The University of Nottingham

The Effects of Green Tea Derived Catechins upon Adipocyte Metabolism

Anisha Mehra, BSc (Hons)

Thesis submitted to the University of Nottingham

for the degree of Doctor of Philosophy

January 2010

Abstract

Tea, from the plant *Camellia Sinensis*, is after water, the most consumed drink in the world. Green tea, produced by steaming freshly harvested leaves to prevent fermentation, is high in polyphenols known as catechins or flavanols. The major flavanols found in green tea include (-) epigallocatechin-3-gallate (EGCG) and (-) epicatechin. Literature reports suggest that green tea flavanols have the potential to exert anti-obesity effects by modulating weight gain and other factors such as lipogenesis, β-oxidation and adipokine release. Key features of obesity and associated insulin resistance are adipokine dysregulation, decreased sensitivity of adipocytes to insulin and subsequently, changes in glucose uptake by cells.

The aims of this thesis were firstly to establish an efficient system of differentiated adipocytes and secondly to use this to investigate the effects of the flavanols EGCG and epicatechin on physiological outcomes such as adipokine release and glucose uptake. Thirdly, the question of whether these processes might be regulated by the ERK1/2 pathway and what may be happening upstream of this was explored. Finally, the effect of flavanol treatment on adipocyte gene expression of genes known to be modulated in obesity was investigated.

Using the well established cell culture model of 3T3-L1 adipocytes, the studies from this thesis show that EGCG and epicatechin are able to modulate the release of the adipokines adiponectin and resistin, dependent on the media glucose concentration. This modulation may be mediated by the ERK1/2 pathway, since flavanol treatment increased ERK1/2 phosphorylation. Uptake of glucose was not altered by any time or concentration of EGCG or epicatechin, and there were no significant changes in adipocyte gene expression following EGCG or epicatechin treatment.

A thorough investigation of adipokine release, ERK signalling, glucose uptake and gene expression, under the influence of flavanol treatment, showed that although molecular changes occurred in the 3T3-L1 system, these did not

translate into functional readouts. Therefore, it appears unlikely that these have major direct effects on adipocyte function.

Publications and Presentations:

Mehra Anisha, Macdonald Ian (2007) Int J Obes **31**:S46-77 - Presented as a poster at European Congress on Obesity (Budapest 2007)

Mehra A, Macdonald IA and Pillay TS (2007). "Variability in 3T3-L1 adipocyte differentiation depending on cell culture dish." Anal Biochem **362**(2): 281-3.

Acknowledgements

Firstly, I would like to thank my supervisors Prof Ian Macdonald and Dr Andrew Bennett. I am grateful for the opportunity that they gave me and for their guidance and support throughout. I also gratefully acknowledge the funding contributions from the BBRSC.

Thank you to Profs Steve Alexander and Dave Kendall for help and advice on radioimmunoassays. Special thanks to everyone in the FRAME lab, especially Elke Gottshalg, Monika Owen, Nicola Bourne, Roya Babaei-Jadidi and Deb Briggs for their invaluable help, advice and support.

I would like to express my gratitude to all of the friends that I have made during my PhD, without whom I would have been lost. Namely Sarah, Veronica, Gillian, Amanda, Dave, Kirsten, Annie, Elke and Marleen. Particular thanks also to Cath, Sham and Luke. Thank you all for supporting me through the tough times and being there to celebrate the good times.

Finally, heartfelt thanks to my family. Rohan for unfailing optimism, inspiration and enthusiasm and Papa for continued faith in me and an endless supply of good advice. Last but not least, Mum, who is always with me, and without whom I would not have come this far.

Contents

List of Figures

List of Tables

List of Abbreviations

ADD1	Adipocyte differentiation determination factor 1
aP2	Adipocyte specific fatty acid binding protein
BAT	Brown adipose tissue
C/EBP	CCAAT/enhancer binding protein
Cat	(+)-Catechin
CREB	cAMP response element binding protein
DEX	Dexamethasone
EC	(-)-Epicatechin
ECG	(-)-Epicatechin gallate
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin-3-gallate
Epi	(-)-Epicatechin
ERK1/2	Extracellular signal-regulated protein kinase 1/2
FABP4	Adipocyte specific fatty acid binding protein
FAS	Fatty acid synthase
FCS	Foetal calf serum
FIZZ	Found in inflammatory zone
HDL	High density lipoprotein
HUVEC	Human umbilical vein endothelial cells
IBMX	Isobutylmethylxanthine
ICAM	Intercellular adhesion molecule
IL-6	Interleukin-6
LDL	Low density lipoprotein
LPL	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MEK	MAPK extracellular signal-regulated kinase kinase
NF-κB	Nuclear factor-κB
PAI-I	Plasminogen activator inhibitor-I
PAK	p 21 activated kinase
PEPCK	Phosphoenol pyruvate carboxykinase
PKA	Protein kinase A
PKC	Protein kinase C
PPARγ	Peroxisome proliferator activated receptor-γ
PPRE	Peroxisome proliferator response element
RELMs	Resistin like molecules
SAA	Serum amyloid A
SREBP	Sterol regulatory element binding protein-1c
TNFα	Tumour necrosis factor-α
TZD	Thiazolidinedione
VCAM	Vascular cell adhesion molecule
WAT	White adipose tissue

Introduction

1 Introduction

1.1 Adipose tissue

Adipose tissue is our main energy storage organ; it has the ability to retain fat following food intake, and release fatty acids and glycerol into the blood during starvation. It is specialised connective tissue, a loose association of adipocytes held in collagen fibres and also containing leukocytes, macrophages and preadipocytes. Humans have two types of adipose tissue, brown adipose tissue (BAT) and white adipose tissue (WAT). WAT is the most predominant, and in non-obese adult humans, makes up 10-25% of total body weight. It has three major functions: to provide thermal insulation, the major role of the subcutaneous layer; mechanical cushioning by surrounding the internal organs; and finally as an efficient storage site of excess energy. 60-80% of white adipose tissue is made up of lipid, 90-95% of which is triglyceride, the remainder comprising of free fatty acids, diglyceride, cholesterol, phospholipid and minute quantities of cholesterol ester and monoglyceride are also present. Water (5-30%) and protein (2-3%) make up the remaining weight of white adipose tissue.

BAT is scarcer and the general contention was that depots decrease significantly in size as humans mature, however recent evidence now suggests that adult humans have depots of BAT in the supraclavicular and neck regions, with some other additional locations (Nedergaard *et al.* 2007). BAT derives its colour from rich vascularisation and its densely packed mitochondria. In hibernating animals and neonates, brown adipose tissue is important for

regulating body temperature via non-shivering thermogenesis. During maturation of non-hibernating animals, including humans, BAT is metabolically less active, however activity can be induced by cold exposure. The lipids stored in brown adipose tissue release their energy directly as heat. (Tong and Hotamisligil 2001)

A new role has emerged for both WAT and BAT as endocrine organs, secreting a range of bioactive polypeptides, known as adipokines.

1.1.1 Adipose Tissue Development

Adipocytes originate from fibroblast-like precursors that commit to preadipocytes and either stay dormant or, proceed to become differentiated adipocytes (Ntambi and Young-Cheul 2000). During terminal differentiation, the fibroblast-like preadipocytes undergo a series of morphological and biochemical changes to eventually accumulate lipid droplets (Tong and Hotamisligil 2001). The size of adipose tissue is a function of adipocyte number and size. An increase in adipose tissue can occur by either hyperplastic growth (increased adipocyte number) or hypertrophic growth (increased adipocyte size), caused by this lipid accumulation.

Adipose tissue differs from other tissues in the body in that it occurs in multiple, dispersed sites around the body, mostly in areas rich in loose connective tissues. Differentiation of preadipocytes to mature adipocytes occurs in clusters, possibly suggesting the expression of a recruitment factor by mature adipocytes (Rosen and Spiegelman 2000). The absence of a clear

molecular marker for adipogenesis, along with the diffuse nature of adipose tissue has made it difficult to isolate and study preadipocytes. The earliest event associated with adipogenesis is a proliferating network of capillaries in an otherwise undistinguished region of subcutaneous tissue. This shows that there is an important link between adipogenesis and angiogenesis, however it is still unclear how they induce each other. Foetal adipocytes and their associated endothelial cells express many of the same cell surface markers, which implies a common line of descent (Rosen and Spiegelman 2000).

The two models that have been frequently used to study adipose tissue are adipocytes differentiated either from immortalised preadipocyte cell lines in culture or obtained from stromal-vascular cells in adipose tissue (Tong and Hotamisligil 2001). The establishment of these *in vitro* cellular models for adipogenesis has greatly advanced the understanding of the molecular basis of differentiation. The most extensively characterised preadipocyte lines are the 3T3-L1 and 3T3-F442A lines, isolated from non-clonal murine Swiss 3T3 cells by Green, Meuth and Kehinde (Green and Meuth 1974; Green and Kehinde 1975). These cells are morphologically indistinguishable from fibroblasts but are already committed to the adipocyte lineage. When treated with pro-differentiative agents (cyclic adenosine monophosphate (cAMP), insulin and glucocorticoids) they undergo differentiation over a 4-6 day period to form mature fat cells. This is thought to represent a valid model of preadipocyte differentiation *in vivo*, as indicated by transplantation studies. When 3T3-F442A preadipocytes were injected subcutaneously into Balb-C athymic mice, normal fat pads developed at the site of injection within 5 weeks (Green and Kehinde 1979). Additionally, evidence shows that the fully differentiated

adipocytes mimic the metabolism of adipocytes isolated from adipose tissue. Preadipocytes in culture also acquire the proteins necessary for lipolysis of triacylglycerol, uptake and intracellular translocation of fatty acids, and regulation of these processes by lipogenic and lipolytic hormones. Finally, detailed electro-micrographic studies have verified that mature 3T3-L1 adipocytes possess the ultrastructural features of adipocytes *in vivo*, such as the occurrence of constellations composed of large triacylglycerol droplets bordered by the endoplasmic reticulum and closely associated with microperoxisomes and mitochondria. These constellations are thought to serve as foci for the biosynthesis, accumulation and metabolism of adipocyte lipid (Novikoff *et al.* 1980).

