of proteins and recalculated per minute [pmol/(mg protein.min)].

2.3.2. L6 Insulin-Stimulated 2-[³H]deoxy-D-glucose Uptake

L6 cells were cultured onto 12-well plates and prepared for experimental use as described in section 2.1.4. To determine the acute effects on glucose uptake, L6 cells were washed twice with 1x PBS then serum starved in 1 ml α -MEM containing 0.1 % fatty acid free (FAF)-BSA (Sigma) for 6 h at 37°C and 5% CO₂. The L6 cells were then washed twice with 1x PBS and subjected to the experimental conditions (i.e. a compound of interest), diluted in 1 ml α MEM containing 0.1% FAF-BSA and incubated for 30 min at 37°C and 5% CO₂. To determine the chronic effects on glucose uptake, L6 cells were washed twice with 1x PBS and α MEM containing 0.1% FAF-BSA, then subjected to the experimental conditions (i.e. a compound of interest), diluted in 1 ml α MEM containing 0.1% FAF-BSA and incubated for 24 h at 37°C and 5% CO₂. Exposure to the experimental conditions was conducted in triplicate for each experiment.

Following exposure to the experimental conditions, L6 cells were washed once with 1x PBS and incubated in 1 ml α MEM containing 0.1% FAF-BSA with or without 100 nM insulin, 10 μ M DMSO or 0.1 μ M cytochalasin B for 30 min at 37°C in 5% CO₂. L6 cells were then washed 3 times with uptake buffer at pH 7.4 containing: 150 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 2.5 mM NaH₂PO₄, 1.2 mM CaCl₂, 10 mM HEPES and 0.1 % FAF-BSA. The glucose uptake reaction was initiated with the addition of 2-[³H]DOG (1 μ Ci/ml) and 2-DOG (10 μ mol/ml) diluted in 1 ml uptake buffer and incubated at 37°C in 5% CO₂ for 15 min. The uptake buffer was then rapidly

aspirated and the L6 cells rinsed 4 times in ice-cold 1x PBS. L6 cells were lysed with 0.1 M NaOH and left at room temperature for 30 min before being separated into two aliquots. One aliquot was used to determine the specific activity of 2-[³H]DOG using Ultima Gold™ scintillation fluid and the other was used for the quantification of protein content with the (BCA) Protein Assay Reagent Kit. After subtraction of non-specific radioactivity, values for glucose uptake in the samples were corrected for quantity of proteins and recalculated per minute [pmol/(mg protein.min)]. Glucose uptake experiments were performed in duplicate to ensure reproducibility of the results.

2.4. WESTERN BLOT

2.4.1. Collection of Protein

Human primary skeletal muscle myotubes or L6 cells were cultured onto 10 cm² Petri dishes and prepared for experimental use as described in sections 2.1.2. and 2.1.4., respectively. Following exposure to the experimental conditions, the cells were placed on ice, washed twice with ice-cold 1x PBS and then lysed in 100 µl lysis buffer, pH 7.4 at 4°C containing: 50 mM Trizma Base (pH 7.4 at 4°), 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM NaPPi, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1mM DTT, 1 mM Benzamidine, 10 µg/ml SBTI, 5 µM leupeptin, 3 µM aprotinin, 5 µM pepstatin and 1 mM PMSF (Hardie, Salt et al. 2000; Janovska, Hatzinikolas et al. 2008) and thoroughly scrapped with a cell scraper. If multiple dishes were used for a single experimental condition, the lysates were combined and centrifuged at 13000 rpm at 4°C for 4 min, then immediately frozen using dry ice and stored at -80°C. Total protein content was quantified with the BCA Protein Assay Reagent Kit.

2.4.2. Western Blot Protocol

To measure levels of phosphorylation of protein, 20 µg of protein were subjected to SDS-PAGE using either pre-cast 1.5 mm 3-8% tris-acetate or 10% bis-tris gels (Invitrogen), then blotted onto polyvinylidene difluoride (PVDF) membranes (Invitrogen). After transfer, the membranes were blocked with either 5% ECL™ Blocking Agent (GE Healthcare, Uppsala, Sweden) or 5% skim milk in tris-buffered

saline/0.1% Tween 20 (TBS/T) for 1 h at room temperature with slow agitation followed by washing 3x with TBS/T.

Membranes were incubated overnight at 4°C with the primary antibody in TBS/T with or without 5% BSA. Primary antibodies were all purchased from Millipore, Sydney Australia. Bound primary antibodies were detected with either Goat Anti-Rabbit IgG, Alkaline phosphatase-conjugated secondary antibody (Millipore) in TBS/T with 5% BSA at room temperature for 1.5 h or Sheep Anti-Rabbit IgG, Alkaline phosphatase-conjugated secondary antibody (Millipore) in TBS/T with 5% skim milk at room temperature for 1 h.

Membranes were developed with a chemifluorescence substrate (ECF Substrate, GE Healthcare), scanned by the Typhoon Imager (GE Healthcare) and quantified using the ImageQuant software (Molecular Dynamics). Results were normalised to the loading control to account for variations in protein load. Differences in the level of chemifluorescent development among membranes were normalised to the band intensities of positive controls.

2.5. TRPV1 KNOCKOUT STUDIES

2.5.1. Animals

All experimental procedures involving the use of animals were approved by the University of Adelaide Animal Ethics Committee and the Institute of Medical and Veterinary Science Animal Ethics Committee. Mice were purchased from Jackson Laboratories (Bar Harbour, ME, USA). 16 male C57BL/6J wild type and 16 male B6.129S4-TRPV1^{tm1Juj}/J knockout mice at 6 weeks of age and at an average weight of 20 g were housed in individual cages and maintained under a 12 h light-dark cycle at a constant room temperature of $24 \pm 1^\circ\text{C}$. Following 2 weeks of acclimatisation, the mice were subjected to intraperitoneal glucose tolerance testing then divided into four groups (n=8/group) and fed either a standard laboratory diet (4.6% fat, 19.6% protein, 4.8 % crude fibre, 14.3 MJ/Kg digestible energy) or a high fat diet (35.5% fat, 19.5% protein, 9.3% crude fibre, 21.4 MJ/Kg digestible energy) *ad libitum* for 18 weeks. Water was also available *ad libitum*. At age 26 weeks, mice were subjected to a second round of intraperitoneal glucose tolerance testing, followed by 1 week recovery then were sacrificed via cardiac puncture. Mice were fasted overnight and provided with free access to water prior to any experimental procedure. Body mass was monitored twice per week for the duration of the studies.

2.5.2. Intraperitoneal glucose tolerance tests

Prior to intraperitoneal glucose tolerance testing, the mice were fasted overnight but still allowed access to water. On the day of the experiment, fasting body weight

was measured. For the measurement of blood glucose, mice were briefly restrained to allow access to the tail vein. Tail veins were pricked with a 25G needle and fresh whole blood (at least 0.6 μ L) was extracted directly onto blood glucose test strips (Abbott, Chicago, IL). Baseline blood glucose was measured and followed by intraperitoneal injection of 20% glucose at 2 g glucose per Kg of fasting body weight. Blood glucose was measured at 15, 30, 60 and 120 min after injection.

2.6. STATISTICAL ANALYSIS

All data are presented as raw values or means \pm SEM where appropriate, and were confirmed to fit the normal Gaussian distribution of variance. Statistical analyses were performed by 2-way ANOVA with Bonferroni post-hoc tests and unpaired *t*-tests with Welch's correction using GraphPad Prism 5.00 (GraphPad Software Inc., San Diego, CA, USA) and by 2-, 3- or 5- way ANOVA using pair wise comparisons with Tukey post-hoc tests using SPSS 17 software from SPSS Inc (Chigaco, IL, USA) unless stated otherwise. An ANOVA is a statistical method for making simultaneous comparisons between two or more means. For example, if an experiment tests 2 unique factors/variables (such as varying concentrations of 2 drugs separately and in combination) and the resultant data meets the standard ANOVA assumptions of independence, normality and equality, then a 2-way ANOVA would be the appropriate measure. If an experiment tests 3 unique factors/variables and the resultant data satisfy the ANOVA assumptions, then a 3-way ANOVA would be performed. Significance was accepted at $P < 0.05$ for all analyses.

CHAPTER 3

SR141716, A SELECTIVE CB₁ INVERSE AGONIST, INCREASES BASAL GLUCOSE UPTAKE IN HUMAN SKELETAL MUSCLE MYOTUBES DERIVED FROM OBESE PATIENTS

3.1. SUMMARY

In vivo, endocannabinoids regulate peripheral glucose metabolism. We measured the effect of the non-specific cannabinoid receptor agonist anandamide (AEA; 0 μ M, 0.2 μ M and 5 μ M) and specific cannabinoid receptor type 1 (CB₁) inverse agonist SR141716 (0 nM, 20 nM, 100 nM and 500 nM) alone and in all combinations on basal glucose uptake, mRNA content and protein phosphorylation of key genes regulating energy metabolism in primary cultures of skeletal muscle myotubes derived from lean and obese humans. In lean-derived myotubes AEA and SR141716 were without effect. In obese-derived myotubes AEA (0.2 μ M) decreased ($P < 0.05$), whereas SR141716 (100 nM) increased ($P < 0.05$), glucose uptake, an effect reversed by AEA. AMPK α 2 mRNA was lower in obese than lean-derived myotubes ($P < 0.05$) and increased with AEA (5 μ M; $P < 0.05$), an effect reversed by SR141716 (100 nM). AMPK α 1 mRNA increased with SR141716 (100 nM; $P < 0.05$), an effect reversed by

AEA (0.2 μ M and 5 μ M). PGC-1 α mRNA decreased with AEA (5 μ M) and SR141716 (100 nM) together ($P < 0.05$). Neither AMPK α nor MEK1/2 protein phosphorylation were affected under any condition. These data are consistent with an 'overactive endocannabinoid system' in skeletal muscle. CB₁ blockade increased basal glucose uptake; this was not mediated by AMPK α or MEK1/2 phosphorylation.

3.2. INTRODUCTION

Obesity is associated with impaired oxidative metabolism in skeletal muscle (Zurlo, Larson et al. 1990; Kelley, Goodpaster et al. 1999). There are a number of possible mechanisms for this effect including increased stores of intramyocellular triglycerides, insulin resistance or changes in growth hormone, sex steroids, muscle fibre type and the recently discovered endocannabinoid system (Jeukendrup 2002; Kelley 2005).

The endocannabinoid system is an endogenous cell signalling system that has a role in both central and peripheral energy metabolism (Pagotto, Marsicano et al. 2006). The most abundant endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are synthesised on demand from arachidonic acid and after receptor binding, are rapidly metabolised by fatty acid amide hydrolase (FAAH) and monoglycerol lipase (MAGL), respectively (Pagotto, Marsicano et al. 2006). The cannabinoid receptors, type 1 (CB₁) and type 2 (CB₂), are 7 transmembrane domain G-protein coupled receptors (Matsuda, Lolait et al. 1990; Munro, Thomas et al. 1993). CB₁, expressed in the hypothalamus and nucleus accumbens, regulates food intake and motivation to eat (Di Marzo, Goparaju et al. 2001). Endocannabinoids also regulate energy balance via peripheral effects on CB₁ in adipose tissue, liver, gastrointestinal tract (Pagotto, Marsicano et al. 2006) and skeletal muscle (Liu, Connoley et al. 2005; Cavauto, McAinch et al. 2007). CB₂, initially thought to only be expressed in cells of the immune system (Galiegue, Mary

et al. 1995), has recently been shown in humans and rodents to be expressed in adipose tissue, pancreas and skeletal muscle (Roche, Hoareau et al. 2006; Bermudez-Silva, Sanchez-Vera et al. 2007; Cavuoto, McAinch et al. 2007).

Obesity is characterised by 'overactivity' of the endocannabinoid system (Engeli, Bohnke et al. 2005), with an increase in plasma and tissue endocannabinoid levels due to increased production, decreased catabolism or a combination of both (Engeli, Bohnke et al. 2005; Sipe, Waalen et al. 2005). Specific inhibitors of CB₁ have been shown to reduce obesity in humans and animals via both central and peripheral mechanisms of action (Bensaid, Gary-Bobo et al. 2003; Van Gaal, Rissanen et al. 2005). Skeletal muscle is a major site of glucose and fatty acid metabolism (Zurlo, Larson et al. 1990). Endocannabinoids have been shown to increase basal glucose uptake in rodent skeletal muscle (Liu, Connoley et al. 2005).

Basal glucose uptake in skeletal muscle is ultimately dependant on translocation of insulin-responsive GLUT4 to the plasma membrane (Klip and Paquet 1990). Among the many factors regulating GLUT4 translocation, AMP-activated protein kinase (AMPK) is a key component acting as a 'master switch' between energy storage and utilisation (Kemp, Mitchelhill et al. 1999). Mitogen-activated protein kinases (MAPKs) such as MEK may regulate glucose uptake in addition to cell growth, differentiation and death (Schaeffer and Weber 1999). Peroxisome proliferator-activated receptor γ co-activator-1 α (PGC-1 α), a transcription factor,

induces gene expression of GLUT4 and has regulatory effects on mitochondrial biogenesis and triglyceride metabolism in skeletal muscle (Puigserver and Spiegelman 2003). In relation to glucose oxidation, pyruvate dehydrogenase kinase 4 (PDK4) inhibits the pyruvate dehydrogenase complex (PDC), a critical component which converts pyruvate to acetyl-CoA in preparation for entry into the Citric Acid Cycle for oxidation (Sugden and Holness 1994).

We have previously demonstrated that CB₁ is expressed in primary cultures of human skeletal muscle myotubes and that the CB₁-specific antagonist AM251 increases mRNA expression of AMPK α 1 (Cavuoto, McAinch et al. 2007). Antagonists of CB₁ have been shown to, at least partially, regulate the MAPK pathway in smooth muscle (Begg, Baydoun et al. 2001).

In this study the effects of the endocannabinoid AEA and the CB₁-specific inverse agonist SR141716 were determined on basal glucose uptake, mRNA of AMPK α 1, AMPK α 2, PDK4 and PGC-1 α and protein phosphorylation of AMPK α and MEK1/2 in primary cultures of human skeletal muscle myotubes derived from lean and obese patients. Human skeletal muscle myotubes retain the metabolic physiology of the donor thus allowing direct comparisons between lean and obese subject groups (Henry, Abrams et al. 1995; Gaster, Petersen et al. 2002; Ukropcova, McNeil et al. 2005). The cell culture model allows for the abnormalities in muscle that exist *in vivo* to be studied *in vitro* without the surrounding nutritional and hormonal

influences that would otherwise exert their effects on muscle function (Cavuoto, McAinch et al. 2007).

3.3. MATERIALS AND METHODS

3.3.1. Human Primary Skeletal Muscle Cell Culture

Primary cultures of human skeletal muscle myotubes were prepared for experimental use as described in Chapter 2.1.2. Subject characteristics are displayed in Table 3.1.

Table 3.1 – Subject Characteristics

	Lean	Obese
Age	42.3 ± 5.0	42.0 ± 8.0
Gender [M, F]	2, 2	2, 2
Weight [kg]	66.9 ± 4.1	126.6 ± 16.0*
BMI [kg/m²]	22.3 ± 0.7	40.4 ± 2.5*
Fasting Blood Glucose [mmol/l]	5.3 ± 0.2	5.2 ± 0.2
Fasting Blood Insulin [μU/l]	3.4 ± 0.3	12.0 ± 1.6*

Table 3.1: Subject characteristics of donors of rectus abdominus muscle biopsy for the culture of human skeletal muscle myotubes used in glucose uptake and western blot. The subject characteristics for myotubes used in 'Real Time' RT-PCR are comparable but not shown. (*) P<0.05 lean vs. obese.

3.3.2. Experimental Protocols

Stock solutions of SR141716 (10 mM) and AEA (10 mM) were prepared in DMSO and ethanol, respectively. For all experiments, myotubes were exposed to SR141716 (0 nM, 20 nM, 100 nM and 500 nM) and AEA (0 μ M, 0.2 μ M and 5 μ M) separately and in combination, resulting in a total of 12 conditions. The concentration of AEA used in the current study was supraphysiological to known plasma concentrations of AEA (2.86 nM) but consistent with other studies (Gasperi, Fezza et al. 2007; Eckardt, Sell et al. 2009). Levels of AEA in human skeletal muscle are currently unknown. Known concentrations of endocannabinoids in a range of tissues are described in Chapter 1.4.5. The concentration of the vehicles for SR141716 (DMSO) and AEA (ethanol) were consistent across all conditions and did not exceed 0.2% of total volume. To measure basal glucose uptake and protein phosphorylation, myotubes were exposed to the SR141716 and AEA conditions for 45 min. For the analysis of 'Real Time' RT-PCR, myotubes were subjected to the SR141716 and AEA conditions for 24 h.

3.3.3. Basal Glucose Uptake

Myotubes (lean, n=4 and obese, n=4) were cultured onto ECM-coated 12-well plates and glucose uptake experiments were conducted as described in Chapter 2.3.1.

3.3.4. RNA Extraction and 'Real Time' RT-PCR

Myotubes (lean, n=3 and obese, n=3) were cultured onto ECM-coated 6-well plates. RNA was extracted and 'Real Time' RT-PCR was performed as described in Chapter 2.2. Primer sequences used for this study are described in Chapter 2.2.5.

3.3.5. Western Blot

Myotubes were cultured on ECM-coated 10 cm² Petri dishes (lean, n=4 and obese, n=4) and western blot experiments were completed as described in Chapter 2.4.

The following antibodies were used: anti-phospho-AMPK α -(Thr¹⁷²) and anti-phospho-MEK1/2-(Ser^{217/221}) (1:1000) in tris-buffered saline containing 0.1% Tween 20 (TBS/T), and anti-AMPK α (1:1000), anti-MEK1/2, (1:1000), anti- β -tubulin (1:2000) and anti- β -actin (1:10 000) in TBS/T with 5% BSA. The secondary antibody was either goat anti-rabbit IgG alkaline phosphatase-linked antibody (1:2000, Millipore, Sydney, Australia) in TBS/T with 5% BSA or sheep anti-rabbit IgG alkaline phosphatase-conjugated (1:5000, Millipore) in TBS/T with 5% skim milk. Results were normalised to either β -actin or β -tubulin to account for variations in protein load.