There are, however, several potential drawbacks to the exclusive use of established preadipocytic cell lines in studies of adipogenesis, including their aneuploid status, which may influence their competence to undergo differentiation, and the fact that they exist out of the context of their normal extracellular matrix and supporting structures. Another important limitation is that they do not allow the assessment of depot-specific differences in fat cell behaviour. *In vivo*, white adipose tissue is located in a variety of locations, including omental, retroperitoneal, and subcutaneous depots. Preadipocytes isolated from different areas have different adipogenic potential. Additionally, the metabolic behaviour of mature fat cells differs from depot to depot.

1.1.2 Regulation of Adipogenesis

Although the signals which initiate *in vivo* adipogenesis are still unknown, a combination of hormones such as insulin, glucocorticoids and cAMP-generating agents have been demonstrated to effectively promote the terminal differentiation of cultured preadipocyte cells. The role of insulin is to increase the percentage of cells that differentiate and increase the amount of lipid accumulation in each fat cell (Rosen and Spiegelman 2000). Insulin affects differentiation by cross-activating the IGF-1 receptor. Activation of the IGF-1 receptor is essential for preadipocytes to acquire their adipocyte morphology and accumulate lipid droplets (Smith *et al.* 1988). This is paralleled with the increase in activity of glycerol-3-P dehydrogenase, a sensitive indicator of adipocyte development (Wise and Green 1979). IGF-1 and insulin also activate several distinct downstream signal transduction pathways including ras and protein kinase B, which may mediate their adipogenic effects (Rosen and Spiegelman 2000). Glucocorticoids, usually in the form of dexamethasone, are used to activate the glucocorticoid receptor. The transcriptional targets involved are unclear, but induction of CCAAT/enhancer binding protein-δ (C/EBPδ) may be a possible mechanism. Addition of methylisobutylxanthine (IBMX) to cultured preadipocytic cell lines has been shown to enhance differentiation (Rosen and Spiegelman 2000). IBMX is a phosphodiesterase inhibitor, which can elevate intracellular levels of cAMP. Protein kinase A (PKA) is the main target of cAMP, and both cAMP and PKA have been implicated in the regulation of lipid accumulation (Spiegelman and Green 1981; Zhang *et al.* 2002).

Thiazolidinediones (TZDs) have also been shown to influence adipocyte differentiation as they are peroxisome proliferator activated receptor-γ (PPARγ) agonists. Several genes implicated in adipocyte differentiation contain PPAR response elements (PPREs), including adipocyte specific fatty acid binding protein (aP2/FABP4) and lipoprotein lipase (LPL). PPARγ knockout mice studies have shown that PPARγ -/+ mice are characterised by decreased adipose tissue mass (Kubota *et al.* 1999; Miles *et al.* 2000).

Upon hormonal stimulation, cultured preadipocytes undergo several rounds of proliferation and then succumb to growth arrest. During this period, the expression of C/EBPβ and C/EBPδ has a temporal rise, followed by the stimulation of expression of PPARγ and C/EBPα. There is a positive feedback loop between PPARγ and C/EBPα where both factors induce the expression of the other. A generally cooperative action between PPARγ and C/EBPα drives the expression of genes that are necessary for the generation and maintenance of the adipogenic phenotype. These genes include, among others, aP2/FABP4, phosphoenolpyruvate carboxykinase (PEPCK), glycerolphosphate dehydrogenase, fatty acid synthase (FAS), acyl CoA carboxylase, GLUT4 and the insulin receptor (Tong and Hotamisligil 2001). Additionally, sterol regulatory element binding protein (ADD1/SREBP1) plays a role in adipogenesis by activating PPARγ transcription and controlling the production of endogenous PPARγ ligands (Kim *et al.* 1998). This is summarised in Figure 1.1.

The cAMP responsive transcription factor CREB has also been found to play a role in adipogenesis. Hormonal induction of 3T3-L1 differentiation activates

CREB phosphorylation and transcriptional activity. The expression of constitutively active CREB in 3T3-L1 cells induces adipocyte differentiation, and the expression of dominant-negative form of CREB blocks adipogenesis. CREB may exert its function by binding to and activating the promoters of C/EBPβ and C/EBPδ and several other genes, such as PEPCK, FAS and aP2/FABP4 (Reusch *et al.* 2000).

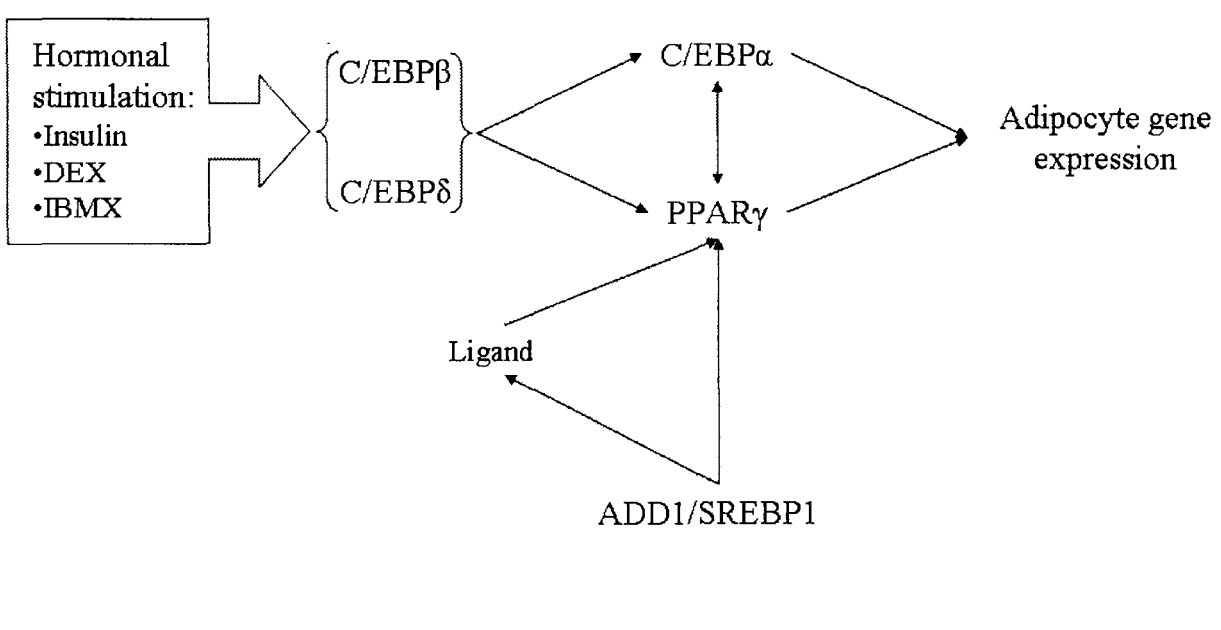


Figure 1.1 Sequential events during adipocyte differentiation.
Hormonal stimulation by insulin, dexamethasone and methylisobutylxanthine, initiates the adipogenic program involving sequential activation of the C/EBP family of transcription factors and PPARγ and the eventual expression of a large set of adipogenic genes such as aP2/FABP4, FAS, PEPCK and leptin. ADD1/SREBP1 is a transcription factor that can activate PPARγ transcription and produce ligands for PPARγ. Adapted from Tong and Hotamisligil (2001).

Terminal differentiation is generally coupled to growth arrest, as in the case in adipocyte differentiation. Both PPARγ and C/EBPα possess anti proliferation properties (Rosen *et al.* 2000). Adipogenesis is also associated with a transient

activation of cyclin expression, and changes in the expression of several cyclin dependent kinase inhibitors such as p18 and p21 (Morrison and Farmer 1999).

ERK1/2 is regulated at various stages throughout the differentiation process and is regulated by insulin and growth factors – it is turned on for proliferation early on, and then switched off to avoid PPARγ phosphorylation. Additionally, ERK1/2 activity is necessary for the expression of the crucial adipogenic regulators C/EBPα, β, and δ (Prusty *et al.* 2002).

1.2 Adipokines

Adipokines are bioactive peptides and proteins secreted by both brown and white adipose tissue; however, as white adipose tissue comprises 10-25% of the total body weight of non-obese humans, adipokines secreted by it are thought to have greater influence than those secreted by brown adipose tissue. Adipokines represent a diverse group of enzymes (adipsin), growth factors (vascular endothelial growth factor), cytokines (tumour necrosis factor-α (TNF α) and interleukin-6 (IL-6) and other hormones (leptin, adiponectin, resistin and acylation stimulation protein), which play a critical role in energy regulation and carbohydrate and lipid metabolism (Mora and Pessin 2002). Only leptin, adiponectin, resistin, adipsin and visfatin are primarily produced by adipocytes and can therefore be properly classified as adipokines (Fantuzzi 2005). Adiponectin and resistin are the adipokines investigated in this study, and so they shall be explored in further detail.

1.2.1 Adiponectin

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (Acrp30), adipose most abundant gene transcript 1 (apM1), adipoQ and gelatin binding protein of 28 kDa (GBP28), was identified as a protein expressed and secreted by differentiated 3T3-L1 adipocytes and also found to be in abundant quantity in serum (Scherer *et al.* 1995). It was independently cloned as AdipoQ by differential display of murine mRNAs among non-adipogenic 3T3-C2 fibroblasts, undifferentiated 3T3-F442A preadipocytes, and differentiated 3T3-F442A adipocytes (Hu *et al.* 1996). It is an approximately 30 kDa polypeptide containing an N-terminal sequence, a variable domain, a collagen-like domain and a C-terminal globular domain (Kershaw and Flier 2004). Post-translational modification by hydroxylation and glycosylation produces multiple isoforms, which assemble into trimers and then into higher-order oligomeric structures composed of four to six trimers (Fantuzzi 2005). Both trimers and oligomers, but not monomers, of adiponectin are present in the circulation (Fantuzzi 2005). A proteolytic cleavage product containing the globular domain of adiponectin also circulates at physiologically significant levels and has biological activity (Kershaw and Flier 2004).

Adiponectin is expressed exclusively in white adipose tissue and adipocytes are the most important source of adiponectin, but serum adiponectin levels do not increase with obesity. Serum adiponectin levels are low in obese subjects but increase with weight loss (Lyon *et al.* 2003). Adiponectin is distinct from other known adipokines in that it appears to improve insulin sensitivity and inhibit vascular inflammation. The mechanism by which the insulin-resistant

state is associated with low adiponectin levels is not clear. TNF α, which is increased in the white adipose tissue of obese subjects, might down-regulate adiponectin production (Fantuzzi 2005). However, adiponectin reduces the production and activity of TNF α, IL-6 and nuclear factor (NF)-κB (Fantuzzi 2005).

In the liver, adiponectin enhances insulin sensitivity, decreases influx of non-esterified fatty acids (NEFAs), increases fatty acid oxidation, and reduces hepatic glucose output. In muscle, adiponectin stimulates glucose utilisation and oxidation of fatty acids. Within the vascular wall, adiponectin inhibits monocyte adhesion by decreasing the expression of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) by either TNF-α or resistin (Fantuzzi 2005). Adiponectin also inhibits macrophage transformation to foam cells by inhibiting expression of scavenger receptors and decreases proliferation of migrating smooth muscle cells in response to growth factors. In addition, adiponectin increases nitric oxide production in endothelial cells and stimulates angiogenesis. All of these effects are mediated via: increased phosphorylation of the insulin receptor; activation of AMP-activated protein kinase in skeletal muscle and liver to increase fatty acid oxidation and reduce hepatic glucose production; and modulation of the NF-κB pathway (Guerre-Millo 2004; Kershaw and Flier 2004).

Adiponectin administration to animals enhances insulin action, and low levels of adiponectin have been proposed to contribute to insulin resistance associated with obesity. Adiponectin gene expression and circulating adiponectin levels

are lower in patients with type 2 diabetes than in non diabetic individuals (Havel 2004). Adiponectin mRNA is decreased three fold in *ob/ob* mice compared to wild type littermates (Haluzik *et al.* 2004).

Adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2) have been identified. These receptors contain seven-trans-membrane domains but are structurally and functionally distinct from G protein coupled receptors. AdipoR1 is expressed primarily in muscle, and functions as a high affinity receptor for globular adiponectin and a low affinity receptor for full-length adiponectin. AdipoR2 is expressed primarily in the liver, and functions as an intermediate affinity receptor for both globular and full-length adiponectin. The tissue specific expression of the receptor subtypes, the properties of the different adiponectin isoforms as well as their circulating levels are all responsible for the biological effects of adiponectin. (Kadowaki *et al.* 2006)

1.2.2 Resistin

Resistin is an adipokine with contrasting roles in mice and man. It was initially shown to be released in large amounts from mouse adipocytes; its release was increased in obese mice and accompanied by insulin resistance, implying that adipocyte-derived resistin linked obesity to diabetes. Resistin is secreted by mature adipocytes and has an inhibitory effect on insulin-stimulated glucose uptake in differentiated 3T3-L1 adipocytes, and the inhibitory effect is prevented by an anti-resistin antibody (Steppan *et al.* 2001). Ablation of the resistin gene in mice decreases fasting glucose through reducing

gluconeogenesis, while resistin administration in these resistin-deficient mice increases hepatic glucose production (Banerjee *et al.* 2004). These studies indicate that resistin impairs insulin sensitivity and may contribute to the development of insulin resistance or diabetes in obese rodents.

Resistin is encoded by the *retn* gene (Steppan *et al.* 2001). Serum levels have been found to be elevated in both obesity (Degawa-Yamauchi *et al.* 2003) and diabetes (McTernan *et al.* 2003; Fujinami *et al.* 2004), implicating that resistin is dysregulated in these conditions. Resistin gene expression is also increased 2.2 fold in obesity (Li *et al.* 2002).

Resistin is not found in human adipocytes, but it is found in the monocytes within adipose tissue (Nagaev and Smith 2001). However, in mice it is thought that resistin is secreted by adipocytes into the blood circulation. Resistin opposes the actions of insulin in adipocytes and liver, causing increased glucose production by the liver and inhibiting differentiation of preadipocytes to adipocytes. Increased dietary fat and obesity increase the expression of resistin in adipocytes.

Studies *in vitro* have shown that resistin is induced during adipocyte differentiation and is secreted into media (Steppan *et al.* 2001). A family of related proteins (called resistin like molecules [RELMs] or found in inflammatory zone [FIZZ]) were revealed via database searching using the resistin cDNA sequence (Steppan *et al.* 2001). FIZZ1 and FIZZ2 are homologous to the mouse RELMα and RELMβ respectively and resistin is alternatively known as FIZZ3 (Steppan *et al.* 2001). Although the other RELM/FIZZ family members exhibit tissue-specific expression, resistin is the

only one of these family members to be expressed in mouse adipocytes (Hartman *et al.* 2002).

Resistin is secreted as a dimer (Banerjee and Lazar 2001), and studies suggest that it forms higher order multimeric complexes with other dimers or the other RELMs (Chen *et al.* 2002), in a similar way to adiponectin. Resistin expression in visceral fat is 15-fold higher than in subcutaneous fat (Gabriely *et al.* 2002). Visceral fat has been identified as a risk factor for insulin resistance and increased levels of visceral fat are correlated with decreased insulin sensitivity (Gabriely *et al.* 2002; Goldstein 2002).

Resistin inhibits the differentiation of 3T3-L1 preadipocytes (Kim *et al.* 2001). It has been suggested that resistin plays a role in inflammatory conditions as it is expressed in human macrophages (Nagaev and Smith 2001; Savage *et al.* 2001) and RELMα/FIZZ1 is up-regulated by inflammation in the mouse. However, resistin is down regulated by inflammatory mediators such as TNFα and lipopolysaccharide (LPS) (Hartman *et al.* 2002).

Resistin is secreted from murine adipocytes and antagonises insulin-stimulated glucose metabolism in skeletal muscle myocytes, hepatocytes, and adipocytes themselves. (Shojima *et al.* 2002). Resistin gene expression is induced during adipocyte differentiation in culture and is restricted to WAT and to BAT *in vivo*. (Walczak and Tontonoz 2002)

1.3 Adipokine Regulation

Adipokine dysregulation is one of the key features of obesity and associated insulin resistance. It is therefore important to try and understand how the adipokines are regulated, how dysregulation is caused and the ways in which this may be corrected.

Adiponectin and resistin are the two adipokines that are the focus of these studies, therefore, only the regulation of these two will be further described.

1.3.1 Regulation of Adiponectin

There is a general hypothesis that adiponectin plays a significant role in linking obesity and insulin resistance, leading ultimately to Type 2 diabetes and atherosclerosis. Several clinical studies have observed that circulating adiponectin levels are reduced in obesity, in which mean adipocyte size is increased, and increased in lean individuals with smaller adipocyte size. A situation of low circulating adiponectin levels then promotes increased cellular lipid content and insulin resistance in skeletal muscle and liver.

Adiponectin gene expression is regulated by several extracellular signalling factors, including insulin, TNFα and β-adrenergic agonists (Havel 2004), although the link between these signals and adiponectin gene expression, in most cases, has yet to be elucidated. PPARγ has been shown to induce adiponectin promoter activity though an unidentified element responsive to PPARγ (Maeda *et al.* 2001). TZDs (thiazolidinediones) are a class of synthetic drugs, which include rosiglitazone, pioglitazone, troglitazone and darglitazone. TZDs were identified clinically as drugs that lower blood glucose and insulin

levels, leading to improved insulin sensitivity in Type 2 diabetic patients (Day 1999; Reginato and Lazar 1999). TZDs are high affinity ligands for PPARγ (Willson *et al.* 2001). TZD agonists of PPARγ increase adiponectin expression and circulating levels in rodents, and plasma adiponectin levels in non-diabetic subjects and in patients with Type 2 diabetes. In addition, the improvement of insulin sensitivity during TZD treatment was related to the increase in circulating adiponectin. It is possible that the effects of TZDs to increase whole body insulin sensitivity and to protect against cardiovascular disease could be mediated by increased adiponectin production (Havel 2004). Another potential mechanism of action of the TZDs is the "lipid steal hypothesis" (Ye *et al.* 2006), in which excessive lipid accumulation in muscle and liver is reduced by redistributing circulating lipids to adipose tissue.

C/EBPα has been reported to regulate adiponectin gene transcription through an intronic enhancer (Qiao *et al.* 2005). SREBP-1c binds to the adiponectin promoter and mediates the insulin-dependent adiponectin expression (Seo *et al.* 2004). Kruppel like factor (KLF)-7 has been reported to inhibit adiponectin gene expression in adipocytes (Kawamura *et al.* 2006).

1.3.2 Regulation of Resistin

Most mouse studies support the notion that resistin is an adipokine regulator of insulin action. However, most human studies show an entirely different picture. Human fat cells, unlike those of mice, do not produce resistin, although segments of human adipose tissue do release it. Resistin was

originally thought to be an adipokine because it is produced by adipocytes and causes insulin resistance in rodent models. However, subsequent human studies failed to link resistin to insulin resistance. In addition, the protein is not produced by human fat cells but by some yet unidentified cell in the stroma of human adipose tissue, which might be the macrophage (Bokarewa *et al.* 2005). In humans, it appears that peripheral blood mononuclear cells and macrophages are the major source of resistin rather than the adipocyte; this may explain why resistin mRNA expression is relatively low in human adipocytes (Yang *et al.* 2003). Moreover, as resistin levels do not consistently correlate with insulin resistance or obesity (Janke *et al.* 2002), the role of human resistin in the pathogenesis of insulin resistance is unclear.

Treatment of 3T3-L1 adipocytes with insulin was found to significantly reduce resistin mRNA and protein levels (Haugen *et al.* 2001; Shojima *et al.* 2002), additionally, *in vivo* studies have shown that insulin administration reduced resistin (Kim *et al.* 2001).

TZDs, by activating PPARγ, cause decreased resistin expression and decreased insulin resistance (Wolf 2004). Treatment of mice with TZDs lowered resistin protein levels, similar to the effects observed *in vitro*, using 3T3-L1 cells (Steppan *et al.* 2001).

Resistin is up-regulated several fold following dexamethasone treatment *in vitro* (Shojima *et al.* 2002), however it is unclear whether this up-regulation is independent of dexamethasone stimulated adipogenesis. Treatment of mice with dexamethasone induces resistin in white adipose tissues (Shojima *et al.*

2002). Additionally, resistin levels are suppressed by adrenaline and somatotrophin (Shojima *et al.* 2002).

The mechanisms of adiponectin and resistin regulation have not been fully elucidated as yet, however they are both altered by TZD treatment and therefore are under the control of PPARγ phosphorylation. PPARγ is a substrate of ERK1/2, and there is evidence to suggest that phosphorylation decreases its transcriptional activity (Hu *et al.* 1996; Adams *et al.* 1997; Camp and Tafuri 1997; Reginato *et al.* 1998; Chan *et al.* 2001). This suppression of PPARγ activity can result in increased adiponectin release and suppression of resistin release in adipocytes, shown recently by Sharma and Staels (Sharma and Staels 2007).