3.3.6. Statistical Analyses

All data are presented as means \pm standard error of the mean (SEM), and were confirmed to fit the normal Gaussian distribution of variance. Experiments were conducted at passage 6; however, cells from the same donor were grown separately from passage 4. Hence, each primary cell line was subject to 2 independent growth phases prior to exposure to the experimental conditions. Each experiment was run twice, separately, for each cell line. Therefore, the two sets of data from the same cell line would have been sufficiently independent for Gaussian distribution. The data was then combined to form an n of 8. Analysis of distribution did not reveal any data points that would skew the results beyond normality. Differences among conditions of SR141716 and AEA were calculated using GraphPad Prism 5.00 (GraphPad Software Inc., San Diego, CA) using a two-way ANOVA with Bonferroni post-hoc tests to compare differences between conditions. Basal values of mRNA expression were analysed using unpaired *t*-tests with Welch's correction. Significance was accepted at $P < 0.05$.

3.4. RESULTS

3.4.1. Basal Glucose Uptake

In myotubes derived from lean subjects, there was no change in basal glucose uptake in response to either AEA or SR141716, alone or in combination (Fig. 3.1A). In myotubes derived from obese subjects, there was a decrease in basal glucose uptake in response to 0.2 μ M, but not 5 μ M AEA ($P < 0.05$; Fig 3.1B). In addition, there was an increase in basal glucose uptake in response to 100 nM SR141716 ($P < 0.05$; Fig. 3.1B). In combination with SR141716, 0.2 μ M but not 5 μ M AEA decreased basal glucose uptake irrespective of the concentration of SR141716 ($P < 0.05$; Fig 3.1B).

Figure 3.1

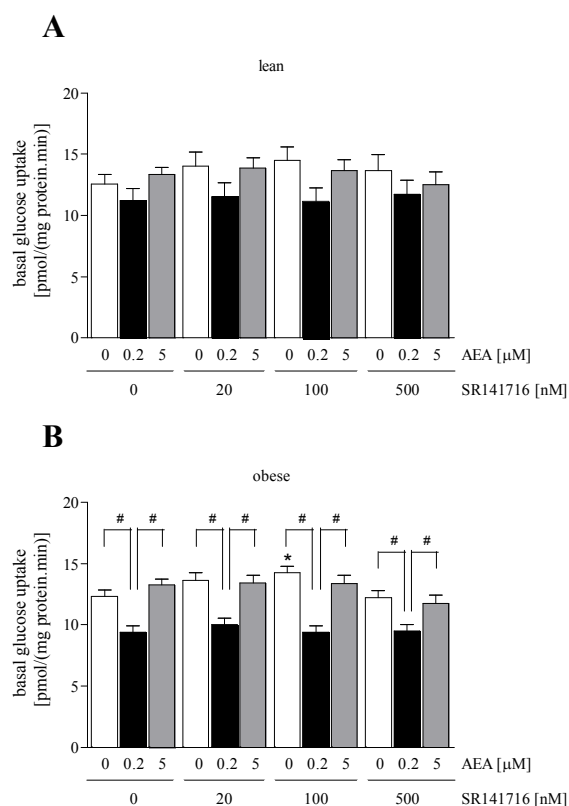


Figure 3.1: Glucose uptake in myotubes derived from lean (A) and obese (B) subjects ($n=4/\text{group}$). Exposure to the SR141716 and AEA conditions was performed in triplicate for a total of 45 min. Glucose uptake experiments were performed twice for each cell line. Open bars (\square) represent the SR141716 conditions, closed bars (\blacksquare) represent SR141716 in combination with 0.2 μ M AEA and grey bars (\blacksquare) represent SR141716 in combination with 5 μ M AEA. Data are shown as means \pm SEM and are expressed as [pmol/(mg protein.min)]. (*) $P<0.05$, condition vs. control (0 μ M SR141716); (#) $P<0.05$, 0.2 μ M AEA vs. 0 μ M AEA.

3.4.2. mRNA Expression

The basal expression of PDK4, PGC-1 α and AMPK α 1 mRNA did not differ significantly between myotubes derived from lean and obese subject groups. AMPK α 2 mRNA expression however, was lower in myotubes derived from obese as compared to lean subjects ($P < 0.05$; Table 3.2).

There was no effect of SR141716 and AEA, separately or in combination, on PDK4 (Fig. 3.2A), PGC-1 α (Fig. 3.2C), AMPK α 1 (Fig. 3.3A) and AMPK α 2 (Fig. 3.3C) mRNA expression in myotubes derived from lean subjects. In myotubes derived from obese subjects, PDK4 mRNA expression was unaltered in response to SR141716 and AEA separately or in combination (Fig. 3.2B). In contrast while PGC-1 α mRNA was unaffected by either AEA or SR141716 alone, the combination of 5 μ M AEA and 100 nM SR141716 decreased PGC-1 α mRNA expression ($P < 0.05$; Fig. 3.2D). Similarly, relative to 100 nM SR141716 alone, PGC-1 α mRNA expression decreased in the presence of 100 nM SR141716 together with either 0.2 μ M or 5 μ M AEA ($P < 0.05$; Fig. 3.2D). AMPK α 1 mRNA expression increased in response to 100 nM SR141716 ($P < 0.05$; Fig. 3.3B), an effect reversed by the addition of AEA (0.2 μ M or 5 μ M) ($P < 0.05$; Fig. 3.3B). AMPK α 2 mRNA expression increased in response to AEA (5 μ M) alone ($P < 0.05$; Fig. 3.3D), an effect reversed by the addition of SR141716 (100 nM) ($P < 0.05$; Fig. 3.3D).

Table 3.2 – Basal mRNA Expression of Genes

Gene	Lean	Obese
PDK4	4.123 ± 0.2122	4.961 ± 1.128
PGC-1α	28.55 ± 7.051	15.27 ± 2.128
AMPKα1	170.6 ± 11.36	176.2 ± 10.58
AMPKα2	1.330 ± 0.2186	0.6733 ± 0.04306*

Table 3.2: Comparison of levels of basal expression of analysed genes between myotubes derived from lean (n=3) and obese (n=3) subjects. Values are means \pm SEM and are presented as a dimensionless quantity after being normalized to cyclophilin A. (*) P<0.05 significant between groups of lean and obese subjects. PDK4, pyruvate dehydrogenase kinase 4; PGC-1 α , peroxisome proliferator-activated receptor γ co-activator-1 α ; AMPK α 1 and AMPK α 2, AMP-activated protein kinase alpha 1 and alpha 2.

Figure 3.2

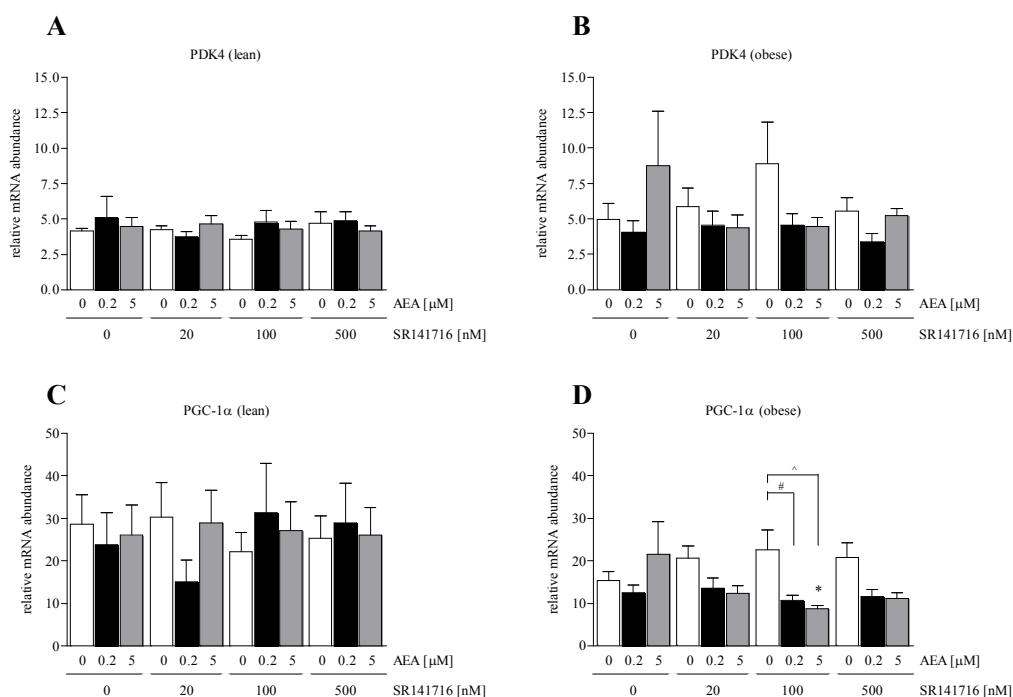


Figure 3.2: Analysis of PDK4 (A and B) and PGC-1 α (C and D) mRNA in myotubes derived from lean and obese subjects ($n=3$ /group). Exposure to the SR141716 and AEA conditions was performed in triplicate for 24 h. Experiments were performed twice for each cell line. Open bars (\square) represent the SR141716 conditions, closed bars (\blacksquare) represent SR141716 in combination with 0.2 μ M AEA and grey bars (\blacksquare) represent SR141716 in combination with 5 μ M AEA. Data are shown as means \pm SEM and are expressed as ratio of target to housekeeping gene. (*) $P<0.05$, condition vs. control (0 μ M SR141716); (#) $P<0.05$, 0.2 μ M AEA vs. 0 μ M AEA; (^) $P<0.05$, 5 μ M AEA vs. 0 μ M AEA. PDK4, pyruvate dehydrogenase kinase 4; PGC-1 α , peroxisome proliferator-activated receptor γ co-activator-1 α .

Figure 3.3

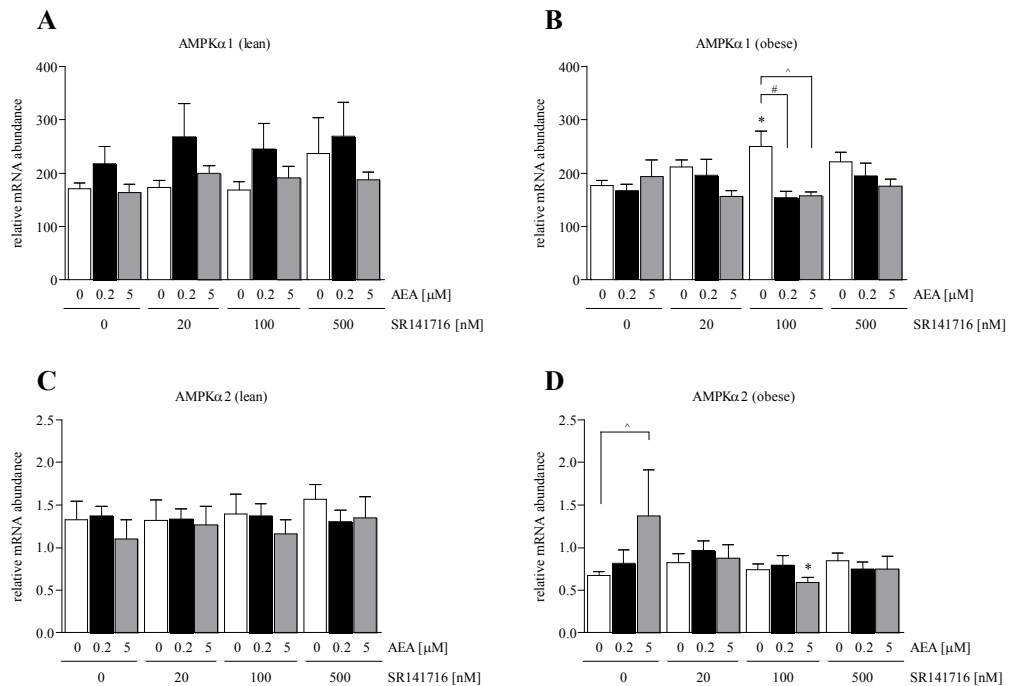


Figure 3.3: Analysis of AMPK α 1 (A and B) and AMPK α 2 (C and D) mRNA in myotubes derived from lean and obese subjects ($n=3/\text{group}$). Exposure to the SR141716 and AEA conditions was performed in triplicate for 24 h. Experiments were performed twice for each cell line. Open bars (\square) represent the SR141716 conditions, closed bars (\blacksquare) represent SR141716 in combination with 0.2 μM AEA and grey bars (\blacksquare) represent SR141716 in combination with 5 μM AEA. Data are shown as means \pm SEM and are expressed as ratio of target to housekeeping gene. (*) $P<0.05$, condition vs. control (0 μM SR141716); (#) $P<0.05$, 0.2 μM AEA vs. 0 μM AEA; (^) $P<0.05$, 5 μM AEA vs. 0 μM AEA. AMPK α 1 and AMPK α 2, AMP-activated protein kinase alpha 1 and alpha 2.

3.4.3. Phosphorylation of AMPK α and MEK1/2

A representative western blot sample is depicted in Figure 3.4. Neither SR141716 nor AEA alone or together had any effect on AMPK α or MEK1/2 protein phosphorylation in myotubes derived from either lean or obese subject groups (Fig. 3.5).

Figure 3.4

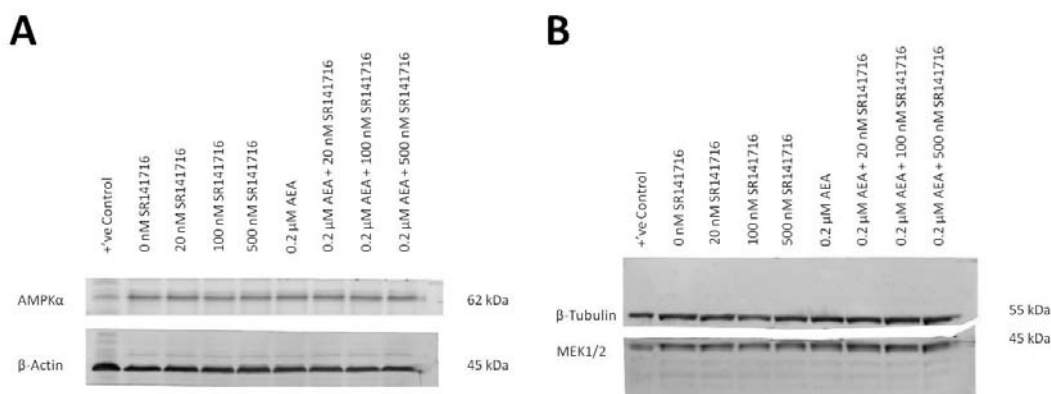


Figure 3.4: Representative western blot for the measurement of AMPK α (A) and MEK1/2 (B) protein phosphorylation. Experimental conditions were analysed on multiple blots for the same cell line. The intensities of the bands were normalised to the positive control (AICAR treated C2C12 cells for AMPK α and TPA treated HeLa cells for MEK1/2) to account for differences between blots. Individual results were then normalised to β -actin (AMPK α) or β -tubulin (MEK1/2) to account for variations in protein load.

Figure 3.5

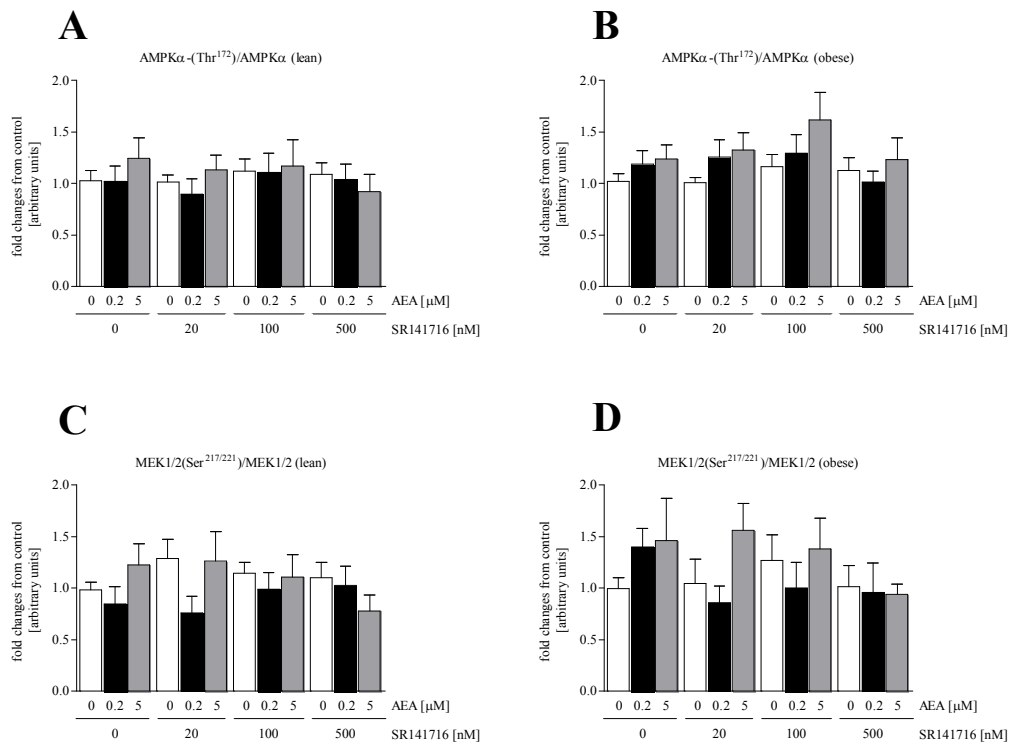


Figure 3.5: Ratio of phosphorylated to total protein of AMPK α (A and B) and MEK1/2 (C and D) in myotubes derived from lean and obese subjects (n=4/group). Exposure to the SR141716 and AEA conditions was performed in duplicate for 45 min. Experiments were performed twice for each cell line. Open bars (\square) represent the SR141716 conditions, closed bars (\blacksquare) represent SR141716 in combination with 0.2 μ M AEA and grey bars (\blacksquare) represent SR141716 in combination with 5 μ M AEA. Data are shown as means \pm SEM and are expressed as fold changes from control. Data are represented as a ratio between values obtained for anti-phospho-AMPK α -(Thr¹⁷²) and anti-AMPK α (A and B) and anti-phospho-MEK1/2-(Ser^{217/221}) and anti-MEK1/2 (C and D). AMPK α , AMP-activated protein kinase α .