1.4 Role of MAPK

In addition to the role of the mitogen activated protein kinase (MAPK) ERK1/2 in mediating adiponectin and resistin release via PPARγ, ERK1/2 also plays a crucial role in adipogenesis, as detailed in 1.1.2. For this reason, the ERK1/2 pathway will be further explored.

MAPKs comprise a family of protein kinases whose function and regulation have been conserved during evolution from unicellular organisms to complex organisms including humans. MAPKs phosphorylate specific serines and threonines of target protein substrates and regulate cellular activities ranging from gene expression, mitosis, movement, metabolism, and programmed death. The MAPK family is an essential part of the signal transduction machinery in

signal transmissions from cell surface receptors and environmental stimulation. Because of the many important cellular functions controlled by MAPKs, they have been studied extensively to define their roles in physiology and human disease.

There are five subfamilies of MAPKs, these are extracellular signal-regulated kinases (ERK) 1 and 2, the c-Jun NH2-terminal kinases (JNK) 1 and 2, p38 enzymes, ERK 3 and 4 and ERK 5.

1.4.1 The ERK1/2 MAPK Pathway

The most well characterised pathway is the ERK1/2 pathway (Ray and Sturgill 1987). This pathway plays an essential role in cell proliferation and its role in normal and pathological adipogenesis has been intensively investigated since 1991, when the first paper demonstrating a link between ERK1/2 and adipocyte differentiation was published (Benito *et al.* 1991). The ERK1/2 pathway is important in regulating the cell cycle and cell proliferation and, as such, is activated by growth factors and insulin. The relevance of this pathway is that obesity is characterised by hypertrophy of adipocytes and the recruitment of new adipocytes from precursor cells. These processes are dependent on adipocyte differentiation. ERK1/2 is regulated at various stages throughout the differentiation process – it is turned on for proliferation early on, and then switched off to avoid PPARγ phosphorylation. Additionally, ERK1/2 activity is necessary for the expression of the crucial adipogenic regulators C/EBPα, β, and δ (Prusty *et al.* 2002). The potential modulation of the ERK1/2 MAPK

pathway by flavanols is therefore significant, considering the role it plays in obesity.

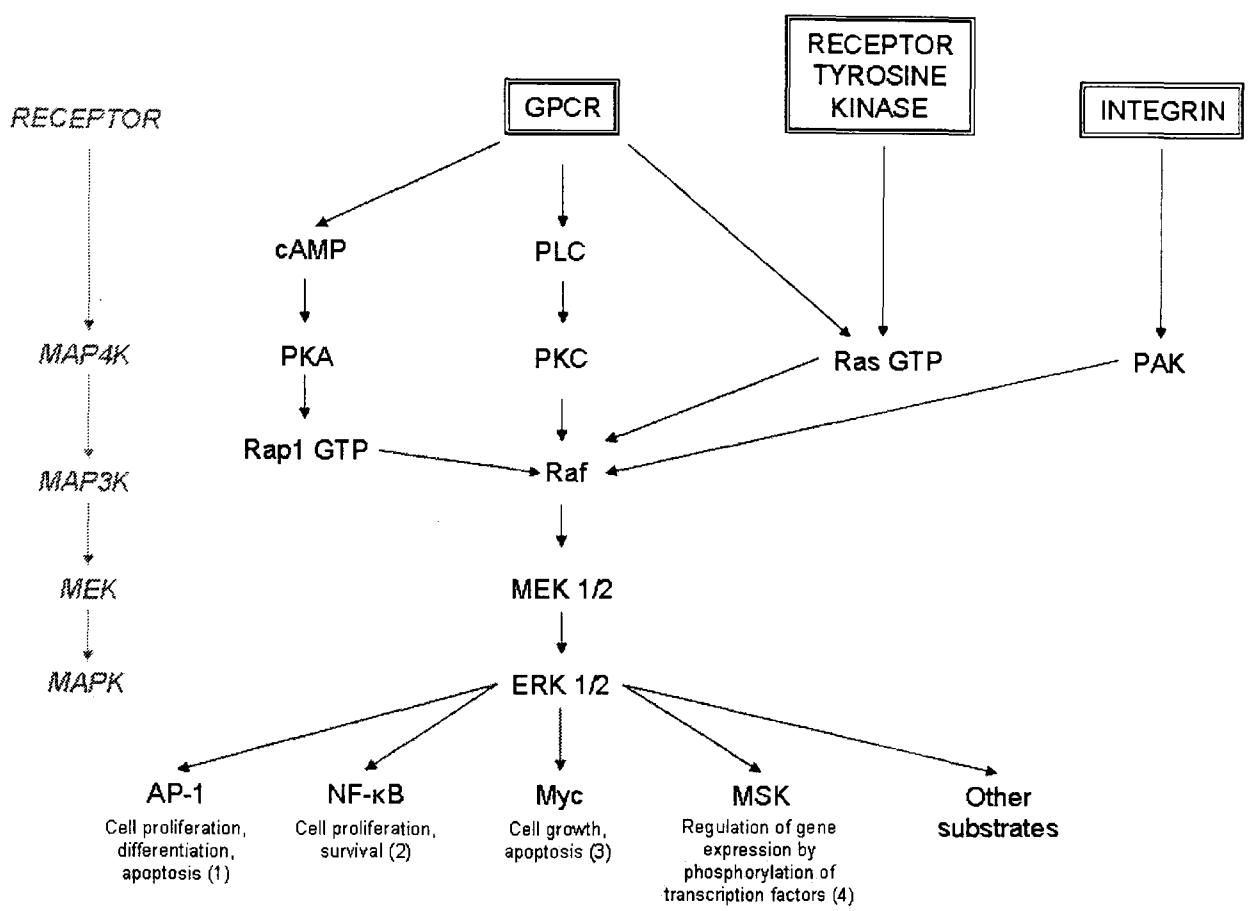


Figure 1.2 Summary of ERK1/2 MAPK pathway, adapted from Qi and Elion (2005).

Ligands bind to cell surface receptor tyrosine kinases, integrins or G-protein coupled receptors (GPCRs). This leads to activation of protein kinase A (PKA), protein kinase C (PKC) via phospholipase C (PLC), p21 activated kinase (PAK) or Ras GTP. This then triggers activation of Raf isoforms, followed by phosphorylation of MEK1/2 and then ERK1/2.

ERK1/2 have many known targets, including key transcription factors such as AP-1 (activating protein-1), NF-κB (nuclear factor- κB), Myc and MSK (mitogen- and stress-activated protein kinase). References are (1) (Karin et al. 1997), (2) (Gilmore 2006), (3) (Telfer et al. 2005), (4) (Hauge and Frodin 2006).

MAPK pathways comprise a four tiered kinase cascade comprising a MAP kinase kinase kinase kinase (MAP4K), a MAP kinase kinase kinase (MAP3K), a MAP kinase kinase (MEK) and finally the MAPK.

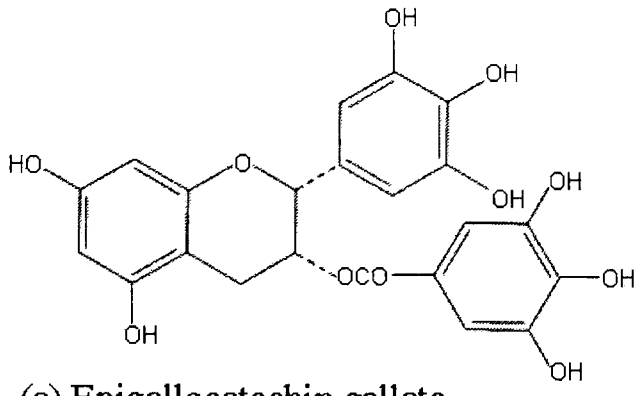
In the ERK1/2 MAPK pathway there are several MAP4Ks; (protein kinase A (PKA), protein kinase C (PKC) and p21 activated kinase (PAK)) which activate the Raf MAP3Ks (A-Raf, B-Raf, C-Raf/Raf-1). Alternatively, the Raf isoforms can be activated by Ras, a small GTP-binding protein. Raf is then recruited to the plasma membrane, followed by phosphorylation of MEK1/MEK2 and then ERK1 and ERK 2. (Qi and Elion 2005). The ERK1/2 MAPK pathway is summarised in Figure 1.2.

1.5 Green Tea Catechins

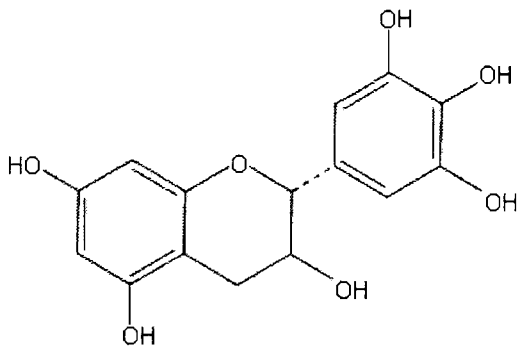
Tea, from the plant *Camellia Sinensis*, is the most consumed drink in the world after water. Depending on the manufacturing process, teas are classified into three major types: 1) non fermented green tea; 2) semi-fermented oolong tea; and 3) fermented black and red teas (Cabrera *et al.* 2006).

Green tea is produced by steaming freshly harvested leaves to prevent fermentation, yielding a dry, stable product. Tea polyphenols, known as catechins or flavanols, usually account for 30-42% of the dry weight of the solids in brewed green tea (Khan and Mukhtar 2007). The four major flavanols are (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG) and (-)-epicatechin. EGCG is the major flavanol and may account for 50-80% of the total catechin in tea. Green tea also

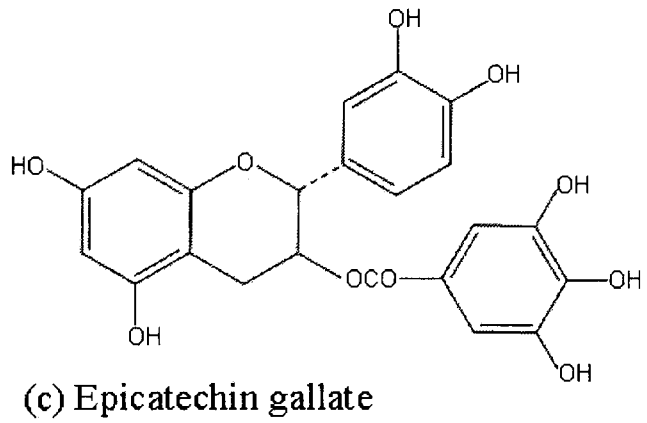
contains (+)-catechin, kaempferol, myricetin and quercetin. The structures of these are shown in Figure 1.3.