3.5. DISCUSSION

It has recently been reported that CB₁ inhibition increases glucose uptake *ex vivo* in isolated soleus muscle from female Lep^{ob}/Lep^{ob} mice (Liu, Connoley et al. 2005). We have now shown that inhibition of CB₁ results in an increase in basal glucose uptake in primary cultures of skeletal muscle myotubes derived from obese, but not lean humans. The observation that SR141716 alone induced an increase in basal glucose uptake suggests endogenous production of cannabinoids in the obese-derived myotubes resulting in an increased 'endocannabinoid tone' (Fig. 3.1). This phenomenon has also been observed *in vitro* in L6 myotubes (Esposito, Proto et al. 2008), *in vivo* in relation to emesis in ferrets (Sharkey, Cristino et al. 2007) and *ex vivo* in soleus muscle from obese mice (Matias, Petrosino et al. 2008).

SR141716 is an inverse agonist at CB₁ and although we assume that the effects of SR141716 are due to binding to CB₁, it is possible that SR141716 is producing its effects as an agonist at another receptor, the expression of which may be increased in obese muscle. In a recent study, 100 nM SR141716 increased basal glucose uptake in L6 myotubes by an effect that was clearly CB₁ dependent as the effect was lost with the knockdown of CB₁ expression (Esposito, Proto et al. 2008). Similarly, in the current study, the maximal effect of SR141716 on all outcome measures was observed at 100 nM in accordance with the previously established *in vitro* dose-response effects of SR141716 (Fig 3.1) (Bensaid, Gary-Bobo et al. 2003). It is

possible that at a higher concentration, SR141716 is an agonist at another cannabinoid receptor with an opposing effect. In our study, the highest concentration of SR141716 (500 nM) had no effect, however, significantly higher concentrations of SR141716 have been shown to decrease glucose uptake in L6 cells (10 μ M) (Esposito, Proto et al. 2008) and in human primary skeletal muscle myotubes from a lean donor (1 μ M) (Eckardt, Sell et al. 2009). SR141716 is known to bind to CB₂ with low affinity (MacLennan, Reynen et al. 1998). The consequence of such binding or whether other receptors may be involved remains unknown.

AEA decreased basal glucose uptake in obese- but not lean-derived myotubes. This effect occurred only at a low, albeit supra-physiological, concentration of AEA (Bluher, Engeli et al. 2006) and was not reversed by SR141716; possibly because it was unable to compete for CB₁ under such conditions, or the effect of AEA was mediated by a receptor other than CB₁. Similarly, the absence of an effect of AEA at a higher concentration may also be due to the involvement of other cannabinoid receptors in the skeletal muscle myotubes. AEA has been shown to bind to CB₁ and CB₂ (Pagotto, Marsicano et al. 2006) and also to the transient receptor potential channel-vanilloid sub family member 1 (TRPV1) (Smart and Jerman 2000). We have recently demonstrated the expression of CB₁, CB₂ and TRPV1 in human and rodent skeletal muscle (Cavuoto, McAinch et al. 2007).

As previously shown (Berggren, Boyle et al. 2008), basal expression of PDK4 and PGC-1 α mRNA was similar in skeletal muscle from lean and obese subject groups. PDK4 mRNA content did not change in response to SR141716 or AEA at any concentration alone or together, in either lean- or obese-derived myotubes (Fig. 3.2). In our previous study using primary human skeletal muscle myotubes, we observed a decrease in PDK4 mRNA in response to the specific CB₁ antagonist AM251 (Cavuoto, McAinch et al. 2007). Possible reasons for the discrepancy between the effects of SR141716 and AM251 include differences in the relative concentrations of SR141716 (20 nM, 100 nM and 500 nM) and AM251 (0.2 μ M and 5 μ M) used, and receptor interaction and binding affinity; SR141716 is an inverse agonist and AM251 is an antagonist (Howlett, Barth et al. 2002). An inverse agonist binds to the same receptor binding site as an agonist and reduces the constitutive activity of the receptor whereas an antagonist binds to the receptor without activating it.

PGC-1 α mRNA did not change in response to SR141716 or AEA alone, but decreased in response to the combination of 100 nM SR141716 and AEA at the highest concentration (Fig. 3.2). In our previous study, a similar concentration of AEA together with AM251 also resulted in a decrease in PGC-1 α mRNA (Cavuoto, McAinch et al. 2007). Taken together, these data suggest that when CB₁-mediated effects are inhibited, AEA has a regulatory effect on PGC-1 α mRNA mediated by other cannabinoid receptors.

The alpha catalytic subunit of AMPK consists of two isoforms, AMPK α 1 and AMPK α 2. Basal AMPK α 1 mRNA was similar in lean- and obese-derived myotubes while AMPK α 2 mRNA was less in obese- as compared to lean-derived myotubes (Table 3.2). In contrast, mRNA of AMPK α 1 and AMPK α 2 in rectus abdominus muscle biopsies from lean and obese donors have been shown to be comparable (Steinberg, Smith et al. 2004). The expression of the AMPK α subunits are muscle fibre type dependent. AMPK α 1 and AMPK α 2 are predominantly expressed in Type I and Type II fibres, respectively (Winder, Hardie et al. 2003). Abdominal wall muscle typically has an even distribution of fibre types (Haggmark and Thorstensson 1979). The current study utilised a primary skeletal muscle cell culture and therefore it is possible that the difference in AMPK α 2 mRNA expression between the studies may be due to alterations in skeletal muscle fibre type or AMPK α isoform expression during culture.

AMPK α 1 but not AMPK α 2 mRNA increased in response to 100 nM SR141716 in myotubes derived from obese subjects, consistent with our previous data using the specific CB₁ antagonist AM251 (Fig. 3.3) (Cavuoto, McAinch et al. 2007). The effect of SR141716 alone on AMPK α 1 mRNA was reversed when combined with AEA, again suggesting effects mediated by other cannabinoid receptors. A decrease in AMPK α 1 mRNA may be deleterious to cellular energy metabolism as AMPK regulates partitioning of fatty acids between storage and oxidation (Kemp, Mitchelhill et al. 1999).

AEA alone increased AMPK α 2 mRNA expression, an effect that was reversed by the addition of 100 nM SR141716 leading to a decrease in AMPK α 2 mRNA relative to control (Fig. 3.3). This result is similar to the observed effect on PGC-1 α mRNA in response to the combination of SR141716 and AEA. The mechanism responsible for the selective effect of AEA and SR141716 on AMPK α 1 and AMPK α 2 mRNA remains to be elucidated, but possible explanations include the effects of more than one type of cannabinoid receptor and muscle fibre-type specificity.

We anticipated that the acute effects of SR141716 and AEA on basal glucose uptake may be mediated either by an AMPK or MEK1/2 pathway. MEK1/2 may be activated independently of AMPK via a calmodulin-dependent protein kinase kinase (CaMKK) pathway (Pierce, Luttrell et al. 2001; Witczak, Fujii et al. 2007). There was no effect of SR141716 or AEA, alone or in combination, on phosphorylation of AMPK α or MEK1/2 after 45 min (Fig. 3.5). A recent study by Esposito *et al.* (2008) has suggested that the effect of SR141716 on basal glucose uptake occurs via phosphatidylinositol-3-kinase (PI3K) and not GLUT4 translocation or GLUT1 which correlates with the AMPK α and MEK1/2 protein data presented in this study.

In summary, the effect of SR141716 to increase basal glucose uptake and mRNA expression of AMPK α 1 in myotubes derived from obese subjects suggests that the beneficial effects of this compound on metabolic activity in obese humans may be mediated at least in part by its effects on skeletal muscle. A limitation of the current

study was the small sample size used which increased the possibility of a type II error in the data. A larger sample size would be appropriate in future experimental designs to ensure more reliable results. Nevertheless, these data suggest a complex physiology of endocannabinoids in skeletal muscle in obesity, involving more than one receptor and intracellular mechanisms of action that remain to be fully elucidated. A hypothesised model for the multiple receptor effects on endocannabinoid-mediated glucose uptake in skeletal muscle is presented in Chapter 7.

CHAPTER 4

ENDOCANNABINOID RECEPTOR EXPRESSION IN SKELETAL MUSCLE

4.1. SUMMARY

The endocannabinoid system is a lipid derived signalling system that has been shown to regulate appetite and energy metabolism. The most abundant endogenous cannabinoid, anandamide (AEA), has been shown to activate the cannabinoid receptor type 1 (CB₁) and type 2 (CB₂) as well as the 'non-cannabinoid' transient receptor potential channel-vanilloid sub-family member 1 (TRPV1), before being rapidly metabolised by fatty acid amide hydrolase (FAAH). We have previously demonstrated the expression of CB₁ and studied the effects of CB₁ activation and inhibition in human skeletal muscle myotubes, however, not all results could be explained by CB₁ mediated effects. This suggests that other receptors which are activated by endocannabinoids may be present in skeletal muscle. In this study we describe the presence of not only CB₁, but also CB₂, TRPV1 and the degrading enzyme FAAH in human and rodent skeletal muscle using reverse transcription polymerase chain reaction (RT-PCR).

4.2. INTRODUCTION

The endocannabinoid, anandamide (AEA) is synthesised from arachidonic acid and has an autocrine or paracrine mode of action before being rapidly metabolised by fatty acid amide hydrolase (FAAH) (Devane, Hanus et al. 1992; Deutsch and Chin 1993). It is a ligand for cannabinoid receptors type 1 (CB₁) and type 2 (CB₂), which are 7 transmembrane domain G-protein coupled receptors (Matsuda, Lolait et al. 1990; Munro, Thomas et al. 1993). AEA is also a ligand for the transient receptor potential channel-vanilloid sub-family member 1 (TRPV1), activation of which leads to cellular depolarisation and increased intracellular calcium (Zygmunt, Petersson et al. 1999; Smart and Jerman 2000; van der Stelt, Trevisani et al. 2005).

Initially, the effects of endocannabinoids were considered to be predominantly central with the effects to increase food intake substantially mediated within the hypothalamus and nucleus accumbens (Williams and Kirkham 1999). Initial pair feeding studies suggested that not all of the effects of CB₁ antagonists on decreasing adiposity could be explained by a reduction in food intake (Bensaid, Gary-Bobo et al. 2003). Effects on metabolism in adipose tissue (Cota, Marsicano et al. 2003), the liver (Osei-Hyiaman, DePetrillo et al. 2005) and skeletal muscle (Liu, Connoley et al. 2005) are now known to be an important component of the mechanism of action of CB₁ antagonists (Engeli and Jordan 2006).

CB₂ receptors were initially thought to be expressed exclusively in cells of the immune system (Munro, Thomas et al. 1993; Schatz, Lee et al. 1997) but have now been observed in the pancreas, adipose tissue and cardiac muscle (Roche, Hoareau et al. 2006; Shmist, Goncharov et al. 2006; Bermudez-Silva, Sanchez-Vera et al. 2007).

CB₁ is expressed in human skeletal muscle and the endocannabinoid AEA modifies the pathways that regulate fatty acid oxidation in human skeletal muscle (Cavuoto, McAinch et al. 2007). However, not all of the effects of AEA can be accounted for by the inhibition of CB₁ with the CB₁-specific antagonist AM251, suggesting the presence of other receptors for endocannabinoids in skeletal muscle (Cavuoto, McAinch et al. 2007). This study has therefore used reverse transcription polymerase chain reaction (RT-PCR) to demonstrate the expression of not only CB₁, but also CB₂, TRPV1 and FAAH in human and rodent skeletal muscle, suggesting that the endocannabinoid system has regulatory effects in skeletal muscle.

4.3. MATERIALS AND METHODS

4.3.1. Human and Rodent Tissue and Cell Culture

The collection of tissue and cell culture samples from both humans and rodents are described in Chapter 2.1.

4.3.2. RNA Extraction and RT-PCR

RNA was extracted and RT-PCR performed as described in Chapter 2.2. Primer sequences used for this study are described in Chapter 2.2.5.

4.4. RESULTS

Using RT-PCR, expression of CB₁ was observed in all human and rodent samples analysed (Figure 4.1). As the open reading frame for CB₁ for both human and rodent contains a single exon, the RNA utilised was extracted and DNase treated as described in Chapter 2.2. Furthermore, negative controls containing extracted RNA but not the reverse transcriptase enzyme for each sample were applied to ensure no DNA contamination. Product sizes of 66 base pairs (bp) for human and 68 bp for rodent were observed as predicted (Chapter 2.2.5., Figure 4.1).

Similarly, the CB₂ open reading frame for human was in a single exon. For rodent, while there were three exons, it was not possible to design primers that spanned the exon junctions, due to potential sequence variability and the small size exons 2 and 3. As such, all samples for CB₂ underwent the same DNase treatment and control set as described in Chapter 2.2. The RT-PCR yield for CB₂ produced was very low; to counter this, the amount of cDNA loaded for RT-PCR was increased 10 times over the standard loading amount. Using this technique, CB₂ was detected in human cell culture, skeletal muscle, intra-abdominal adipose tissue, but not in subcutaneous adipose tissue (Figure 4.1). In rodent tissue, CB₂ expression was detected in all samples (Figure 4.1). Product sizes for human (141 bp) and rodent (369 bp) matched predicted values (Chapter 2.2.5., Figure 4.1).

Figure 4.1

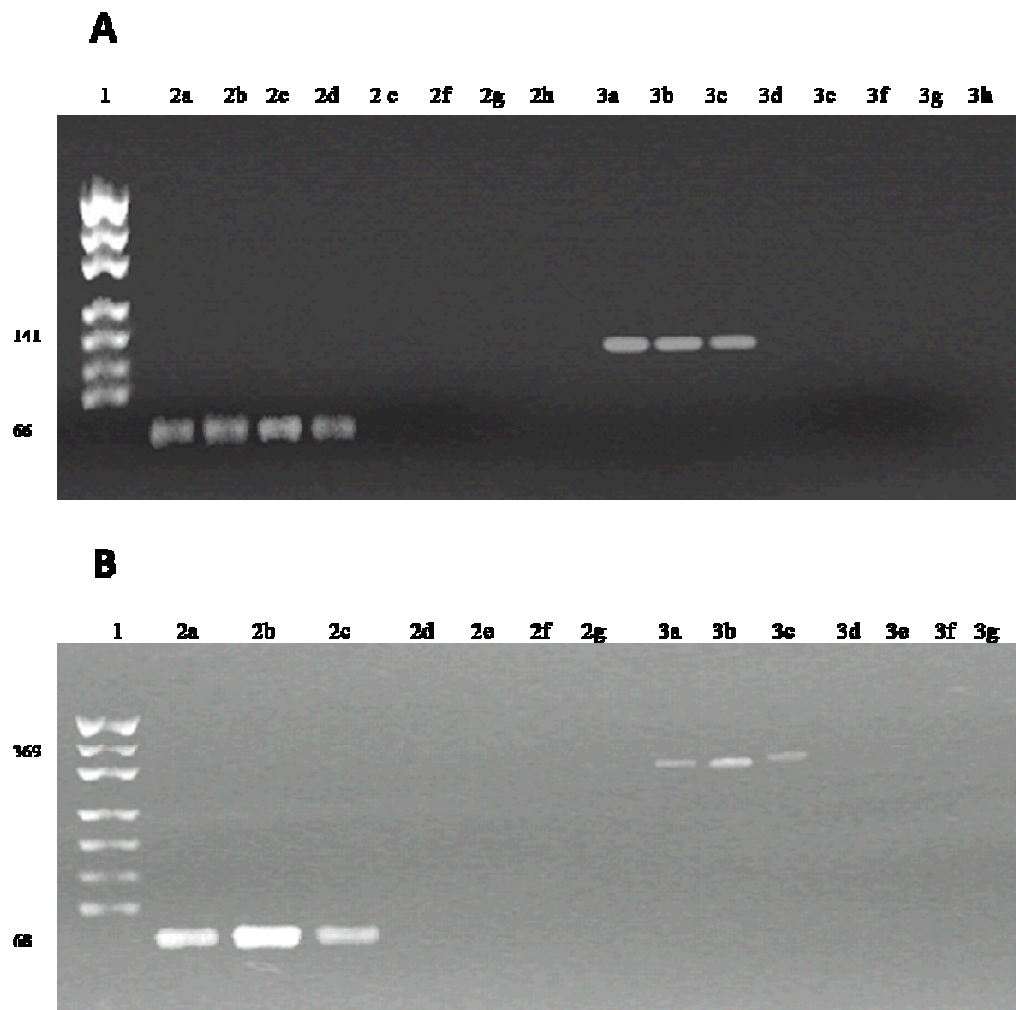


Figure 4.1: Gel electrophoresis of RT-PCR products. Products were analysed on a 2.5% agarose gel stained with ethidium bromide. A: Human tissue samples; lane: (1) pUC 19 DNA size marker, (2a-h) CB₁, (3a-h) CB₂. Samples were loaded alphanumerically: (a) primary skeletal muscle myotubes, (b) rectus abdominus muscle, (c) intra-abdominal adipose tissue, (d) subcutaneous adipose tissue, (e) reverse transcriptase negative (rt-) myotubes, (f) rt- skeletal muscle, (g) rt- combined adipose tissue, (h) RT-PCR template negative. B: Rodent tissue samples; lane: (1) pUC 19 DNA size marker, (2a-g) CB₁, (3a-g) CB₂. Samples were loaded alphanumerically: (a) L6 cells, (b) mixed hind limb muscle, (c) adipose tissue, (d) rt- L6 cells, (e) rt- skeletal muscle, (f) rt- adipose tissue, (g) RT-PCR template negative.