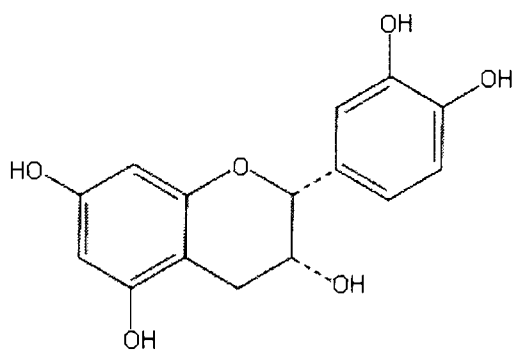
(a) Epigallocatechin gallate



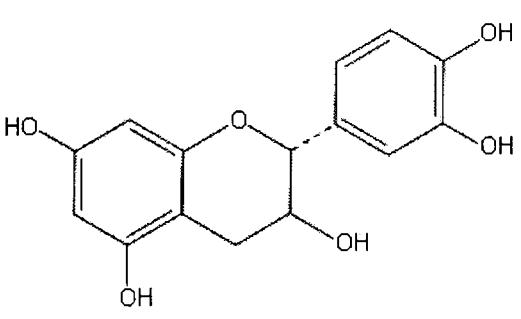
(b) Epigallocatechin



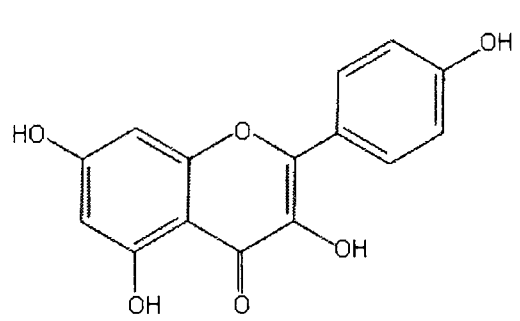
(c) Epicatechin gallate



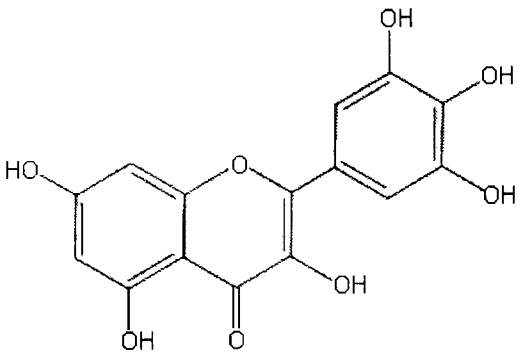
(d) Epicatechin



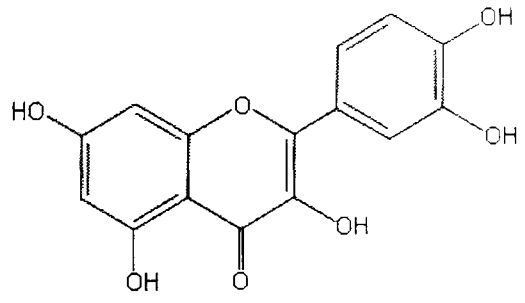
(e) Catechin



(f) Kaempferol



(g) Myricetin



(h) Quercetin

Figure 1.3 Chemical structures of the major flavanols and flavonols found in green tea.

Flavonoids belong to a vast group of polyphenolic compounds that are widely distributed in all foods of plant origin. Plant polyphenols have been of interest to scientists for decades, originally owing to their importance in plant physiology, specifically for their roles in plant pigmentation and flavour. Polyphenols are involved in plant growth and reproduction, provide resistance to pathogens and predators, and protect crops from disease and preharvest seed germination.

1.5.1 Nomenclature

The catechins belong to a group known as the flavanols, a subclass of the flavonoid polyphenols. More than 5000 different flavonoids have been described. The six major subclasses of flavonoids are the flavanols, flavanones, flavones, isoflavones, flavonols and anthocyanidins. Table 1.1 lists the flavonoid subclasses, the compounds within that class, and primary food sources. The common structure of flavonoids involves two aromatic rings linked through three carbons that typically form an oxygenated heterocycle. Flavonoids are classified into categories according to the oxidation level of the central heterocyclic ring. Within each subclass, individual compounds are characterised by specific hydroxylation and conjugation patterns (Beecher 2003).

It has been calculated that a cup of green tea, comprising 2.5 g of green tea leaves/200 ml of water, may contain 90 mg of EGCG (Wu and Wei 2002). Pharmacokinetic studies by Ullmann *et al* reveal that a bulk dose of 100 mg

EGCG resulted in a maximum plasma concentration of 189.52 ng/ml (0.4 μM) and a bulk dose of 1600 mg EGCG was required to produce a maximum plasma concentration of 2911.35 ng/ml (6.35 μM) free EGCG (Ullmann *et al.* 2003)

Chow *et al* carried out a Phase I pharmacokinetic study in humans to determine the systemic availability of EGCG after a single oral dose administration of 200, 400, 600 or 800 mg. The maximum plasma concentrations were 73.7, 111.8, 169.1 and 438.5 ng/ml (0.16, 0.24, 0.37 and 0.96 μM) for each dose level respectively, 4 h after administration (Chow *et al.* 2001).

Table 1.1 Flavonoid subclasses, compounds and food sources (adapted from Beecher, 2003).

Subclass	Compounds	Primary food sources
Flavanols	(+)-Catechin, (+)-gallocatechin, (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechingallate, (-)-epigallocatechingallate,	Green tea, black tea, grapes and red wines
Flavanones	Hesperetin, naringenin, eriodictyol	Oranges, grapefruits, lemons
Flavones	Luteolin, apigenin	Apples, celery, celeriac, lemons, parsley
Isoflavones	Genistein, diadzein, glyceitein, biochanin A, formononentin	Soybeans, legumes
Flavonols	Quercetin, kaempferol, myricetin, isorhamnetin	Onions, apples, teas, berries, olives, bananas, lettuce, plums, red wine
Anthocyanidins	Cyandin, delphinidin, malvidin, pelargonidin, peonidin, petunidin	Blueberries, raspberries, strawberries, cranberries

1.5.2 Flavanol Content of Green Tea and Bioavailability

A study by Yang *et al* investigating the time dependent plasma concentrations and urinary excretion of 1.5 g decaffeinated tea solids dissolved in 500 ml water demonstrated that the maximum plasma concentrations of EGCG, EGC and EC were 326, 550 and 190 ng/ml respectively and these were observed 1.4-2.4 hours after ingestion of the tea preparation. When the dosage was increased from 1.5 to 3 g, the maximum plasma concentrations increased 2.7-3.4 fold, but increasing the dose to 4.5 g did not significantly increase the maximum plasma concentrations, suggesting a saturation point may have been reached. The half-life of EGCG was much longer than that of EGC or EC (5-5.5 hours compared to 2.5-3.4 hours), and EGC and EC, but no EGCG were excreted in the urine (Yang *et al.* 1998).

Investigation into the degradation of 0.5 g catechin per day over a 3 week period in male Wistar rats revealed that only 3.1% of the ingested catechin was excreted unchanged in the faeces, suggesting the catechin had either been absorbed, degraded or metabolised (Bravo *et al.* 1994).

1.5.3 Flavanol Metabolism

Flavanols are extensively metabolised *in vivo* – the bioactive forms of the flavanols in plants are glycosides (bound to sugar), when they are not attached to sugar moieties they are referred to as the aglycone form. Except for flavanols, flavonoid molecules do not occur in plants as aglycones; the most

frequently occurring forms are the glycoside derivatives in plants. Glycosylation increases the polarity of the flavonoid molecule, which is necessary for storage in plant cell vacuoles (Aherne and O'Brien 2002).

Upon intestinal absorption, conjugates and metabolites are formed from the glycosides. In particular, there is strong evidence for the extensive phase I deglycosylation and phase II metabolism of the resulting aglycones such as epicatechin to glucuronides, sulphates and O-methylated forms during transfer across the small intestine and then again in the liver. Further transformation has been reported in the colon, where the enzymes of the gut microflora degrade flavanols to simple phenolic acids, which may also be absorbed and subsequently further metabolised in the liver (Williams *et al.* 2004).

Following these phase I and II deglycosylations and metabolisms, there are three forms of intracellular metabolism that flavanols may undergo. These are 1) conjugation with thiols, particularly GSH, 2) oxidative metabolism, and 3) P450 related metabolism. These metabolic modifications will alter the flavanols' classical antioxidant nature, i.e. their ability to donate electrons to stabilise radical species and bind transition metals. Circulating metabolites of flavanols, such as glucuronides and O-methylated forms, and intracellular metabolites, for example flavanol-GSH adducts, have a reduced ability to donate hydrogen and are less effective scavengers of reactive oxygen and nitrogen species relative to their parent aglycone forms. Concentrations of flavanols and their metabolite forms accumulated in the plasma are lower (high nanomolar, low micromolar) than those recorded for small molecule antioxidant nutrients such as ascorbic acid and α-tocopherol. Consequently,

flavanols are unlikely to express beneficial actions *in vivo* through outcompeting antioxidants such as ascorbic acid which are present at higher concentrations (high micromolar). Accumulating evidence suggests that the cellular effects of flavanols may be mediated by their interactions with specific proteins central to intracellular signalling cascades. (Williams *et al.* 2004)

1.5.4 Interactions with ERK1/2 Pathway

There is evidence to suggest that flavanols can influence the phosphorylation status of ERK1/2. A study by Hung *et al* in 3T3-L1 preadipocytes showed that 50 μM EGCG was able to decrease ERK1/2 phosphorylation after 30 min, and this was maintained at 1 and 4 hour treatment times, and through this was able to decrease proliferation of the preadipocytes (Hung *et al.* 2005). As obesity is the result of both increased adipocyte size (hypertrophy) and increased adipocyte number (hyperplasia) the anti mitogenic effect suggests that EGCG may be able to modulate obesity.

In human umbilical vein endothelial cells (HUVECs) 30-50 μM EGCG was able to inhibit angiotensin II induced ERK1/2 phosphorylation and therefore prevent increases in protein and mRNA levels of vascular cell and intercellular adhesion molecules (VCAMs and ICAMs) (Chae *et al.* 2007). Studies in human keratinocytes showed that ECG was able to inhibit hydrogen peroxide-induced ERK1/2 phosphorylation, thus protecting keratinocytes from lipid peroxidation and cell death (Huang *et al.* 2007).

The effects of a wide range of flavonoids have been investigated in neurons, where they are thought to have a neuroprotective effect, interacting with MAPK pathways as they are involved in signalling to neuronal survival, regeneration and death. The flavanol effects on ERK1/2 phosphorylation have been investigated in primary cortical neurons where epicatechin at 100-300 nM was able to stimulate ERK1/2 phosphorylation (Schroeter *et al.* 2007). The flavonol quercetin, inhibits ERK1/2 phosphorylation in the same system after 30 min incubation at concentrations of 0.3-30 μM (Spencer *et al.* 2003). A further study using cortical neurons showed that hesperetin, a flavanone, was able to increase ERK1/2 phosphorylation at 100 nM (Vauzour *et al.* 2007).

It is clear that the various flavonoids are able to alter the phosphorylation status of ERK1/2, but not necessarily in the same direction. It is therefore difficult to predict what effect the green tea catechins might have on the phosphorylation status of ERK1/2 in 3T3-L1 adipocytes, because although they decrease phosphorylation in preadipocytes, these cells are very different to the mature, differentiated adipocytes.

It has been suggested that the 67 kDa Laminin Receptor (LR) is a receptor for EGCG, however, this was discovered on cancer cells (Tachibana *et al.* 2004) and receptors for laminin have been found on muscle, epithelial and nerve cells, macrophages, neutrophils and hepatocytes (Mecham 1991), but to date, none have been identified on adipocytes. Laminin serves as a major adhesion substrate for invasive cancer cells, and human melanoma cells expressing a reduced level of 67 kDa LR demonstrate a less aggressive phenotype and a diminished attachment to laminin, an important part of metastasis (Givant-

Horwitz *et al.* 2004). In this study by Givant-Horwitz *et al*, human melanoma cells expressing reduced levels of 67 kDa LR showed increased phosphorylation of ERK1/2, JNK and p38 MAPK, and this was accompanied by a significant reduction of the mRNA of the enzyme MKP-1, which dephosphorylates ERK1/2, JNK and p38 MAPK. This suggests that a 67 kDa LR-related signal transduction pathway, which is associated with the MAPK pathway, does exist and suggests a potential mechanism for EGCG-mediated MAPK modulation.

1.5.5 Anti-Obesity Effects (*in vivo* studies)

Green tea has been regarded for centuries to possess significant health promoting effects, and was traditionally used to improve blood flow, eliminate alcohol and toxins, improve resistance to disease, relieve joint pain, to clear urine and improve its flow (Balentine *et al.* 1997). In recent years, research has focused on the anti-cancer, -cardiovascular disease and -obesity effects. EGCG is believed to be the active constituent of green tea, and studies have been carried out using intraperitoneal and intravenous administration, and dietary supplementation of pure EGCG, a mixture of green tea catechins, and ordinary green tea. A number of studies have been carried out in mice, rats and humans and all show that EGCG/green tea extract/green tea is able to reduce body weight and other obesogenic factors, even when subjects are on a high fat diet.

Intraperitoneal injection of 85 mg/kg EGCG was injected into lean and obese Zucker rats and significantly reduced food intake, body weight, blood levels of leptin, resistin, insulin, insulin-like growth factor I, glucose, cholesterol and triglyceride (Kao *et al.* 2000). Dietary supplementation using TEAVIGO™, an extract from green tea which contains more than 94% EGCG but less than 0.1% caffeine, prevented the increase in body weight and adipose tissue mass induced by feeding a high-fat diet to C57BL/6J mice. This was accompanied by reduced expression of FAS and acetyl-coenzyme A carboxylase-1 mRNA in adipose tissue, suggesting that EGCG reduces lipogenesis in adipose tissue. In obese Sprague-Dawley rats, dietary supplementation resulted in a significant weight loss within 4 weeks (Wolfram *et al.* 2005). Another study using dietary supplementation with TEAVIGO™ in diet-induced obese New Zealand black mice resulted in a dose-dependent attenuation of body fat accumulation. Leptin gene expression in white adipose tissue was also reduced. Acute oral administration over 3 days had no effect on body temperature, activity and energy expenditure, whereas the respiratory quotient during their activity period was decreased, suggesting that EGCG decreased lipogenesis and increased fat oxidation. Food intake was not altered (Klaus *et al.* 2005). The effects of green tea extract on the endurance capacity, energy metabolism and fat oxidation in BALB/c mice was investigated over a 10 week period. Mice had increased swimming time to exhaustion after being fed green tea extract, they also had higher rates of fat oxidation and increased β-oxidation activity in skeletal muscle. Supplementation with TEAVIGO™ also enhanced endurance capacity in the mice. Food intake was not significantly different between the groups (Murase *et al.* 2005). Another study by the same authors compared the

effects of diets containing low fat, high fat or high fat supplemented with tea catechins on C57BL/6J mice over an 11 month period. The tea catechin supplementation resulted in a significant reduction of high-fat diet-induced body weight gain, visceral and liver fat accumulation, and the development of hyperinsulinaemia and hyperleptinaemia. No mention is made of food intake measurements in this study (Murase *et al.* 2006). In a study investigating the effect of powdered green tea in Zucker rats fed a 50% sucrose diet containing 15% butter, it was found that green tea significantly attenuated body weight gain and decreased adipose tissue mass, while food intake was not affected (Hasegawa *et al.* 2003). 3 weeks of green tea drinking by Wistar rats significantly reduced adipose tissue weight compared to water drinking controls, however body weight was not affected. Plasma levels of free fatty acids and of total cholesterol were reduced and the LDL/HDL ratio was also decreased. Additionally, skeletal muscle glucose uptake was significantly increased and adipose tissue glucose uptake was significantly decreased. Food intake was unaffected. Furthermore, PPARγ expression was decreased in adipose tissue (Ashida *et al.* 2004). In mice fed a high fat diet, who were supplemented with EGCG (3.2 g/kg diet), corresponding to 10 cups (200 ml) of green tea (containing 2 g leaves per cup) per day, for 16 weeks, reduced body weight gain, percentage body fat and visceral fat weight compared with mice without EGCG treatment (Bose *et al.* 2008).

A study by Nagao *et al*, where obese adults ingested green tea containing either 583 mg or 96 mg catechins per day, for 12 weeks, revealed that decreases in body weight, body mass index, body fat ratio, body fat mass, waist circumference, hip circumference, visceral fat area, and subcutaneous fat area

were greater in the high catechin group (Nagao *et al.* 2007). A further study by the same authors was carried out on patients with Type 2 diabetes who were not receiving insulin therapy. Patients who ingested green tea containing 583 mg catechins per day had a significantly decreased waist circumference after 12 weeks compared to those who ingested only 96 mg catechins per day. Additionally, adiponectin levels were increased in the high catechin group (Nagao *et al.* 2009). Food intake measurements were not taken in either study.

1.5.6 Anti-Obesity Effects (*in vitro* studies)

Relatively little is known about the underlying mechanisms of the actions of the anti-obesity effects of flavanols but molecular and cellular studies have revealed that they are able to increase lipolytic activity, decrease lipogenic activity, increase fat oxidation and thermogenesis, and modulate the activity and expression of lipoproteins. More relevant to these studies, flavanols are also able to decrease cell numbers and decrease hormone stimulated proliferation of preadipocytes and their differentiation into adipocytes (Kao *et al.* 2006).

As previously mentioned, EGCG has an ERK1/2-dependent antimitogenic effect on preadipocytes, indicated by decreased cell number and decreased incorporation of bromodeoxyuridine in dose-, time-, catechin-, and growth phase dependent manners (Hung *et al.* 2005). Gallated catechins, especially EGCG are more active than other catechins, suggesting that EGCG may act differently from EC, EGC and ECG in regulating preadipocyte growth. EGCG

contains the largest number of hydroxyl groups on its three aromatic rings among the tea catechins, and these hydroxyl groups may be important for hydrogen bonding with other molecules. EGCG has both gallyl and galloyl groups which have some conformational flexibility, that may also be important for interactions with other molecules.

Adipocyte differentiation is tightly regulated by several transcription factors, it is initiated by C/EBPβ and C/EBPδ, which act together to induce PPARγ2, a key regulator for adipogenesis, and C/EBPα. C/EBPα and PPARγ together promote adipocyte differentiation by activating expression of adipose-specific genes and are also crucial for maintenance of each other's expression at high levels. CG and EGC at 30 μM and EGCG at 10 and 30 μM significantly down-regulate expression of PPARγ2 and C/EBPα during adipocyte differentiation (Furuyashiki *et al.* 2004). There is some evidence to suggest that PPARγ activity is inhibited by phosphorylation by activated ERK1/2 (Hu *et al.* 1996; Adams *et al.* 1997; Camp and Tafuri 1997; Reginato *et al.* 1998; Chan *et al.* 2001), suggesting that the anti-adipogenic effects of green tea catechins may be mediated, at least in part, via the ERK1/2 inhibition of PPARγ and C/EBPα.

A decrease in preadipocyte number can either be due to inhibition of mitogenesis, or induction of apoptosis. Using concentrations of EGCG below 50 μM, the cell viability of day 3 preadipocytes remained at 90-100% during the 48hr treatment, whereas EGCG at doses of 100-400 μM reduced the cell viability of preadipocytes by 15-30%, induced the appearance of DNA

fragmentation, and increased the activity of the caspase-3 protein, an apoptotic enzyme (Wu *et al.* 2005).