The human and rodent primers for FAAH and TRPV1 were designed across multiple exons meaning DNase treatment and extensive control testing was not required. In human samples, FAAH was detected in all tissues at the predicted product size (111 bp); similarly FAAH was detected in all rodent tissues at the expected product size (87 bp) (Chapter 2.2.5., Figure 4.2). TRPV1 was also detected in all human and rodent tissues at the expected product sizes (112 bp and 228 bp respectively) (Chapter 2.2.5., Figure 4.2).

Figure 4.2

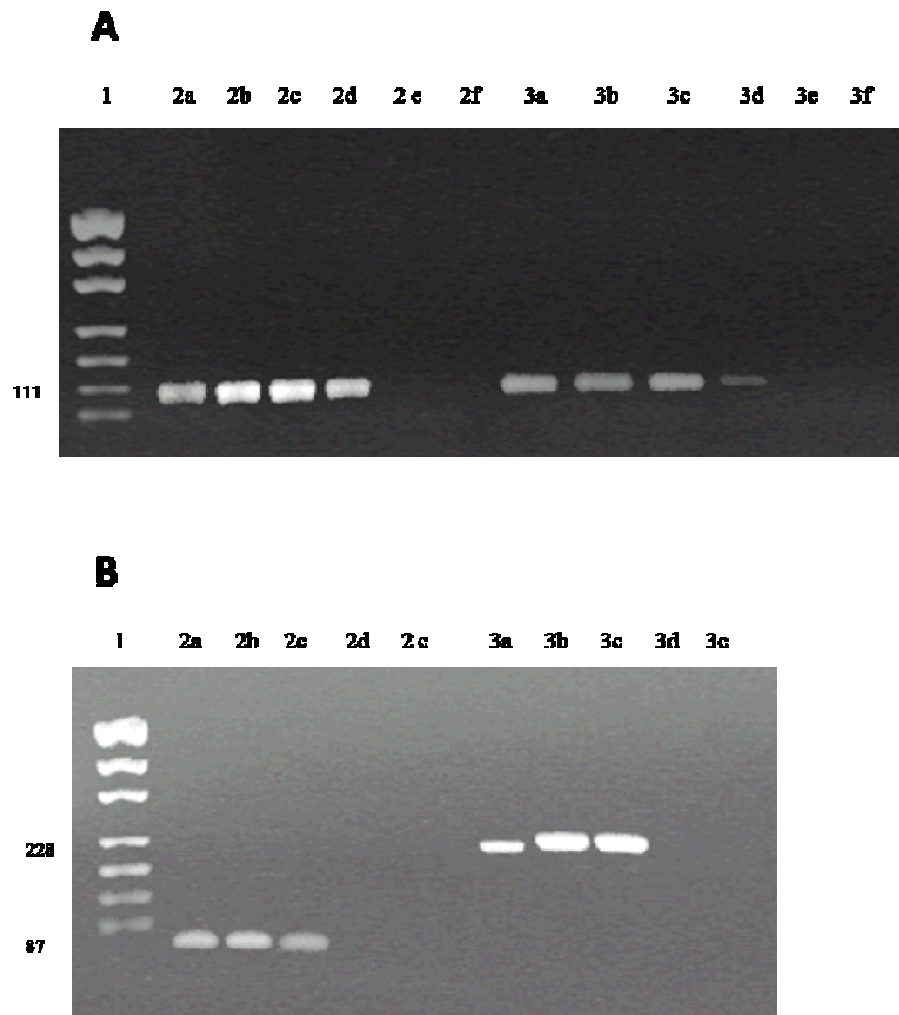


Figure 4.2: Gel electrophoresis of RT-PCR. Products were analysed on a 2.5 % agarose gel stained with ethidium bromide. A: Human tissue samples; lane: (1) pUC 19 DNA size marker, (2a-f) FAAH, (3a-f) TRPV1. Samples were loaded alphanumerically: (a) primary skeletal muscle myotubes, (b) rectus abdominus muscle, (c) intra-abdominal adipose tissue, (d) subcutaneous adipose tissue, (e) reverse transcription template negative, (f) RT-PCR template negative. B: Rodent tissue samples; lane: (1) pUC 19 DNA size marker, (2a-e) FAAH, (3a-e) TRPV1. Samples were loaded alphanumerically: (a) L6 cells, (b) mixed hind limb muscle, (c) adipose tissue, (d) reverse transcription template negative, (e) RT-PCR template negative.

4.5. DISCUSSION

When CB₁ was first cloned in 1990 (Matsuda, Lolait et al. 1990), it was thought to be expressed only in the brain (Herkenham, Lynn et al. 1990). This has since changed to include the peripheral organs such as the thyroid gland, adrenal gland, liver, adipose tissue, the gastrointestinal tract and skeletal muscle (Galiegue, Mary et al. 1995; Pertwee 2001; Porcella, Marchese et al. 2002; Bensaid, Gary-Bobo et al. 2003; Cota, Marsicano et al. 2003; Pagotto, Marsicano et al. 2006). Until recently, the expression of CB₁ had only been reported in skeletal muscle of rodent (Pagotto, Marsicano et al. 2006), but expression of CB₁ has also been demonstrated in human primary skeletal muscle myotubes (Cavuoto, McAinch et al. 2007). In this study, the presence of CB₁ in both human and rodent adipose tissue and skeletal muscle is confirmed. The observation that CB₁, as well as CB₂, TRPV1 and FAAH are expressed in primary cultures of human skeletal muscle myotubes and L6 cells indicates that their expression is myocyte specific and not the result of contaminating adipose tissue or another infiltrating cell type.

Initially, the expression of CB₂ was thought to be limited to cells of the immune system (Munro, Thomas et al. 1993). Recently however, CB₂ has been observed in brain microglia cells under inflammatory conditions (Nunez, Benito et al. 2004), on sensory nerve fibres and adnexal structures in human skin (Stander, Schmelz et al. 2005), in neonatal cardiomyocytes (Shmist, Goncharov et al. 2006) and in zebrafish

muscle (Rodriguez-Martin, Herrero-Turrion et al. 2007). Here, for the first time, the expression of CB₂ in both human and rodent skeletal muscle as well as both human primary skeletal muscle myotubes and rodent L6 cells (albeit at low levels) is demonstrated. The expression of CB₂ in human fat was detected solely in intra-abdominal and not subcutaneous adipose tissue; however, a recent study has detected low levels of CB₂ expression in subcutaneous adipose tissue (Pagano, Pilon et al. 2007). It may be that the constitutive expression of the receptor is very low, and the expression of the receptor is regulated and increased only in response to specific physiological or pathological situations, for example during exercise or inflammation; this remains to be determined.

FAAH, the enzyme responsible for the breakdown of AEA, has been shown to be highly conserved between humans, rodents and mice (Giang and Cravatt 1997). Recent studies have observed that FAAH mRNA expression is down regulated in obesity which is associated with higher levels of circulating AEA (Engeli, Bohnke et al. 2005). Also, polymorphisms in the gene encoding FAAH have been linked with obesity (Sipe, Waalen et al. 2005; Aberle, Fedderwitz et al. 2007). Therefore, the confirmation of the presence of FAAH in human and rodent skeletal muscle substantiates this enzyme as a possible important regulator of whole body energy balance as skeletal muscle is the primary site for fatty acid oxidation and glucose metabolism (Zurlo, Larson et al. 1990; Jeukendrup 2002).

The initial cloning of TRPV1 occurred in 1997, when it was shown to be a receptor for capsaicin (Caterina, Schumacher et al. 1997). Shortly thereafter, it was discovered that AEA could also activate TRPV1 (Zygmunt, Petersson et al. 1999). This has since raised speculation regarding a pathway that links TRPV1 to FAAH through regulation of AEA (Van Der Stelt and Di Marzo 2004). Interestingly, it has been shown in goldfish retinal amacrine cells that TRPV1 and FAAH co-localise when TRPV1 is activated, suggesting an auto regulatory function (Zimov and Yazulla 2007). It has previously been shown that TRPV1 is expressed in the sarcoplasmic reticulum of rodent skeletal muscle, and is likely responsible for release of Ca^{2+} into the cell (Xin, Tanaka et al. 2005). Here, it is shown that TRPV1 is also expressed in human skeletal muscle and adipose tissue. As TRPV1 and FAAH are both expressed in human and rodent skeletal muscle, the pathway linking them may be involved in regulating the metabolism of endocannabinoids in muscle.

There is increasing evidence that the endocannabinoid system has a role in the regulation of whole body energy balance (Pagotto, Marsicano et al. 2006). In skeletal muscle, it has previously been shown that endocannabinoid activity regulates mRNA expression of key genes involved in nutrient oxidation (Cavuto, McAinch et al. 2007). These observations on mRNA expression could not be explained solely by CB_1 mediated effects, making it likely that the effects of AEA in the primary cultures of human myotubes was being mediated by more than one

receptor (Cavuoto, McAinch et al. 2007). In this study, the expression of CB₂ and TRPV1 in human and rodent skeletal muscle are described as potential receptor sites for the previously observed effects of AEA (Cavuoto, McAinch et al. 2007). Drugs targeting CB₁, CB₂, FAAH and TRPV1 for the treatment of obesity, pain and inflammation are currently under development (Vandevoorde and Lambert 2005; Pagotto, Marsicano et al. 2006; Cheng and Hitchcock 2007; Szallasi, Cortright et al. 2007). The expression of CB₂, FAAH and TRPV1 along with CB₁ in human and rodent skeletal muscle is therefore an important consideration in the development of these therapeutic targets, as endocannabinoids may have a complex role in skeletal muscle biology.

CHAPTER 5

ENDOCANNABINOIDS ENHANCE INSULIN-STIMULATED GLUCOSE UPTAKE IN RODENT L6 SKELETAL MUSCLE MYOTUBES; AN EFFECT MEDIATED BY CB₁, CB₂ AND TRPV1

5.1. SUMMARY

In lean subjects, basal and insulin-stimulated glucose uptake in skeletal muscle is increased by endocannabinoids. Conversely, in obese rodents basal and insulin-stimulated glucose uptake is improved by blocking cannabinoid receptor type 1 (CB₁). The studies in Chapter 3 suggest that CB₁ does not mediate all of the effects of endocannabinoids on human skeletal muscle glucose uptake. Additionally, cannabinoid receptor type 2 (CB₂) and transient receptor potential channel-vanilloid sub-family member 1 (TRPV1) mRNA are also expressed in human and rodent skeletal muscle (Chapter 4). This study aimed to determine the role of the principle endogenous cannabinoids anandamide (AEA) and 2-arachidonylglycerol (2-AG) and their receptors CB₁, CB₂ and TRPV1 on basal and insulin-stimulated glucose uptake in rodent skeletal muscle cells.

An acute (30 min) exposure to AEA (0 μ M to 15 μ M) and 2-AG (0 μ M to 15 μ M) had no effect on basal or insulin-stimulated glucose uptake, whereas chronic (24 h) exposure to AEA (5 μ M and 15 μ M) and 2-AG (5 μ M) increased insulin-stimulated glucose uptake ($P < 0.05$). Based on these data, 5 μ M 2-AG for 24 h was utilised for the second phase of this study. The chronic effect of 2-AG on basal and insulin-stimulated glucose uptake was inhibited by the CB₁ inverse agonist SR141716 (100 nM, 500 nM; $P < 0.001$), the CB₂ inverse agonist SR144528 (100 nM, 1 μ M; $P < 0.01$) and the TRPV1 antagonist SB366791 (500 nM, 5 μ M; $P < 0.01$) separately, with no additive effects observed when these compounds were combined. Taken together, these data suggest that multiple receptors may mediate the effects of endocannabinoids on both basal and insulin-stimulated glucose uptake in skeletal muscle.

5.2. INTRODUCTION

Recent studies have suggested a role for endocannabinoids in the regulation of skeletal muscle glucose uptake in the pathogenesis of obesity related insulin resistance (Eckardt, Sell et al. 2008; Esposito, Proto et al. 2008). The available data indicate that cannabinoid receptor type 1 (CB₁) mediates the effects of endocannabinoids on glucose uptake in skeletal muscle (Esposito, Proto et al. 2008; Eckardt, Sell et al. 2009). However, the studies in Chapter 3 suggest that the effects of endocannabinoids on skeletal muscle glucose uptake are mediated by more than one receptor and that mRNA of cannabinoid receptor type 2 (CB₂) and transient receptor potential channel-vanilloid sub-family member 1 (TRPV1) are both expressed in human and rodent skeletal muscle (Cavuoto, McAinch et al. 2007).

Recent studies have suggested a role for CB₂ in the regulation of glucose homeostasis. In rats, the CB₂-specific agonist JWH 133 (1 µg/kg) improved glucose tolerance in response to an intraperitoneal glucose load, whereas the CB₂ antagonist AM630 (50 µg/kg) had the opposite effect (Bermudez-Silva, Sanchez-Vera et al. 2007). Lower concentrations of both compounds were without effect. *In vitro*, 2-arachidonoylglycerol (2-AG; 10 µM) decreased insulin secretion in cultures of pancreatic islets of Langerhans from mice via regulation of intracellular calcium concentration (Juan-Pico, Fuentes et al. 2006). It was inferred by these investigators that this effect was mediated by CB₂ without evidence that this was the case. While

these data suggest an effect of endocannabinoids on glucose induced insulin secretion, the effects of CB₂ on skeletal muscle glucose uptake *in vitro* remain unknown.

There is also evidence that TRPV1 mediates effects on glucose homeostasis. The TRPV1 agonist resiniferatoxin (50 µg/kg body weight) increased both insulin secretion as well as sensitivity in obese Zucker rats (Moesgaard, Brand et al. 2005). Although the effects of TRPV1 on glucose metabolism have been considered to be neuronally mediated (Moesgaard, Brand et al. 2005), the expression of TRPV1 on skeletal muscle cells raises the possibility that TRPV1 may have a regulatory role in skeletal muscle glucose uptake.

This study therefore aimed to determine which of the receptors mediate the effects of the endocannabinoids on skeletal muscle glucose uptake. We hypothesised that CB₂ and TRPV1 which is also a receptor for the endocannabinoids anandamide (AEA) and 2-AG (Smart and Jerman 2000; McVey, Schmid et al. 2003; Golech, McCarron et al. 2004) are able to mediate glucose uptake in skeletal muscle. This study was carried out in L6 cells, a rodent skeletal muscle culture model that we showed expressed CB₁, CB₂ and TRPV1 that have a reproducible insulin response for measurement of glucose uptake (Klip, Li et al. 1984; Klip and Paquet 1990).

5.3. MATERIALS AND METHODS

5.3.1. Chemicals

AEA, 2-AG, SB366791 and all cell culture reagents were purchased from Sigma Aldrich (St. Louis, MO). SR141716 and SR144528 were kindly provided by Sanofi-Aventis (Paris, France). 2-[³H]deoxy-D-glucose and Ultima GoldTM scintillation fluid were purchased from Perkin Elmer (Boston, MA). The BCA Protein Assay Reagent Kit was purchased from Thermo Scientific (Rockford, IL). AEA and 2-AG were dissolved into a 10 mM stock solution of ethanol and acetonitrile, respectively. SR141716, SR144528 and SB366791 were dissolved into 10 mM solutions of DMSO.

5.3.2. Cell Culture (L6)

L6 myotubes were cultured in 12-well plates and prepared for experimental use as described in section 2.1.4.

5.3.3. Experimental Protocol

To measure the acute effects of the endocannabinoids on glucose uptake, L6 myotubes (following 3 days of differentiation) were washed twice with 1x PBS then serum starved in 1 ml α -MEM containing 0.1 % fatty acid free (FAF)-BSA for 6 h at 37°C and 5% CO₂. The L6 myotubes were then washed twice with 1x PBS and subjected to either AEA (0 μ M, 5 μ M, 10 μ M and 15 μ M) or 2-AG (0 μ M, 5 μ M,

10 μM and 15 μM) diluted in 1 ml α -MEM containing 0.1% FAF-BSA and incubated for 30 min at 37°C and 5% CO_2 .

To measure the chronic effects of the endocannabinoids on glucose uptake, L6 myotubes (following 3 days of differentiation) were washed twice with 1x PBS and α -MEM containing 0.1% FAF-BSA, then subjected to either AEA (0 μM , 5 μM , 10 μM and 15 μM) or 2-AG (0 μM , 5 μM , 10 μM and 15 μM) diluted in 1 ml α -MEM containing 0.1% FAF-BSA and incubated for 24 h at 37°C and 5% CO_2 .

The second phase of the study investigated the chronic effects of blockers for CB_1 (SR141716), CB_2 (SR144528) and TRPV1 (SB366791). L6 myotubes (following 3 days of differentiation) were washed twice with 1x PBS and α -MEM containing 0.1% FAF-BSA, then subjected to SR141716 (0 nM, 100 nM and 500 nM), SR144528 (0 nM, 100 nM and 1 μM) and SB366791 (0 nM, 500 nM and 5 μM) alone and in combination, with or without 5 μM 2-AG, diluted in 1 ml α -MEM containing 0.1% FAF-BSA and incubated for 24 h at 37°C and 5% CO_2 .

Exposure to the experimental conditions was conducted in triplicate for each experiment.

5.3.4. Basal and Insulin-Stimulated 2-[³H]deoxy-D-glucose Uptake

Basal and insulin-stimulated glucose uptake experiments were completed as described in section 2.3.2.

5.3.5. Statistical Analysis

Data are expressed as mean \pm SEM of the raw data. Statistical analyses were performed by 2-, 3- or 5-way analysis of variance (ANOVA) using pair wise comparisons. Analyses which contained an interaction between relevant factors were subjected to a Tukey post-hoc test. Statistical analyses were completed using SPSS 17 software from SPSS Inc (Chicago, IL). Significance was accepted at $P < 0.05$ for all analyses.