There is evidence to suggest that flavanols are able to modulate the differentiation of adipocytes, another mechanism behind the potential anti-obesity effects that flavanols have. A study by Kao *et al* (2000) revealed that 10 μM EGCG was able to inhibit preadipocyte proliferation by 50% and inhibit insulin-induced increases in cell number by 34% and the triacylglycerol content by 54% during a 9 day period of differentiation. Even in the presence of the prodifferentiative agents dexamethasone, IBMX and insulin, 10-100 μM EGCG was also to reduce cell number and triacylglycerol content (Kao *et al.* 2000). This suggests, therefore, that the *in vitro* effect of EGCG on adipocytes may be mediated by modulation of hormone stimulated cell proliferation and differentiation. Additionally, it was shown that EGCG reduces the levels of adipogenesis-related transcription factors such as C/EBPβ and PPARγ, in differentiating 3T3-L1 preadipocytes treated with dexamethasone, IBMX and insulin (Kao *et al.* 2006). The study mentioned earlier by Furuyashiki *et al* (2004) showed that 10-30 μM EGCG decreased lipid accumulation by 25-50% in differentiating 3T3-L1 adipocytes (Furuyashiki *et al.* 2004). Another study has shown that 50-200 μM EGCG could reduce the size and number of lipid droplets in differentiating 3T3-L1 adipocytes when it was added with the induction medium from days 0-6 of adipogenesis (Lin *et al.* 2005).

The adipokine resistin is known to cause insulin resistance and decrease adipocyte differentiation (Steppan *et al.* 2001) and has thus been proposed as a biomarker of insulin resistance and adipose tissue mass. It is possible that

EGCG exerts its effects through the modulation of resistin. In 3T3-L1 adipocytes, EGCG suppressed resistin mRNA and protein in a time and dose-dependent manner, via an ERK1/2 dependent pathway, as levels of pERK1/2 were also reduced (Liu *et al.* 2006).

1.6 Glucose Uptake

Glucose uptake into cells is dependent on the activity of glucose transporters (GLUTs) which are membrane spanning proteins. GLUT1 is ubiquitously expressed and functions to maintain basal glucose uptake. GLUT4 is expressed by muscle and fat cells, and under basal conditions is sequestered in an intracellular compartment, however following stimulation by insulin, moves to the cell surface (Bryant *et al.* 2002). The major physiological action of insulin is to promote glucose uptake into muscle and adipose tissue by triggering GLUT4 translocation. It is defects in this system that contribute to the development of insulin resistance in Type 2 diabetes and obesity, as it is primarily the presence of GLUT4 in the plasma membrane that determines glucose utilisation in these tissues (Kahn 1996). Upon binding of insulin to its receptor, there is a net shift in the subcellular distribution of GLUT4 to the plasma membrane (Bryant *et al.* 2002). Once in the plasma membrane, GLUT4 facilitates diffusion of glucose into the cell, resulting in a 20-30 fold increase in the rate of glucose uptake in the presence of insulin. The effect of insulin on GLUT4 trafficking is mediated, at least in part by phosphatidylinositol-3-kinase (PI-3 kinase), but the downstream effectors of

this enzyme, as well as the subcellular compartments that are mobilised, are poorly defined (Watson and Pessin 2001). It is unclear whether the insulin-stimulatable GLUT4 compartment is part of a regulated pathway for protein secretion. GLUT4 is present in the trans Golgi network, the site where most secretory vesicles form (Huang and Czech 2007). There is evidence to suggest that ERK1/2 activation up-regulates GLUT1 expression resulting in increased basal uptake in the absence of insulin (Fingar and Birnbaum 1994; Fujishiro *et al.* 2003).

There are reports in the literature that suggest that flavonoids are able to modulate both basal and insulin-mediated glucose uptake. Kaempferol and quercetin, flavonoids extracted from *Euonymus alatus*, significantly improved imsulin-mediated glucose uptake when added to differentiated 3T3-L1 adipocytes at 5-50 μM for 3 days (Fang *et al.* 2008). The addition of (-)-catechin to differentiated 3T3-L1 adipocytes at 50 μM for 24 hours also increased insulin-mediated glucose uptake (Cho *et al.* 2007).

Berberine, an isoquinoline alkaloid, isolated from Chinese medicinal herbs such as *Coptidis rhizoma* and *Cortex phellodendri*, enhanced basal glucose uptake in normal and insulin-resistant 3T3-L1 adipocytes when added at 5 μM for 6 hours (Kim *et al.* 2007). This study also demonstrated that berberine increased expression of GLUT-1 but not GLUT-4 in 3T3-L1 adipocytes and that this was through an AMP-activated protein kinase and ERK mediated mechanism. The ERK1/2 pathway has been demonstrated to play a role in glucose transport, through treatment of 3T3-L1 adipocytes with the MEK

inhibitors PD98059 and U0126, which both inhibited insulin-mediated glucose uptake (Harmon *et al.* 2004).

In comparison to the studies by Cho *et al*, Fang *et al* and Kim *et al*, investigation of genestein, an isoflavone derivative, showed significant inhibition of insulin-mediated glucose uptake when incubated at 50 μM, 15 min prior to insulin treatment in 3T3-L1 adipocytes (Bazuine *et al.* 2005). In isolated rat adipocytes, myricetin, quercetin and catechin-gallate showed significant inhibition of insulin-mediated glucose uptake when incubated in the range of 10-100 μM (Strobel *et al.* 2005). Interestingly, in this same study, catechin had no effect on glucose uptake.

Studies *in vivo*, in which rats have been given green tea instead of drinking water, for 3 (Ashida *et al.* 2004) or 12 weeks (Wu *et al.* 2004), have investigated the effects of flavonoids on glucose uptake. The study by Ashida *et al* showed that green tea significantly reduced glucose uptake, accompanied by a decrease in translocation of GLUT4 in adipose tissue, while it significantly stimulated glucose uptake with GLUT4 in skeletal muscle. However, Wu *et al* reported that basal glucose uptake was significantly higher in rats in the green tea group, versus rats who had been drinking water.

Evidence to date, on the effects of flavonoids on basal and insulin-mediated glucose uptake, suggests that the different flavonoids vary in their modulation of this process. As the characteristic of insulin resistance is the decrease of insulin sensitivity and glucose uptake in peripheral tissues, there is a possibility that certain flavonoids may be able to ameliorate insulin resistance.

1.7 Summary

In summary, it appears that the green tea and cocoa flavanols have the potential to exert anti-obesity effects and there is already a wealth of literature suggesting that they are able to modulate weight gain and other obesogenic factors such as lipogenesis, β-oxidation and adipokine release. There is also evidence that the flavanols are able to modulate signalling pathways in adipocytes and preadipocytes but as yet definite links between the signalling pathways and physiological outcomes have not been established. Key features of obesity and associated insulin resistance are adipokine dysregulation, decreased sensitivity of adipocytes to insulin and subsequently changes in glucose uptake by cells. The aims of this thesis were therefore to firstly establish an efficient system of differentiated adipocytes and secondly to use this to investigate the effects of the flavanols on physiological outcomes such as adipokine release and glucose uptake. Thirdly, the question of whether these processes might be regulated by the ERK1/2 pathway and what may be happening upstream of this was explored. Finally, the effect of flavanol treatment on adipocyte gene expression of genes known to be modulated in obesity was investigated.

Materials & Methods

2 Materials & Methods

All materials were obtained from Sigma (Poole, UK) unless otherwise stated.

2.1 3T3-L1 Cell Culture

2.1.1 Materials

FCS (foetal calf serum) and NCS (newborn calf serum) were obtained from PAA Laboratories (Somerset, UK).

6 well plates were supplied by Corning Ltd (Sunderland, UK), Nalge Ltd (Hereford, UK), Orange Scientific (Belgium), Becton, Dickinson, Falcon (Oxford, UK), Iwaki, Asahi Techno Glass Corporation (Tokyo, Japan). Tissue culture flasks were supplied by Sarstedt Ltd. 35mm dishes, 12 and 24 well plates were supplied by Nalge Ltd.

Oil Red O stain was obtained from British Drug Houses Ltd (Poole, England).

3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC).

2.1.2 Solutions

Medium 1: Dulbecco's Modified Eagle Medium (DMEM) high glucose with 10% NCS, supplemented with 2mM glutamine, 1% antibiotic/antimycotic mix and 0.5% gentamicin.

Medium 2: DMEM high glucose with 10% FCS, supplemented with 2mM glutamine, 1% antibiotic/antimycotic mix and 0.5% gentamicin and containing 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 μM dexamethasone and 166 nM insulin.

Medium 3: DMEM high glucose with 10% FCS, supplemented with 2mM glutamine, 1% 100X antibiotic/antimycotic mix (10000 U/ml penicillin G, 10 mg/ml streptomycin, 25 μg/ml amphotericin B) and 0.5% 10mg/ml gentamicin and containing 166 nM insulin.

Medium 4: DMEM high glucose with 10% FCS, supplemented with 2mM glutamine, 1% antibiotic/antimycotic mix and 0.5% gentamicin.

Oil red O stain: 1% Oil red O in isopropanol, diluted 3:2 in PBS.

2.1.3 Cell Culture

Cells were seeded at either 5 x 10^4 cells/T75 flask, or 1 x 10^5 cells/35mm well and grown to confluence in medium 1. 48 hr after reaching confluence, differentiation was induced by incubation in medium 2 for 48 h. This start of differentiation was represented as post induction day 0 (PID 0). Cells were

then incubated in medium 3 for 48 h. Differentiated cells were maintained in medium 4 until needed for treatment (see Figure 2.1). Cells were grown at 37°C, 5% humidity and 10% CO_2.