5.4. RESULTS

5.4.1. Effect of Endocannabinoids on Basal and Insulin-Stimulated Glucose Uptake in Skeletal Muscle

Basal and insulin-stimulated glucose uptake displayed a “u” shaped dose response curve on exposure to AEA for 30 min with a decrease in response to 10 μ M AEA as compared to 5 μ M and 15 μ M AEA ($P < 0.05$; Fig. 5.1A). After 24 h, basal glucose uptake increased in response to 15 μ M AEA ($P < 0.05$; Fig 5.1B) and insulin-stimulated glucose uptake increased in response to 5 μ M and 15 μ M AEA relative to control ($P < 0.01$; Fig 5.1B). After 24 h, insulin-stimulated glucose uptake displayed a “u” shaped dose response curve on exposure to AEA with a decrease in response to 10 μ M AEA as compared to 5 μ M and 15 μ M AEA ($P < 0.05$; Fig 5.1B).

Basal and insulin-stimulated glucose uptake were unaltered in response to a 30 min exposure to 2-AG (Fig. 5.1C). After 24 h, basal and insulin-stimulated glucose uptake increased in response to 5 μ M 2-AG ($P < 0.05$; Fig 5.1D), and decreased in response to 10 μ M 2-AG as compared to 5 μ M and 15 μ M 2-AG ($P < 0.05$; Fig. 5.1D).

Figure 5.1

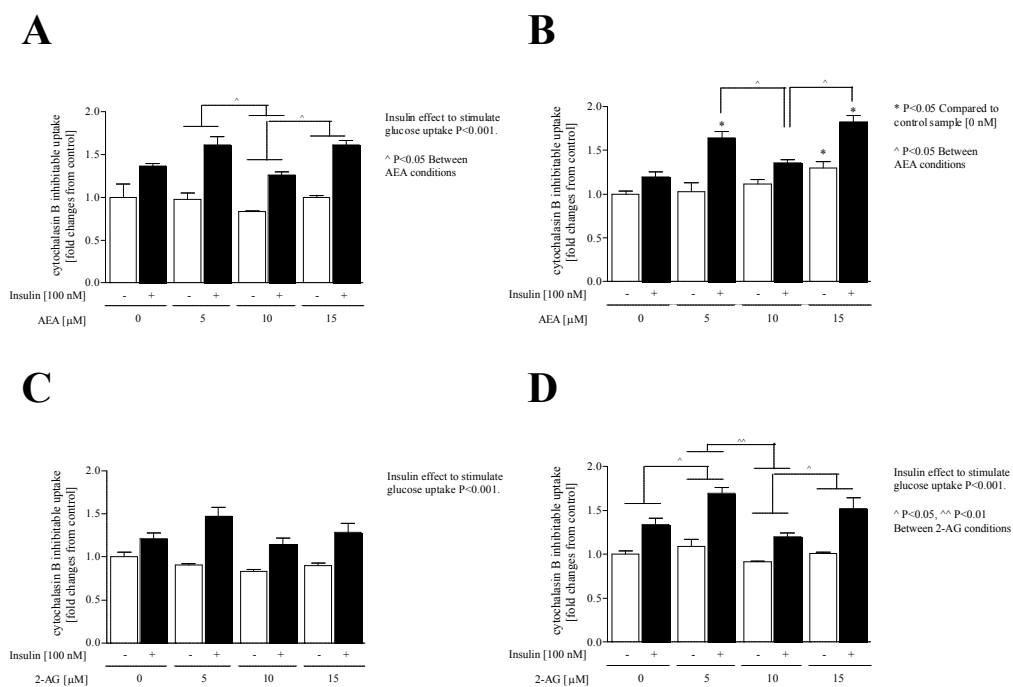


Figure 5.1: Basal and insulin-stimulated glucose uptake in L6 cell culture in response to AEA (A and B) and 2-AG (C and D). Experimental conditions were performed in triplicate for 30 min (A and C) and 24 h (B and D), followed by 30 min insulin-stimulation. Glucose uptake experiments were performed twice and independently of each other. Open bars (□) represent basal glucose uptake and closed bars (■) represent insulin-stimulated glucose uptake. Data are shown as means ± SEM (n=3) and are expressed as fold changes from control.

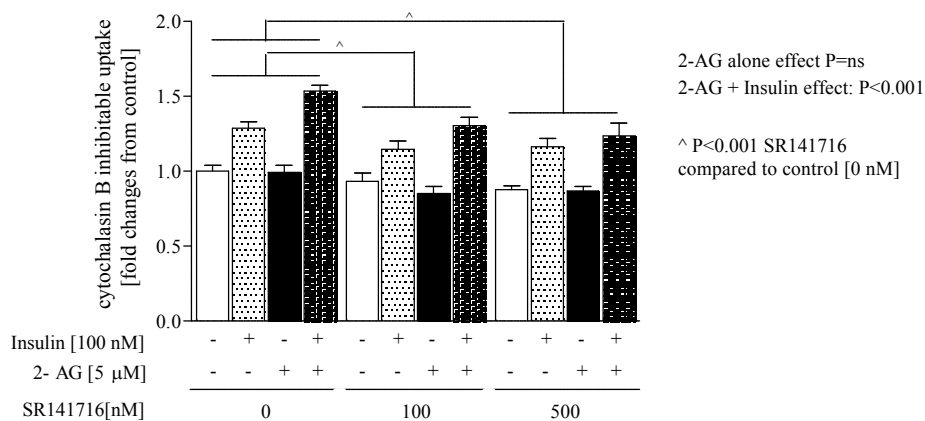
5.4.2. Effect of Chronic Inhibition of the Receptors for Endocannabinoids on Basal and Insulin-Stimulated Glucose Uptake in Skeletal Muscle

For the following experiments, we measured basal and insulin-stimulated glucose uptake in response to 24 h exposure to chemical inhibitors of CB₁ (SR141716), CB₂ (SR144528) and TRPV1 (SB366791) in the presence or absence of 5 μM 2-AG. Basal and insulin-stimulated glucose uptake in the presence and absence of 5 μM 2-AG decreased in response to 100 nM and 500 nM SR141716 ($P < 0.001$; Fig. 5.2A). Insulin-stimulated, but not basal, glucose uptake decreased in response to 100 nM and 1 μM SR144528 in the presence and absence of 5 μM 2-AG ($P < 0.01$; Fig. 5.2B). Basal and insulin-stimulated glucose uptake in the presence of 5 μM 2-AG decreased in response to 500 nM and 5 μM SB366791 ($P < 0.01$; Fig. 5.2C).

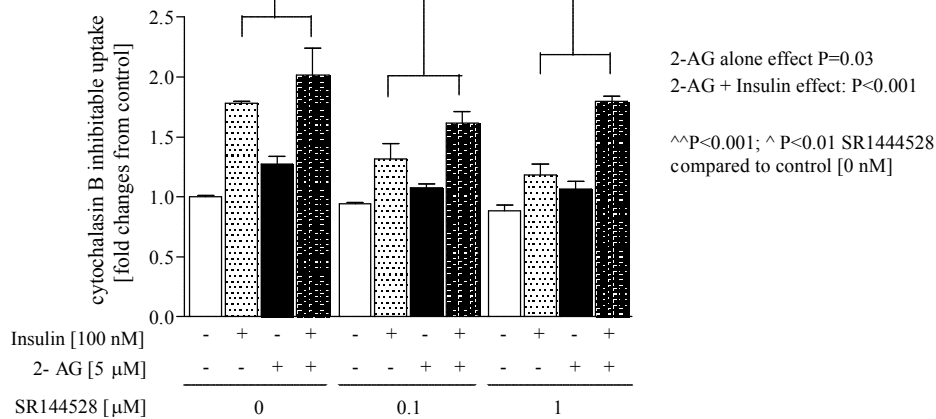
The combination of SR141716, SR144528 and SB366791 did not have an additive effect to decrease basal and insulin-stimulated glucose uptake in presence or absence of 5 μM 2-AG (Fig 5.3).

Figure 5.2

A



B



C

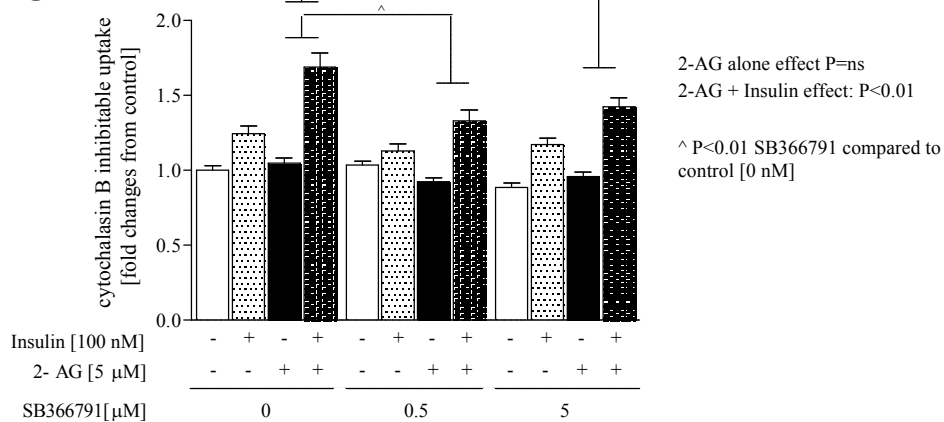


Figure 5.2: Basal and insulin-stimulated glucose uptake in L6 cell culture in response to SR141716 (A), SR144528 (B) and SB366791 (C). Experimental conditions were performed in triplicate for 24 h, followed by 30 min insulin-stimulation. Glucose uptake experiments were performed twice and independently of each other. Open bars (□) represent basal glucose uptake, speckled bars (▨) represent insulin-stimulated glucose uptake, closed bars (■) represent 2-AG exposure during basal glucose uptake and tiled bars (▩) represent 2-AG exposure during insulin-stimulated glucose uptake. Data are shown as means \pm SEM and are expressed as fold changes from control.

Figure 5.3

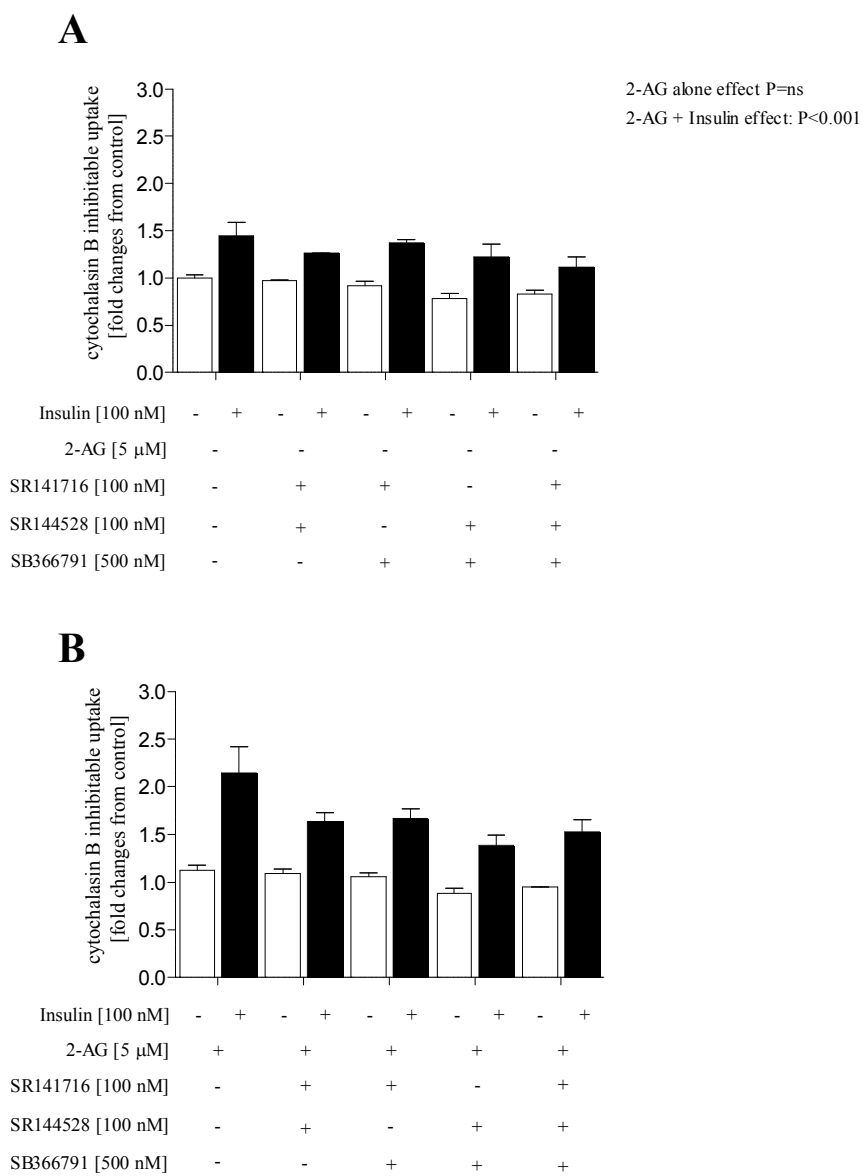


Figure 5.3: Basal and insulin-stimulated glucose uptake in L6 cell culture in response SR141716, SR144528 and SB366791. (A) Effects mediated without 2-AG; (B) effects mediated with 2-AG. Experimental conditions were performed in triplicate for 24 h, followed by 30 min insulin-stimulation. Glucose uptake experiments were performed twice and independently of each other. Open bars (□) represent basal glucose uptake and closed bars (■) represent insulin-stimulated glucose uptake. Data are shown as means \pm SEM and are expressed as fold changes from control.

5.5. DISCUSSION

These data show that chronic but not acute exposure to AEA and 2-AG induces an increase in insulin-stimulated glucose uptake in L6 myotubes; the effect of 2-AG appeared to be greater than that of AEA, although direct comparisons were not performed in the current study. There was a trend for AEA to acutely increase insulin-stimulated glucose uptake, although this may be due to a low repeat number of the experimental conditions, possibly causing a Type II error. The absence of an effect of AEA on basal glucose uptake in the current study is consistent with data from 3T3-L1 adipocyte cell culture studies (Gasperi, Fezza et al. 2007; Pagano, Pilon et al. 2007), but is in contrast to the effects of AEA on a single line of human primary skeletal muscle myotubes where both basal and insulin-stimulated glucose uptake increased (Eckardt, Sell et al. 2009). Each of these aforementioned studies utilised a different cell type and cellular conditions and therefore cell specific variations in responsiveness may account for the discrepancies observed.

In L6 cells, the effect of both AEA and 2-AG on insulin-stimulated glucose uptake was biphasic across the dose response curve (beginning with an initial increase in insulin-stimulated glucose uptake). Similar effects have been observed in primary human skeletal muscle culture from obese, but not lean subjects (Chapter 3). In that study, basal glucose uptake decreased in response to 0.2 μM AEA and was unchanged from control samples at 5 μM AEA (Chapter 3). Biphasic effects have

also been reported in response to SR141716. A study utilising L6 cell culture reported that the effects of SR141716 on basal glucose uptake may be biphasic as glucose uptake increased in response to 100 nM SR141716 and decreased in response to 10 μ M SR141716 (Esposito, Proto et al. 2008). Endocannabinoids are known to be involved in biphasic dose response curves in other biological systems; for example, food intake in humans and rodents (Berry and Mechoulam 2002), voltage-gated currents from synaptic terminals in goldfish retinas (Fan and Yazulla 2003) and blastocyst activation and uterine receptivity during human pre-implantation embryo development (Wang, Xie et al. 2006). A possible explanation for this is that effects of the endocannabinoids on a particular cellular function may be mediated by more than one endocannabinoid receptor, and our observation that all three receptors are present in skeletal muscle is consistent with this notion. AEA and 2-AG are agonists at both CB₁ and CB₂; 2-AG is more potent at both of these receptors than AEA (Sugiura, Kodaka et al. 1999; Gonsiorek, Lunn et al. 2000). Additionally, 2-AG is the predominant endocannabinoid synthesised in skeletal muscle (Cote, Matias et al. 2007; Matias, Petrosino et al. 2008).

The current study is, as far as we can determine, the first to report effects of 2-AG on basal and insulin-stimulated glucose uptake in skeletal muscle and demonstrate that CB₁, CB₂ and TRPV1 are all involved to some extent.

In the absence of agonist, the effect of the CB₁ inverse agonist SR141716 to decrease basal and insulin-stimulated glucose uptake is consistent with the observations of Eckardt *et al.* (2009). The CB₂ inverse agonist SR144528 decreased insulin-stimulated, but not basal glucose uptake. In the absence of 2-AG, the TRPV1 antagonist SB366791 had no effect on basal or insulin-stimulated glucose uptake. Possible explanations for these data is that the autocrine effects of endocannabinoids present in culture occur at thresholds that differ between the receptors, or that TRPV1 does not mediate effects on glucose metabolism. This latter explanation seems less likely given the effects of the TRPV1 antagonist to ameliorate the effects of 2-AG on glucose uptake in L6 cells in the current study. Threshold effects on receptor activity have been reported in studies investigating calcitonin gene-related peptide (CGRP) release in primary sensory neurons in response to AEA (Ahluwalia, Urban et al. 2003). CGRP release was mediated primarily by CB₁ in response to <1 μM AEA. At concentrations above 1 μM AEA, CGRP release was mediated predominately by TRPV1 (Ahluwalia, Urban et al. 2003).

The increase in basal and insulin-stimulated glucose uptake induced by 2-AG was attenuated by SR141716 and SB366791. The observation that SR144528 decreased the effect of 2-AG on insulin-stimulated, but not basal glucose uptake suggests that CB₂ activity is insulin-dependent. Various combinations of each compound did not produce effects that were different from each alone which might suggest common

intracellular mechanisms of action or receptor co-localisations to mediate these effects.

Although SR141716 and SR144528 specifically inhibit CB₁ and CB₂ (Shire, Calandra et al. 1999), there is some evidence from isolated Langendorff-perfused rat hearts that SR141716 acts as a partial agonist at CB₂ and SR144528 acts as a partial agonist at CB₁ (Krylatov, Maslov et al. 2005). These effects may be dose dependent. SB366791, a selective TRPV1 antagonist, is also a partial antagonist of both CB₁ (12% inhibition) and CB₂ (16% inhibition) (Gunthorpe, Rami et al. 2004). It is therefore possible that as opposed to a common intracellular mechanism of action, the lack of an additive effect of the cannabinoid receptor inhibitors was due to these compounds impairing the action of one another when used in combination. There may also be interactions between these receptors. A recent study has reported effects of co-localisation of CB₁ and TRPV1 where the cannabinoid receptor agonist HU-210 produced a TRPV1 mediated increase in intracellular calcium in human embryonic kidney (HEK) cells; an effect which only occurred when both CB₁ and TRPV1 was present and was inhibited by SR141716 (Hermann, De Petrocellis et al. 2003).

The effect of SR141716 on skeletal muscle glucose uptake appears to depend on phenotype and cellular conditions. SR141716 increases glucose uptake in skeletal

muscle from obese rodents (Liu, Connoley et al. 2005; Nogueiras, Veyrat-Durebex et al. 2008) and in primary skeletal muscle cell culture from obese but not lean humans (Chapter 3). SR141716 decreased basal glucose uptake in primary human skeletal muscle cell culture from a lean healthy donor without change in GLUT1 and GLUT4 (Eckardt, Sell et al. 2009). The reason for this discrepancy is unknown but at the very least highlights the differences between the responsiveness of skeletal muscle from lean and obese donors.

There is limited data on the effects of CB₂ inhibitors on cellular glucose uptake. We report, for the first time, effects of CB₂ on insulin-stimulated glucose uptake in skeletal muscle cell culture. A recent study reported a biphasic effect of the CB₂ specific inverse agonist AM630 (0.5 μM increased and 1 μM decreased) on basal glucose uptake in human intestinal endothelium derived Caco-2 cells (Goncalves, Araujo et al. 2008). Another study which investigated the role of the CB₁ and CB₂ on the regulation of insulin sensitivity in L6 cell culture reported no effect of the CB₂-specific agonist JWH015 (100 nM) on insulin-stimulated activation of MAP kinase (ERK1/2) or protein kinase B (PKB) (Lipina, Stretton et al. 2009). This suggests that CB₂ was unlikely to mediate effects on skeletal muscle growth, differentiation or metabolism. The data presented in the current study imply CB₂ may at least be involved in the regulation of insulin-stimulated glucose uptake in skeletal muscle which is unlikely to be regulated by any of the aforementioned pathways. However,

recent data has suggested that cannabinoid receptors may signal via the phosphoinositide 3-kinase (PI3K) pathway (Pagano, Pilon et al. 2007).

2-AG has been considered not to be an agonist at TRPV1. The review by Van Der Stelt and Di Marzo (2004) is frequently cited in evidence albeit that it makes no mention of 2-AG. There is emerging data suggesting that 2-AG is an agonist at TRPV1 (McVey, Schmid et al. 2003; Golech, McCarron et al. 2004). In the current study, the effects of the TRPV1 antagonist SB366791 to selectively inhibit 2-AG mediated basal and insulin-stimulated glucose uptake provides further evidence that 2-AG may act as an agonist at TRPV1. Further studies are required to determine the pharmacology of the interaction between 2-AG and TRPV1 and any potential interaction of TRPV1 with CB₁ or CB₂.

There is increasing interest in TRPV1 as a potential therapeutic target. TRPV1 is involved in the regulation of food intake and intermediary metabolism. Agonists of TRPV1 reduce food intake and increase satiety and energy expenditure (Yoshioka, St-Pierre et al. 1999; Westerterp-Plantenga, Smeets et al. 2005; Snitker, Fujishima et al. 2009). Mice deficient in TRPV1 are resistant to diet induced obesity, suggesting a regulatory role for TRPV1 in the accumulation of weight (Motter and Ahern 2008). There are 2 possibilities for this phenomenon: 1. TRPV1 agonists produce an initial increase in energy expenditure and prolonged exposure results in desensitisation of TRPV1; 2. The TRPV1 agonists are exerting their effects via a TRPV1-independent

pathway. The data in the current study suggests that the effects of TRPV1 on basal or insulin-stimulated glucose uptake are dependent on an elevated level of endocannabinoid signalling. We postulate that TRPV1 may mediate basal and insulin-stimulated glucose uptake under conditions of increased endocannabinoid activity, such as obesity (Engeli, Bohnke et al. 2005). The precise role of TRPV1 in the regulation of glucose metabolism *in vivo* requires further study and will be addressed in Chapter 6.

In summary, the data presented in the current study shows, for the first time that the effects of the endocannabinoid system on skeletal muscle glucose uptake are not mediated exclusively by CB₁ but also by CB₂ and TRPV1. This presents an opportunity to further investigate the effects of the endocannabinoid system on energy metabolism beyond CB₁-mediated effects and may contribute to a better understanding of the role of the endocannabinoid system on energy metabolism in obesity.

CHAPTER 6

TRPV1 MEDIATES DISCORDANT EFFECTS ON THE REGULATION OF FAT MASS AND GLUCOSE METABOLISM

6.1. SUMMARY

The transient receptor potential vanilloid sub-family member 1 (TRPV1) is a neuronally expressed receptor for which capsaicin, the pungent component of hot peppers, is a natural ligand. TRPV1 has a major role in thermal inflammatory hyperalgesia. TRPV1 is involved in the regulation of energy balance and transgenic mice deficient in TRPV1 are resistant to diet induced obesity (Mottet and Ahern 2008). TRPV1 may also have a role in glucose metabolism. Endocannabinoids induce an increase in glucose uptake in skeletal muscle and the endocannabinoids anandamide (AEA) and possibly 2-arachidonoylglycerol (2-AG) are TRPV1 agonists. The studies in Chapter 5 demonstrated that TRPV1 is involved in endocannabinoid induced skeletal muscle glucose uptake *in vitro*. Transgenic mice deficient in TRPV1 clear glucose more efficiently than wild-type mice in response to a glucose challenge under chow conditions (Razavi, Chan et al. 2006). In order to determine the effects of high fat feeding on glucose tolerance in TRPV1^{-/-} mice, wild-type and TRPV1^{-/-} mice were fed either a standard chow diet or high fat diet (HFD) for 18

weeks (n=8/group). Wild-type mice on the HFD gained more weight than wild-type mice on the chow ($P < 0.05$), whilst TRPV1^{-/-} mice were resistant to weight gain on the HFD. At baseline, TRPV1^{-/-} mice cleared glucose more efficiently than wild-type mice ($P = 0.02$). After 18 weeks of high fat feeding, glucose tolerance was impaired in both wild-type and TRPV1^{-/-} mice as compared with chow fed wild-type mice ($P = 0.002$ and $P = 0.043$, respectively). Thus, TRPV1^{-/-} mice are protected from diet induced obesity, but not impaired glucose tolerance. Taken together, these data have implications for the potential therapeutic use of TRPV1 antagonists.

6.2. INTRODUCTION

The transient receptor potential vanilloid sub-family member 1 (TRPV1) was first identified as a neuronally expressed receptor for capsaicin, the pungent component of hot peppers (Caterina, Schumacher et al. 1997). TRPV1 has a role in thermal inflammatory hyperalgesia and is therefore a therapeutic target for pain relief (Pingle, Matta et al. 2007). TRPV1 also has a role in cardio-protection as TRPV1^{-/-} mice have impaired cardiac recovery in response to ischemia-reperfusion injury (Wang and Wang 2005; Zhong and Wang 2007; Huang, Rubinstein et al. 2009). Additionally, there is emerging data suggesting that TRPV1 has a role in energy balance and intermediary metabolism (Suri and Szallasi 2008).

In human and animal studies, the use of TRPV1 agonists have beneficial effects on energy metabolism (Knotkova, Pappagallo et al. 2008). Whilst TRPV1 agonists initially activate TRPV1, long term exposure leads to desensitisation of the receptor (Szallasi and Blumberg 1999). Therefore, the pharmacological effects of TRPV1 agonists may be mediated via inhibition of TRPV1 signalling, however, the possibility that these agents exert their effects independently of TRPV1 cannot be excluded (Hsu and Yen 2007; Kang, Kim et al. 2007). For example, in humans, dietary supplementation with capsaicin increased satiety and decreased energy intake relative to control groups (Yoshioka, St-Pierre et al. 1999; Westerterp-Plantenga, Smeets et al. 2005; Snitker, Fujishima et al. 2009). In rats, ablation of TRPV1-positive

nerve fibres by systemic capsaicin administration prevented aging-associated obesity (Melnyk and Himms-Hagen 1995). Mice deficient in TRPV1 are protected against diet induced obesity (Motter and Ahern 2008).

TRPV1 may also have a role in glucose regulation. The endocannabinoids, which form part of a lipid derived cellular signalling system (Pertwee and Ross 2002), increase glucose uptake in skeletal muscle *in vitro* (Chapter 5). In addition, studies from Chapter 5 reported that TRPV1 is involved in endocannabinoid induced skeletal muscle glucose uptake *in vitro*. In obese Zucker rats, the TRPV1 agonist, resiniferatoxin (RTX) increased insulin secretion and sensitivity, suggesting a regulatory role for TRPV1 in glucose homeostasis (Moesgaard, Brand et al. 2005). In addition, 8 week old TRPV1^{-/-} mice on a chow diet have better glucose tolerance than wild-type mice (Razavi, Chan et al. 2006), however, it is not known whether TRPV1^{-/-} mice maintain improved glucose tolerance in response to high fat feeding.

Anandamide (AEA) (Devane, Hanus et al. 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura, Kondo et al. 1995) are the predominant endocannabinoids, although their tissue distribution and relative affinity for various receptors varies (Mechoulam, Fride et al. 1998). They are both degraded by the metabolising enzyme fatty acid amide hydrolase (FAAH) (Pagotto, Marsicano et al. 2006). AEA (Van Der Stelt and Di Marzo 2004) and possibly 2-AG (Smart and Jerman 2000;

McVey, Schmid et al. 2003; Golech, McCarron et al. 2004) are agonists at TRPV1. Studies utilising goldfish retina amacrine cells have demonstrated that TRPV1 and FAAH may co-localise, possibly acting as a regulator of AEA content (Zimov and Yazulla 2007).

In obesity, the increase in 'endocannabinoid tone' may, at least in part, be due to a decrease in circulating levels of FAAH thereby allowing for prolonged effects of AEA and 2-AG on energy metabolism in the periphery (Engeli, Bohnke et al. 2005). Studies from Chapter 4 have demonstrated that TRPV1 and FAAH mRNA is present in human and rodent skeletal muscle, however, it is not known whether mRNA content of TRPV1 and FAAH in skeletal muscle is altered in obesity.

The aims of these studies were to:

1. Evaluate glucose tolerance in TRPV1^{-/-} mice after high fat feeding.
2. Measure TRPV1 and FAAH mRNA content in skeletal muscle from lean and obese rats.

We propose that on a high fat diet (HFD), mice deficient in TRPV1 will have better tolerance to glucose than wild-type mice on the HFD, but poorer tolerance in comparison to wild-type mice on the standard chow diet. Furthermore, mRNA

content of FAAH will be decreased and that of TRPV1 increased in rodent skeletal muscle in obesity.

The design of this study was limited to meet the time requirements of the Doctor of Philosophy (PhD) candidature and only glucose tolerance was examined. Skeletal muscle tissue samples were obtained from a previous study of diet induced obesity in rodents.

6.3. MATERIALS AND METHODS

All experimental procedures in this study were approved by the University of Adelaide and the Institute of Medical and Veterinary Science Animal Ethics Committees.

6.3.1. Wild-type and TRPV1^{-/-} Mice

16 male C57BL/6J wild-type and 16 male B6.129S4-TRPV1^{tm1Jul}/J knockout mice were utilised in this study as described in Chapter 2.5.1.

6.3.2. Genotyping of TRPV1^{-/-} Mice

Liver cDNA was used for validation of TRPV1^{-/-} mice. Liver samples were obtained from the mice immediately after sacrifice. The extraction of RNA and reverse transcription to cDNA was completed as described in Chapter 2.2.1 and Chapter 2.2.2., respectively. RT-PCR was performed and PCR products were visualised as described in Chapter 2.2.3. PCR primers used are described in Chapter 2.2.5.

6.3.3. Intraperitoneal Glucose Tolerance Tests

Intraperitoneal glucose tolerance testing was performed as described in Chapter 2.5.2.

6.3.4. TRPV1 and FAAH mRNA content by Fibre-Type in Rodent Skeletal Muscle

Extensor digitorum longus (EDL) and soleus (SOL) muscle were obtained from male Wistar rats (n=12/group) aged 20 weeks as described in Chapter 2.1.3. RNA was extracted, reverse transcribed into cDNA and 'Real Time' RT-PCR was performed as described in Chapter 2.2. Primer sequences are shown in Chapter 2.2.5. BLAST searches for TRPV1 and FAAH primers confirmed homologous binding to desired mRNA of human or rodent samples for all chosen genes. TRPV1 and FAAH mRNA expression was referenced against the housekeeping gene POLR2C from QIAGEN (QT01577268; Melbourne, Australia).

6.3.5. Statistical Analysis

Analysis of mRNA content in rodent skeletal muscle was performed using 2-way ANOVA. Body weights were analysed using a maximum likelihood mixed effects model that included fixed effects for diet and time. Within-subject correlation was modelled using a first order autoregressive covariance structure over time. Post-hoc comparisons between groups at each time point were tested using simple main effects with the Holm adjustment for multiple comparisons. Intraperitoneal glucose tolerance tests were analysed using repeated measures ANOVA and area under the curve (AUC) comparisons. Statistical analyses were completed using SPSS 17 software from SPSS Inc (Chicago, IL). Significance was accepted at $P < 0.05$.

6.4. RESULTS

6.4.1. Genotyping of TRPV1^{-/-} Mice

PCR product sizes of 984 base-pairs (bp) for wild-type and 600 bp for TRPV1^{-/-} mice were observed as predicted. A representative gel photo is shown in Figure 6.1.

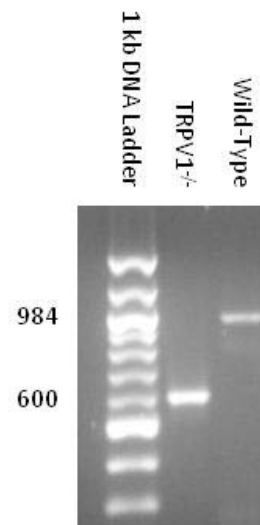
Figure 6.1

Figure 6.1: Representative sample of TRPV1 genotyping. RT-PCR products were analysed on a 2.5% agarose gel stained with ethidium bromide.

6.4.2. Wild-Type and TRPV1^{-/-} Mice Body Weight

As expected, wild-type mice on the HFD gained more weight than wild-type mice fed chow ($P < 0.01$; Figure 6.2A). Chow fed TRPV1^{-/-} mice gained more weight than chow fed wild-type mice ($P < 0.05$; Figure 6.2C). TRPV1^{-/-} mice fed a HFD gained less weight than chow fed TRPV1^{-/-} mice ($P < 0.05$; Figure 6.2B), but were of similar weight to chow fed wild-type mice (Figure 6.2D).

Figure 6.2

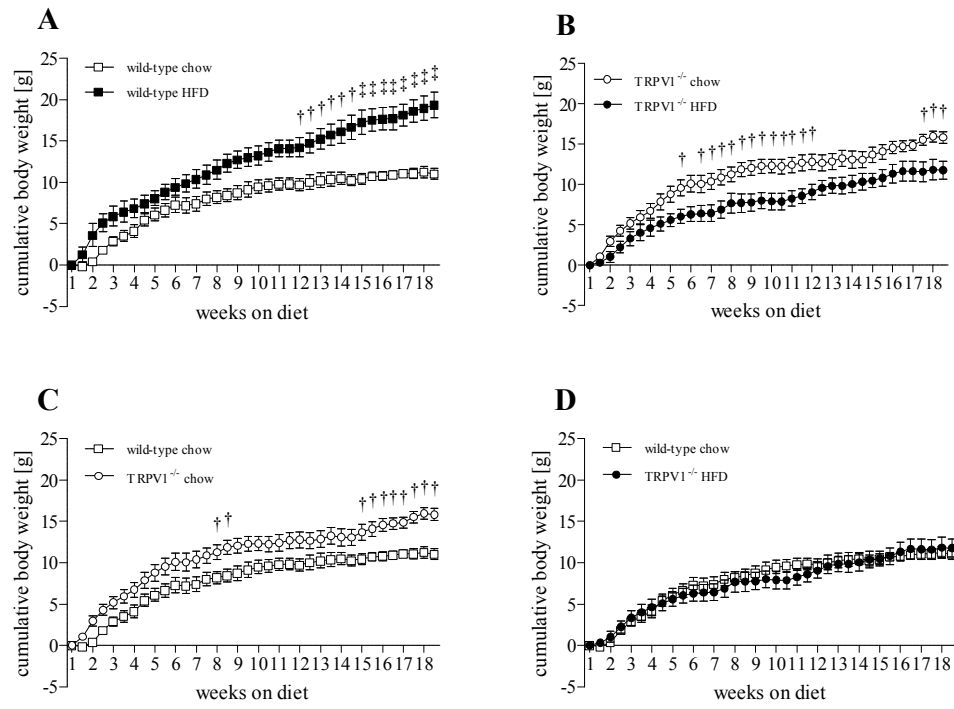


Figure 6.2: Cumulative body weight of wild-type and TRPV1^{-/-} mice during 18 weeks of diet on either a standard chow diet (chow) or a high fat diet (HFD) (n=8/group). The depicted data are from a single experiment of 32 mice. (A) wild-type chow vs. wild-type HFD, (B) TRPV1^{-/-} chow vs. TRPV1^{-/-} HFD, (C) wild-type chow vs. TRPV1^{-/-} chow, (D) wild-type chow vs. TRPV1^{-/-} HFD. Open data points (□ or ○) represent mice fed chow, closed data points (■ or ●) represent mice fed a HFD. Data are shown as means ± SEM and are expressed as cumulative weight gain. (†) P<0.05, (‡) P<0.001.

6.4.3. Basal Glucose Tolerance in Wild-Type and TRPV1^{-/-} Mice

Intraperitoneal glucose tolerance tests were performed on wild-type and TRPV1^{-/-} at baseline. There was no difference in fasting body weight prior to injection of the glucose load (Figure 6.3A). TRPV1^{-/-} mice cleared the glucose load more efficiently than wild-type mice ($P=0.023$; Figure 6.3B). AUC values were not significantly different between wild-type and TRPV1^{-/-} mice ($P>0.05$, Table 6.1).

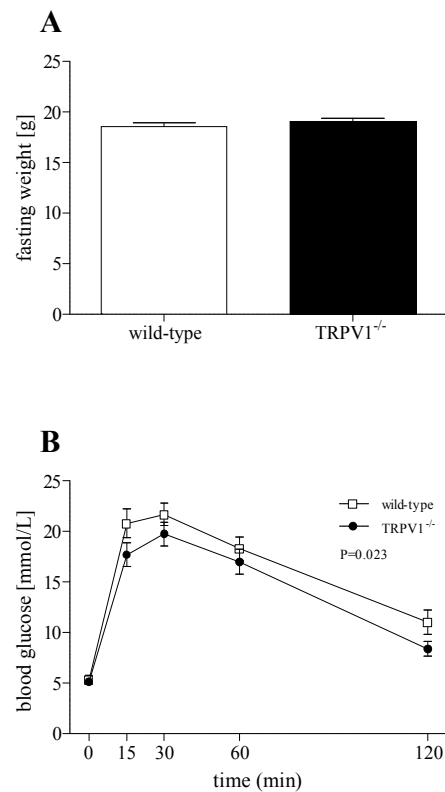
Figure 6.3

Figure 6.3: Glucose tolerance in wild-type and TRPV1^{-/-} mice at 8 weeks of age. (A) Fasting body weight of wild-type and TRPV1^{-/-} mice at 8 weeks of age and prior to study initiation (n=16/group). (B) Intraperitoneal glucose tolerance test was performed using 2 g/kg glucose after overnight fasting and blood samples were drawn from tail veins after 0, 15, 30, 60 and 120 min. Data are shown as means \pm SEM. P=0.023 wild-type vs. TRPV1^{-/-}.

Table 6.1: Basal Glucose Tolerance AUC Values (Arbitrary Units)

Strain	n	Mean \pm SEM
Wild-Type	16	1993.92 \pm 79.95
TRPV1 ^{-/-}	15	1765.35 \pm 80.68

6.4.4. Effect of High Fat Feeding on Glucose Tolerance in Wild-Type and TRPV1^{-/-}

Mice

Intraperitoneal glucose tolerance tests were performed on wild-type and TRPV1^{-/-} mice after 18 weeks of diet. Wild-type mice fed a HFD had a higher fasting body weight than chow fed wild-type mice ($P < 0.05$; Figure 6.4A). There was a trend suggesting that high fat fed TRPV1^{-/-} mice had lower fasting body weights compared to chow fed TRPV1^{-/-} mice, although this was not significant (Figure 6.4A). Wild-type and TRPV1^{-/-} mice fed a HFD displayed impaired glucose tolerance as compared to chow fed wild-type mice ($P = 0.002$; Figure 6.4B and $P = 0.043$; Figure 6.4E). There was no difference between TRPV1^{-/-} mice fed a HFD versus a chow diet (Figure 6.4C) or between chow fed wild-type and TRPV1^{-/-} mice (Figure 6.4D). There was no statistical difference in AUC for any combination of strain or diet ($P > 0.05$, Table 6.2).

Figure 6.4

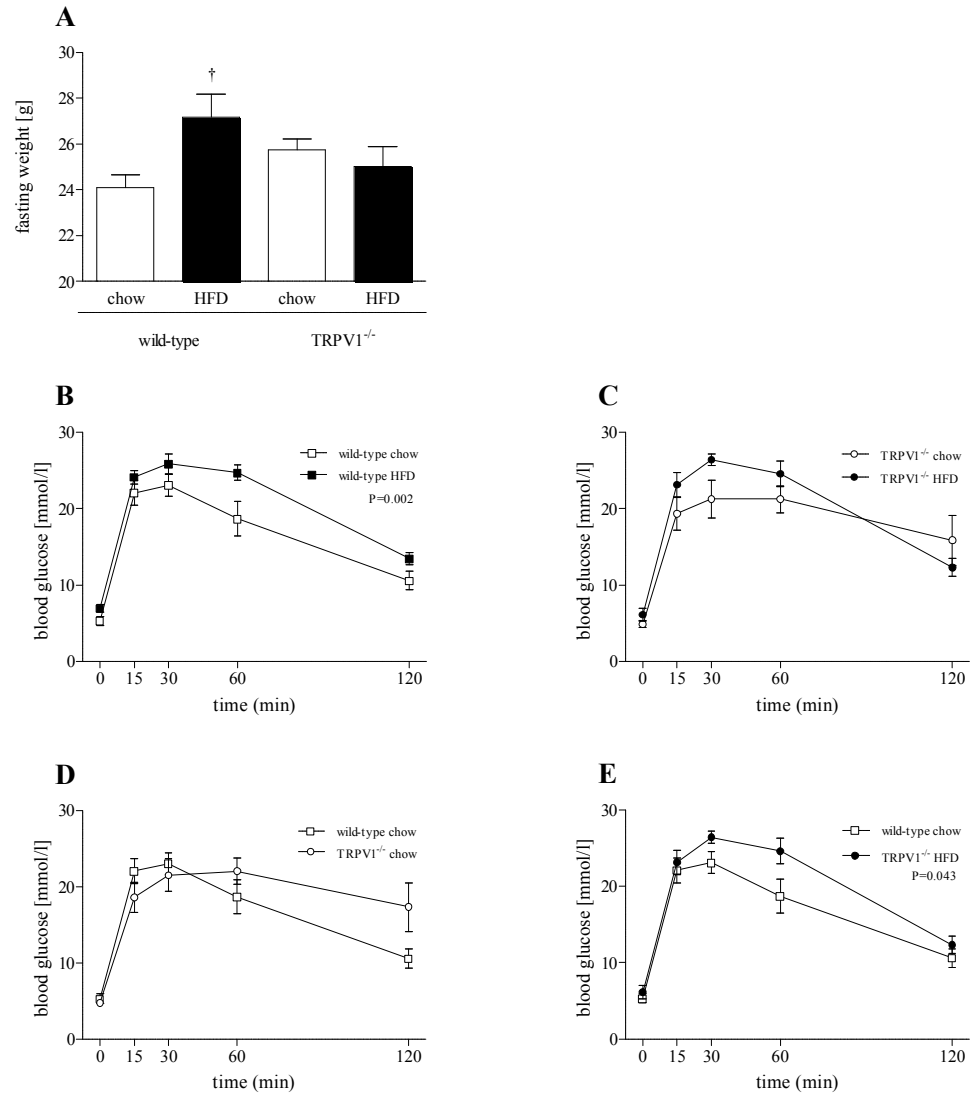


Figure 6.4: Effect of high fat feeding on glucose tolerance in wild-type and TRPV1^{-/-} mice after 18 weeks. (A) Fasting body weight of wild-type and TRPV1^{-/-} mice after 18 weeks of diet (n=8/group). (B-E) Intraperitoneal glucose tolerance test was performed using 2 g/kg glucose after overnight fasting and blood samples were drawn from tail veins after 0, 15, 30, 60 and 120 min. (B) effect of diet on wild-type mice, (C) effect of diet on TRPV1^{-/-} mice, (D) strain effect on mice fed a standard chow diet (chow), (E) TRPV1^{-/-} mice on a high fat diet (HFD) vs. chow fed wild-type mice. Data are shown as means ± SEM. (A) (†) P<0.05, chow vs. HFD.

Table 6.2: AUC Values of the Effect of High Fat Feeding on Glucose Tolerance in Wild-Type and TRPV1^{-/-} Mice (Arbitrary Units)

Strain	Diet	n	Mean ± SEM
Wild-Type	Chow	8	2201.72 ± 126.55
	HFD	8	2513.06 ± 69.38
TRPV1 ^{-/-}	Chow	8	2314.03 ± 190.12
	HFD	7	2084 ± 318.00

6.4.5. TRPV1 and FAAH mRNA content by Fibre-Type in Rodent Skeletal Muscle

FAAH mRNA content was not affected by diet but was significantly lower in SOL as compared to EDL in chow fed rats ($P > 0.05$; Figure 6.5A). Similarly, TRPV1 mRNA content was not altered by diet but was higher in SOL than EDL in both chow and HFD rats ($P < 0.01$; Figure 6.5B).

Figure 6.5

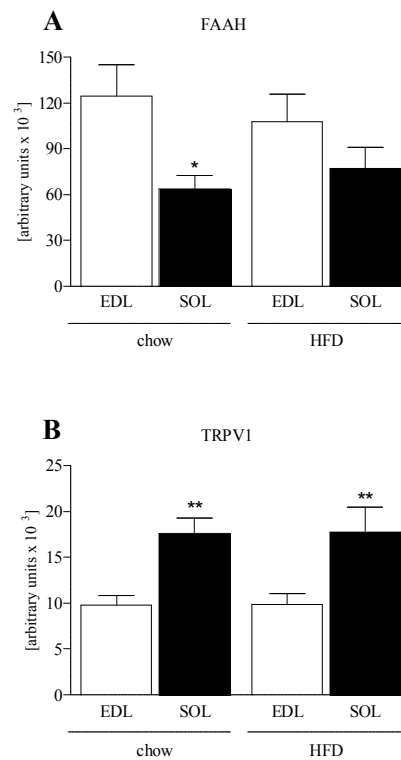


Figure 6.5: Analysis of FAAH (A) and TRPV1 (B) mRNA in skeletal muscle from male Wistar rats (n=12/group). Open bars (□) represent extensor digitorum longus (EDL) muscle and closed bars (■) represent soleus (SOL) muscle. Data are shown as means ± SEM and are expressed as ratio of target to housekeeping gene. (**) P<0.01, (*) P<0.05, EDL vs. SOL. FAAH, fatty acid amide hydrolase and TRPV1, transient receptor potential vanilloid type 1.

6.5. DISCUSSION

There is emerging evidence suggesting that TRPV1 has a role in metabolic diseases such as obesity and diabetes (Suri and Szallasi 2008). The data presented in the current study are consistent with previous findings that 8 week old, chow fed TRPV1^{-/-} mice display better glucose tolerance than wild-type mice (Razavi, Chan et al. 2006) and that TRPV1^{-/-} mice are resistant to diet induced obesity (Motter and Ahern 2008). We extended this research to show that the high fat fed TRPV1^{-/-} mice are not, however, protected against impaired glucose tolerance.

There are a number of possible reasons for the differential weight gain between wild-type and TRPV1^{-/-} mice in response to high fat feeding. Previous studies have reported that TRPV1^{-/-} mice have a higher resting body temperature than wild-type mice suggesting that mice deficient in TRPV1 have increased energy expenditure (Motter and Ahern 2008). The precise molecular mechanism for this effect is unclear. One possibility is increased expression of the uncoupling proteins which has been observed in capsiate fed mice (Masuda, Haramizu et al. 2003). It is unlikely this also occurs in TRPV1^{-/-} mice. Variations in energy intake may also account for the observed differential weight gain. Previous studies have demonstrated that oleoylethanolamide, an endocannabinoid that is a TRPV1 agonist, reduces acute (30 min) food intake in wild-type but not TRPV1^{-/-} mice suggesting a role for TRPV1 in appetite regulation (Wang, Miyares et al. 2005). It has also been reported that

TRPV1^{-/-} mice and wild-type mice exhibit comparable levels of energy intake and intestinal lipid absorption (Motter and Ahern 2008). It is therefore possible that TRPV1^{-/-} mice do not compensate for increased energy expenditure by increasing food intake, presenting a possible mechanism for resistance to diet induced obesity.

An unexpected finding of this study was that on the chow diet, TRPV1^{-/-} mice gained more weight than wild-type mice. This is unlikely to be accounted for by food intake as a previous study reported similar food intake between wild-type and TRPV1^{-/-} mice (Motter and Ahern 2008). Therefore, food intake was not measured in the current study. An alternate explanation may relate to intermediary metabolism and energy efficiency. There is some evidence suggesting that TRPV1 may act as an inhibitor of insulin secretion from pancreatic beta cells (Akiba, Kato et al. 2004). At baseline, TRPV1^{-/-} mice had better glucose tolerance than wild-type mice suggesting that either insulin secretion and/or sensitivity are increased in TRPV1^{-/-} mice. Insulin levels were not measured in the current study, therefore, it is unknown if insulin activity was elevated in TRPV1^{-/-} mice. To better understand the role of insulin in TRPV1^{-/-} mice, future studies should measure insulin levels and energy intake and expenditure at regular intervals during chow and high fat feeding.

At 26 weeks age, high fat fed TRPV1^{-/-} mice had impaired glucose tolerance relative to chow fed wild-type mice. It is unclear whether insulin secretion in

TRPV1^{-/-} mice is impaired in response to high fat feeding or if other factors such as the onset of insulin resistance, contributed to the impairment of glucose tolerance in these mice. Further analysis and investigation is required to resolve this.

The studies described in Chapter 5 demonstrated that TRPV1 mediates glucose uptake in L6 skeletal muscle cell culture, at least after 24 h. It is not known whether this also occurs in skeletal muscle from humans and if there is any fibre-type dependence. In skeletal muscle from both lean and obese rodents, TRPV1 mRNA content is higher in oxidative SOL than glycolytic EDL muscle. Oxidative skeletal muscles have a greater degree of insulin sensitivity and responsiveness than glycolytic muscles (Song, Ryder et al. 1999). Obesity and T2DM are associated with a decrease in the proportion of Type I muscle fibres (Hickey, Carey et al. 1995; Helge, Fraser et al. 1999). Furthermore, primary skeletal muscle cultures from obese humans are insulin resistant and also resistant to the effects of endocannabinoids to increase glucose uptake (Chapter 3). Despite the absence of obesity, high fat feeding in TRPV1^{-/-} mice may still be associated with increased 'endocannabinoid tone'. Insulin resistance may be compounded by the consequence of impaired signalling at other cannabinoid receptors such as CB₁ (Cota, Sandoval et al. 2009).

Endocannabinoids are metabolised by FAAH (Giang and Cravatt 1997). In skeletal muscle from obese rodents, FAAH mRNA content is similar in SOL and EDL and does

not appear to be decreased relative to skeletal muscle from lean rodents. It is unclear what occurs with FAAH expression in high fat fed TRPV1^{-/-} mice. If under conditions of high fat feeding FAAH activity is decreased in SOL skeletal muscle, endocannabinoid activity will be increased possibly leading to insulin resistance. This remains to be determined.

In summary, these data suggest that whilst TRPV1^{-/-} mice are protected from diet induced obesity, they are not protected from impairment of glucose tolerance. This has implications for the potential therapeutic use of TRPV1 antagonists. Further studies are required to determine the mechanisms behind this effect and in particular, any role of the endocannabinoid system.

6.6. FUTURE DIRECTIONS

The data presented in the current study highlight the need for further characterisation of the TRPV1^{-/-} phenotype. Whilst it has been previously reported that TRPV1^{-/-} mice are resistant to diet induced obesity (Motter and Ahern 2008), the observation that TRPV1^{-/-} mice on standard chow weigh more than those on the HFD suggests that this knockout model contains a more complex physiology than originally described. Future studies should measure food intake and energy expenditure as well as insulin levels and tolerance. It would also be of interest to investigate skeletal muscle-specific TRPV1^{-/-} mice to determine whether changes in glucose tolerance and weight gain may be attributed solely to changes in muscle physiology. These studies will help to ascertain whether defects in insulin secretion or resistance are the cause of this impairment in glucose tolerance in high fat fed TRPV1^{-/-} mice. Furthermore, to determine the role of the endocannabinoid system on glucose tolerance in TRPV1^{-/-} mice, circulating levels of endocannabinoids, particularly 2-AG should be assessed and levels of cannabinoid receptor expression in key metabolic tissues, such as pancreas, liver, adipose tissue and skeletal muscle should be measured.

CHAPTER 7

CONCLUSIONS

7.1. SUMMARY OF FINDINGS

The studies completed within this thesis provide new data on the role of the endocannabinoid system on skeletal muscle glucose utilisation during obesity. Inhibition of cannabinoid receptor type 1 (CB₁) with the selective inverse agonist SR141716 decreases body weight and improves cardiometabolic risk factors in obese humans (Pi-Sunyer, Aronne et al. 2006). In diet induced obese rodents, administration of SR141716 decreased triglyceride content and increased glucose uptake and insulin sensitivity (Herling, Kilp et al. 2008; Nogueiras, Veyrat-Durebex et al. 2008; Cota, Sandoval et al. 2009). Furthermore, in genetically obese *Lep^{ob}/Lep^{ob}* mice, glucose uptake and oxygen consumption in isolated soleus muscle was increased in response to SR141716 (Liu, Connoley et al. 2005). Despite these observations, the effect of SR141716 on glucose uptake in human skeletal muscle was unknown.

Therefore, the studies described in Chapter 3 investigated the effects of SR141716 and AEA on basal glucose uptake and mRNA and protein content of key genes in nutrient oxidation in primary cultures of human skeletal muscle myotubes

from lean and obese individuals. Primary cultures reflect the muscle physiology of the donor, allowing for *in vitro* study of different metabolic characteristics that exist *in vivo* (Gaster, Petersen et al. 2002; Gaster, Rustan et al. 2004; McIntyre, Halse et al. 2004).

Basal glucose uptake in cultures from obese individuals was increased in response to an acute (30 min) exposure to 100 nM SR141716. The acute effects of SR141716 did not appear to be mediated by either AMP-activated protein kinase (AMPK) or MEK1/2 pathways. However, chronic (24 h) exposure to 100 nM SR141716 increased AMPK α 1 mRNA content. This suggested that CB₁-mediated effects may occur via more than one pathway and are time-dependant. The observation that the effects of SR141716 occurred only in myotubes derived from obese individuals suggested that the endocannabinoid system was more active in these cultures than those from lean individuals. This is consistent with the previously reported data that the endocannabinoid system is overactive in obesity (Engeli, Bohnke et al. 2005; Cote, Matias et al. 2007).

In contrast to SR141716, AEA decreased basal glucose uptake in obese- but not lean-derived myotubes, an effect which was not reversed by SR141716. We speculated this was either because SR141716 was unable to compete for CB₁ under these conditions, or that AEA acts via another receptor.

We tested this hypothesis by examining receptor expression of CB₁, cannabinoid receptor type 2 (CB₂) and transient receptor potential channel-vanilloid sub family member 1 (TRPV1) in human and rodent skeletal muscle. We observed, for the first time, that CB₁, CB₂ and TRPV1 are expressed in skeletal muscle (Chapter 4) (Cavuoto, McAinch et al. 2007). The expression of receptors for AEA other than CB₁ led to the hypothesis that the effects of the endocannabinoids on skeletal muscle glucose uptake may occur via multiple receptors.

We next examined the relative roles of the endocannabinoid receptors on basal and insulin-stimulated skeletal muscle glucose uptake (Chapter 5). Firstly, we established a time course effect as we previously showed that the effects of AEA on glucose utilisation may be time dependant (Chapter 3). AEA (5 µM and 15 µM) and 2-AG (5 µM) increased insulin-stimulated glucose uptake after 24 h, however the effects appeared to be biphasic. In addition, AEA and 2-AG had no effect at 30 mins. This suggested, along with the glucose uptake data reported from Chapter 3, that the endocannabinoids have a beneficial effect on glucose uptake under normal physiological conditions, but are deleterious during conditions of physiological stress, such as obesity.

Next, we wanted to investigate which receptors were mediating the effect of the endocannabinoids on insulin-stimulated glucose uptake. Inhibitors of CB₁

(SR141716), CB₂ (SR144528) and TRPV1 (SB366791) were utilised. 2-AG was used as a positive control as its expression is up-regulated in the periphery in obesity (Cote, Matias et al. 2007). The increase in insulin-stimulated glucose uptake by 5 µM 2-AG was inhibited by SR141716 (100 nM and 500 nM), SR144528 (100 nM and 1 µM) and SB366791 (500 nM and 5 µM) alone. Noticeably, the effects of SB366791 were observed only in the presence of 2-AG, suggesting that either the autocrine effects of endocannabinoids present in culture occurred at thresholds that differ between the receptors or that TRPV1 did not mediate effects on glucose metabolism. When combined, SR141716, SR144528 and SB366791 had no additive effect on glucose uptake. This suggests a possible common intracellular pathway between the receptors; however, it is also possible that these compounds competed for the same receptor as each compound used may partially bind to another receptor. At any rate, these data suggested that 2-AG-mediated glucose uptake in skeletal muscle may occur via CB₁, CB₂ or TRPV1.

TRPV1 may co-localise with fatty acid amide hydrolase (FAAH), the degradative enzyme for AEA, in amacrine cells (Zimov and Yazulla 2007). Data from rodent skeletal muscle (Chapter 6) showed that TRPV1 and FAAH mRNA is expressed in a fibre-type dependant manner. In skeletal muscle from rodents fed a high fat diet (HFD), mRNA content of FAAH is not fibre-type specific suggesting that FAAH expression is dysregulated in obesity. TRPV1 mRNA content remained unchanged.

This data, the glucose uptake data from Chapter 5 and the emerging data that TRPV1 has a role in obesity and diabetes (Suri and Szallasi 2008) suggested that TRPV1 may have a role in glucose utilisation in obesity.

To test this hypothesis *in vivo*, we next examined glucose tolerance under chow and high fat conditions in mice deficient in TRPV1 (Chapter 6). TRPV1 may be desensitised with a potent agonist such as capsaicin resulting in beneficial effects on satiety and energy intake (Westerterp-Plantenga, Smeets et al. 2005). Furthermore, TRPV1^{-/-} mice are resistant to diet induced obesity, suggesting that TRPV1 is important in weight regulation (Motter and Ahern 2008). At baseline, TRPV1^{-/-} mice clear a glucose load more efficiently than wild-type mice (Razavi, Chan et al. 2006). The studies in Chapter 6 described, for the first time, that TRPV1^{-/-} mice, while resistant to diet induced obesity, are not protected against impaired glucose tolerance in comparison to wild-type mice. An unexpected observation was that TRPV1^{-/-} mice on the chow diet gained more weight than wild-type mice on the chow diet. The reason for this was unclear.

A visual representation of the proposed mechanisms behind endocannabinoid-mediated glucose uptake in skeletal muscle under lean (Figure 7.1), obese (Figure 7.2) and TRPV1^{-/-} (Figure 7.3) conditions is shown below.

Figure 7.1

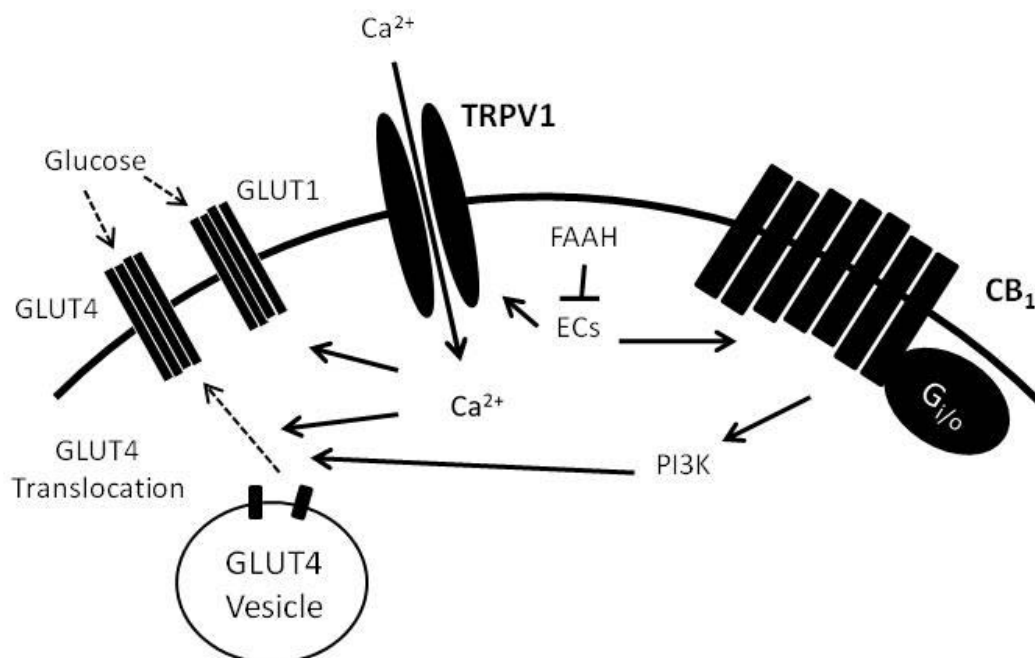


Figure 7.1: Proposed mechanism of EC signalling to promote glucose uptake in skeletal muscle under normal conditions. Under normal, insulin sensitive conditions, the effects of ECs are brief and may mediate basal and insulin-stimulated glucose uptake in response to cellular stress via CB₁ (Esposito, Proto et al. 2008) and TRPV1 (Chapter 5). It has been suggested that TRPV1 may co-localise with FAAH as a mechanism for limiting intracellular EC effects (Zimov and Yazulla 2007), although it is unclear if this mechanism exists in skeletal muscle. ECs, endocannabinoids; CB₁, cannabinoid receptor type 1; FAAH, fatty acid amide hydrolase; PI3K, phosphoinositide 3-kinase; TRPV1, transient receptor potential channel-vanilloid sub-family member 1.

Figure 7.2

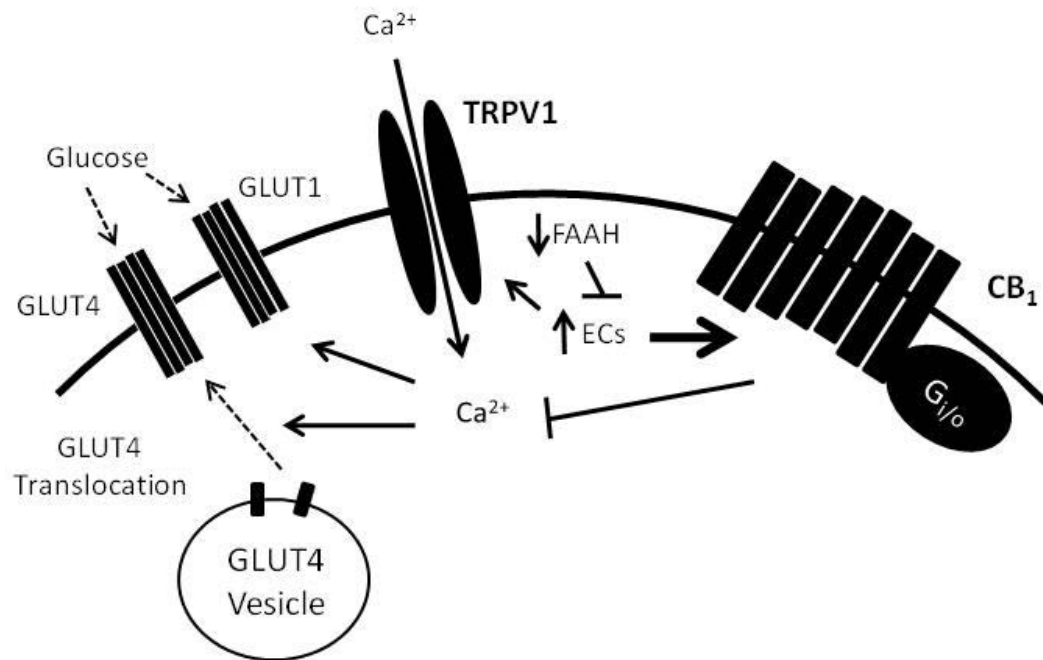


Figure 7.2: Proposed mechanism of EC signalling to promote glucose uptake in skeletal muscle under obese conditions. In obesity, FAAH is decreased contributing to increased 'EC tone' (Engeli, Bohnke et al. 2005). In skeletal muscle from obese animals, CB₁ mRNA content is increased (Pagotto, Marsicano et al. 2006), but TRPV1 mRNA content is similar to that of lean animals (Chapter 6). It has recently been demonstrated that ECs are one of the many factors released by adipocytes that may induce skeletal muscle insulin resistance, an effect reversible by the CB₁-specific inverse agonist SR141716 (Eckardt, Sell et al. 2008). Moreover, high fat fed mice deficient in CB₁ do not develop resistance to insulin (Ravinet Trillou, Delgorge et al. 2004). We speculate that the EC mechanism exists to facilitate glucose uptake to a regional tissue (in this case skeletal muscle) under acute stress, but that when the EC signalling mechanism is chronically activated insulin resistance occurs. ECs, endocannabinoids; CB₁, cannabinoid receptor type 1; FAAH, fatty acid amide hydrolase; PI3K, phosphoinositide 3-kinase; TRPV1, transient receptor potential channel-vanilloid sub-family member 1.

Figure 7.3

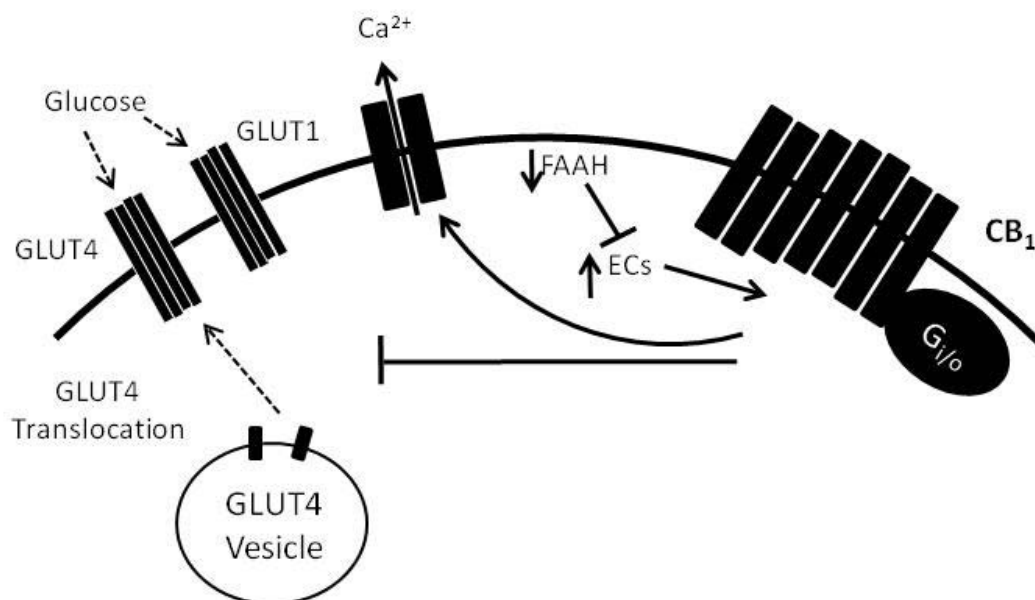


Figure 7.3: Proposed mechanism of EC signalling to promote glucose uptake in skeletal muscle of TRPV1^{-/-} mice. In chow fed TRPV1^{-/-} mice, glucose tolerance is better than that of chow fed wild-type mice, possibly due to increased secretion of insulin (Razavi, Chan et al. 2006), and the fact that TRPV1 is not required for the transient effects of CB₁ to be mediated. We hypothesise that high fat fed mice are resistant to diet induced obesity (Motter and Ahern 2008) but may have increased 'EC tone' due to the combined effects of the high fat diet on EC production and a decrease in FAAH. Therefore, despite the absence of obesity, glucose tolerance deteriorates as demonstrated in Chapter 6. ECs, endocannabinoids; CB₁, cannabinoid receptor type 1; FAAH, fatty acid amide hydrolase; TRPV1, transient receptor potential channel-vanilloid sub-family member 1.

7.2. IMPLICATIONS OF FINDINGS

CB₁ is a therapeutic target for the reduction of body weight in obese individuals. Human trials have reported that the selective CB₁ inverse agonist SR141716 reduces body weight and improves cardiometabolic risk factors (Pi-Sunyer, Aronne et al. 2006). In diet induced obese rats, inhibition of CB₁ in the periphery is beneficial to intermediary metabolism independent of decreased energy intake (Nogueiras, Veyrat-Durebex et al. 2008). Despite this, the central side effects of SR141716 have precluded its use as a therapeutic agent. However, the development of cannabinoid receptor blockers that do not cross the blood brain barrier ensures that this area of investigation will be of increasing interest and applicability to the international research community.

The studies described in this thesis suggest that endocannabinoids may promote skeletal muscle glucose uptake in a lean (L6) culture model but may impair skeletal muscle glucose uptake in a primary culture model derived from obese patients. Whilst the deleterious effects of endocannabinoids on energy metabolism in obesity are well described, the potential beneficial effects of endocannabinoids on energy metabolism under lean conditions are only just emerging and present a novel mechanism in understanding the physiology of the endocannabinoid system.

The expression of CB₂ and TRPV1 in addition to CB₁ in skeletal muscle suggested that endocannabinoid signalling in this tissue may occur via more than one

receptor. Our observation that 2-AG-mediated insulin-stimulated glucose uptake may occur separately via CB₁, CB₂ and TRPV1, presents a novel mechanism through which the endocannabinoid system may regulate glucose utilisation, particularly in obesity.

Furthermore, the emerging role of TRPV1 in obesity and T2DM (Suri and Szallasi 2008) suggests that TRPV1 may be a therapeutic target in the treatment of metabolic diseases. However, the observation that TRPV1^{-/-} mice are not protected against impaired glucose tolerance after high fat feeding, in concert with reduced body weight suggests that TRPV1 antagonists may not be beneficial in the treatment of obesity where insulin resistance and a risk of T2DM exists.

The studies presented in this thesis offer new perspectives on the endocannabinoid system and TRPV1 signalling in obesity. Further studies are required to evaluate the relevance of these findings to human metabolic diseases. A better understanding of the metabolic effects of the endocannabinoids and their receptors in the disease state will allow for more reliable therapeutic solutions to be designed and implemented.

7.3. LIMITATIONS OF THE STUDIES WITHIN THIS THESIS

The glucose uptake, 'Real Time' RT-PCR and western blot experiments completed in Chapter 3 could have utilised greater subject numbers to reduce the possibility of Type II errors.

The determination of the expression of receptors for endocannabinoids in Chapter 4 could have also measured PCR abundance (using 'Real-Time' PCR) as well as protein content.

An increase in the repeat number of the insulin-stimulated glucose uptake experiments completed in Chapter 5 may have reduced the possibility of Type II errors.

The body weight and glucose tolerance test data from the wild-type and TRPV1^{-/-} mice before and after 18 weeks diet (Chapter 6) would have been easier to interpret had food intake and insulin levels been measured for the duration of the study.

7.4. FUTURE STUDIES

Despite a rapid increase in knowledge relating to the physiology of the endocannabinoid system, many questions remain unanswered:

- Studies are required, particularly in humans, to determine the effects of endocannabinoids and endocannabinoid receptor blockers on basal metabolic rate and diet-induced thermogenesis, during energy balance, and in response to caloric restriction and weight loss.
- The mechanism by which CB₁ blockade increases glucose uptake in skeletal muscle from obese rodents or humans requires elucidation.
- The extent to which the effects of cannabinoid receptor antagonists/inverse agonists are direct, or mediated via changes in plasma adiponectin levels, adiponectin responsiveness, or leptin signalling also remains to be determined.
- The relative roles of the CB₁, CB₂ and TRPV1 receptors on skeletal muscle energy utilisation require clarification.
- Further characterisation of the TRPV1^{-/-} mouse is required to better understand the metabolic profile of this phenotype.
- A better understanding of TRPV1 effects on glucose utilisation, particularly in the liver during obesity is required.

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