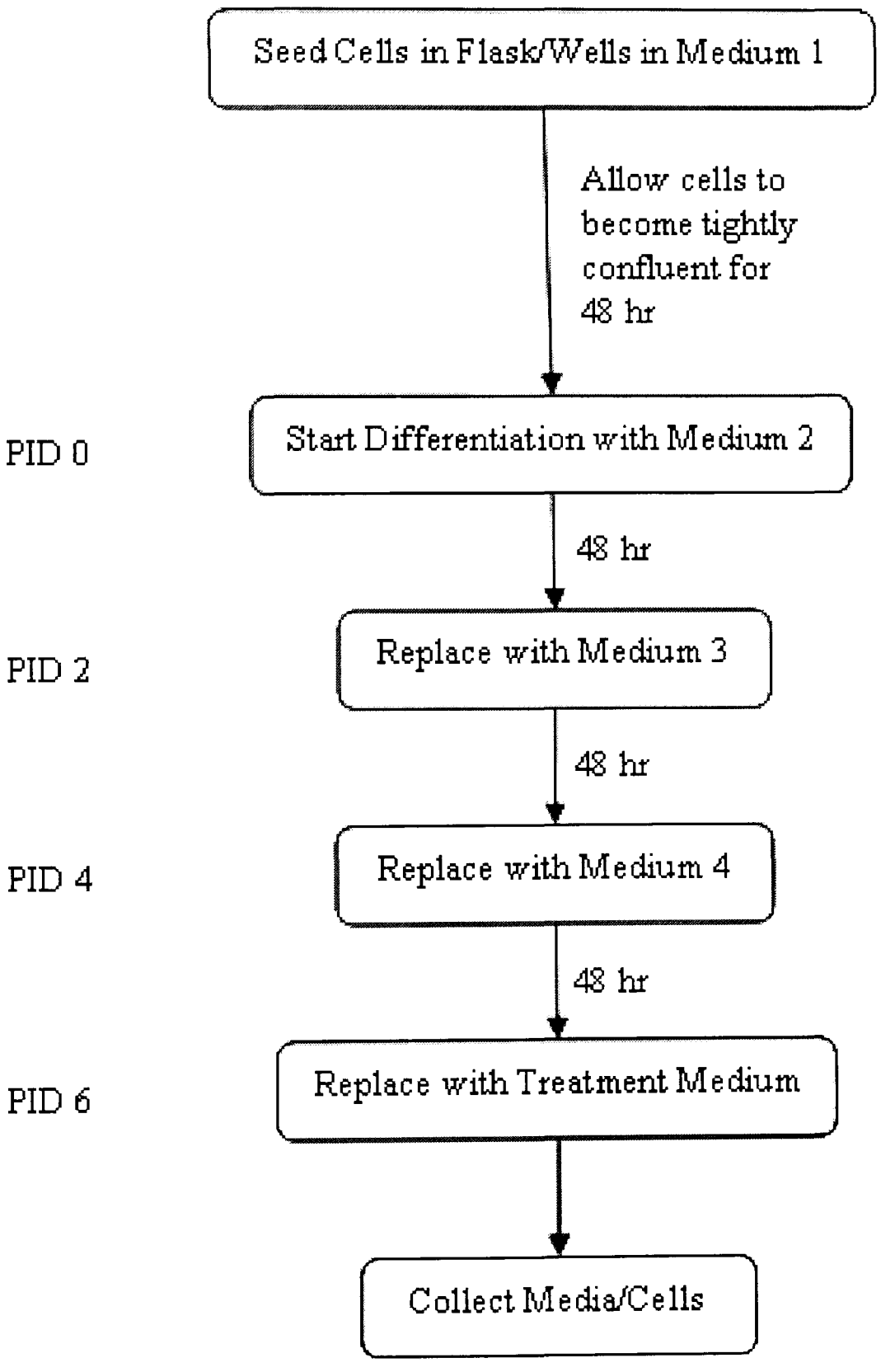


Figure 2.1 Diagrammatic overview of 3T3-L1 differentiation protocol.

2.1.4 Oil Red O Staining

Cells were stained using Oil Red O to determine whether the lipid content of cells had increased, and were therefore differentiated adipocytes. Following media removal, cells were washed three times with PBS and fixed with 10% formalin solution at room temperature, for 10 minutes. Cells were then washed thrice with PBS and stained with Oil Red O for 1 hr at room temperature. Cells were then washed thrice with PBS, and fresh PBS was added to cover the cell surface and prevent dehydration.

2.2 ELISA

2.2.1 Materials

Mouse Adiponectin Duoset and Resistin Duoset ELISA kits were obtained from R&D Systems Europe Ltd (Abingdon, UK).

2.2.2 Method

Collected media samples were assayed for mouse adiponectin and mouse resistin using ELISA duosets in 96 well plates. Initially, samples were assayed at a range of dilutions, in order to determine the optimum dilution for adipokine measurement. Finally, samples assayed for adiponectin and resistin were diluted 1:100. All samples and standards were prepared and assayed in

triplicate. Wavelength of 96 well plates was measured and analysed using Dynex Revelation 4.22 (Dynex Technologies Ltd, Worthing, UK).

2.3 Western Blotting

2.3.1 Materials

Complete Protease Inhibitor Complex was supplied by Roche (West Sussex, UK). BCA Protein Assay Reagent was supplied by Pierce (Thermo Fisher Scientific Inc, Illinois, USA). Mini Protean 3 system was supplied by BioRad (Hemel Hempstead, UK). 30% Bis-Acrylamide was supplied by Severn Biotech (Kidderminster, UK). PageRuler Prestained Protein Ladder Plus was supplied by Fermentas (York, UK). Nitrocellulose membrane, ECL reagents and photographic film were supplied by GE Healthcare (Amersham, UK). Developing and Fixing solutions were supplied by Kodak (Hemel Hempstead, UK).

Antibodies

Anti-cyclophilin B was supplied by Abcam (Cambridge, UK). Anti ERK, pERK and PPARγ antibodies were supplied by Cell Signaling Technology (New England Biolabs, Hitchin, UK). Anti-Dok-1 antibody was supplied by Santa Cruz (California, USA). Swine anti-rabbit and goat anti-mouse secondary antibodies were supplied by Dako (Ely, UK). 680 anti-rabbit, 680

anti-mouse, 800 anti-rabbit and 800 anti-mouse secondary antibodies were supplied by Li-Cor Biosciences (Cambridge, UK).

2.3.2 Solutions

Phosphate Protective Lysis Buffer

50mM HEPES, 10% (v/v) glycerol, 1mM EDTA, 10mM NaF, activated* 1mM Na_3VO_4, 150 mM NaCl, 1% (v/v) triton X-100, pH 7.5, supplemented with Complete Protease Inhibitor Cocktail (1 tablet/10 ml lysis buffer).

*activation process for Na_3VO_4: 200mM Na_3VO_4 was dissolved in HPLC purified water, and adjusted to pH 10 using HCl. This solution was activated by boiling until the solution changed from yellow to colourless, cooled to room temperature and the pH readjusted to 10. This was repeated before finally being stored at –20 °C. Activation depolymerises the vanadate converting it to a more potent inhibitor of protein tyrosine phosphatises.

4x Sample Buffer

0.250M Tris, 40% v/v glycerol, 4% w/v sodium dodecyl sulphate (SDS), 20% v/v ß-mercaptoethanol and 0.002% bromophenol blue.

Lowry Reagents

A: 0.1 M NaOH containing 2% (w/v) Na_2CO_3, 2% (w/v) NaK tartrate, 1% $CuSO_4*5H_2O$.

B: 10% Folin-Ciocalteu phenol in 0.1 M NaOH.

Electrophoresis Buffer

25mM Tris, 192mM glycine, 3mM SDS.

Transfer Buffer

25mM Tris, 192mM glycine 20% v/v methanol.

Tris Buffered Saline with Tween (TBS-T)

25mM Tris, 125mM NaCl, 0.1% v/v Tween 20 pH 7.6.

Blocking Buffer/Antibody Buffer

5% (w/v) skimmed milk powder in TBS-T.

2.3.3 Sample Preparation Using Phosphate Protective Lysis Buffer

Medium was aspirated from cells and cells were given 3 x PBS washes before being harvested in 1.5ml PBS. This cell suspension was centrifuged at 6000rpm for 5 min at 4°C to pellet the cells. The PBS supernatant was removed, 150 µl lysis buffer was added per well and the cells lysed by sonication. Finally, the cell lysate was centrifuged at 10000rpm for 5 min at 4°C. 20 µl of lysate was aliquoted to measure protein concentration using the Lowry assay and 43 µl of 4X sample buffer was added to the remaining 130 µl of cell lysate for Western blotting.

2.3.4 Sample Preparation Using TriReagent

Culture medium was aspirated from cells and cells were given a PBS wash before being harvested in 1 ml TriReagent and incubated at room temperature for 5 min. 0.2 ml 1-bromo-3-chloropropane was added to this cell suspension, which was vortexed and centrifuged at 10000 rpm for 10 min at 4°C to separate into aqueous phase, interphase and organic phase containing RNA, DNA and protein respectively. The aqueous phase was removed for RNA isolation, interphase discarded and 950 µl isopropanol added to the organic phase to precipitate the protein. Following vortexing, this homogeneous solution was centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the protein pellet was washed with 0.3M guanidine hydrochloride in 95% ethanol and centrifuged at 7200 rpm for 5 min at 4°C, this wash and centrifugation was repeated a further 2 times. Following removal of the final wash, 1 ml 100% ethanol was added to the pellet and incubated at room temperature for 20 min. Following centrifugation at 7500 rpm for 10 min at 4°C, the supernatant was removed and 100 µl 1% SDS, 7M urea buffer added, samples were sonicated, followed by boiling at 99°C for 10 min to aid solubilisation.

2.3.5 Protein Determination

2.3.5.1 Lowry Assay

In a 96 well microassay plate, bovine serum albumin (BSA) (1mg /ml) was diluted with 0.1M NaOH in a volume up to 200 µl to achieve a series of protein concentrations 5-80 µg to be used as standards, in duplicate. 200 µl of 0.1M NaOH in duplicate was used as blank. 5 µl of protein extracted was made up to 200 µl with 0.1M NaOH and used for the assay in duplicate. 50 µl of reagent A was added to each well and incubated for 5 minutes at room temperature, followed by adding 50 µl of reagent B to each well and left for at least 15 minutes at room temperature. The absorbance of protein samples and standard BSA was measured at a wavelength of 630 nm.

Following calculation of the protein levels, all sample protein levels were normalised to 2µg/µl using a 1:3 mixture of 4X sample buffer and lysis buffer and stored at –20°C.

2.3.5.2 BCA Protein Assay

Protein determination was carried out according to manufacturer's instructions, using 1 µl sample per assay.

2.3.6 Electrophoresis

Samples were defrosted, heated to 95°C for 5 minutes, mixed briefly and spun in a microcentrifuge for 1 minute. Samples, of 50 μg protein each were then loaded on to 16% polyacrylamide gels for electrophoresis. PageRuler Prestained Protein Ladder Plus, a standard of marker proteins of known molecular weight, was run alongside the samples to show the distance travelled by protein of each weight. An internal control, made up of a pool of all samples was run on each gel such that a comparison could be made between gels as well as within each gel. Gels to be processed using the ECL system were run in duplicate, such that the total and phosphorylated forms of proteins could be detected, avoiding the need for stripping antibodies from membranes and re-incubating with antibodies. Gels to be processed using the Odyssey system were not run in duplicate as different secondary antibodies, that were visible at different wavelengths were bound to the total and phosphorylated forms, such that both forms could be visualised on the same gel and could be differentiated between. The gels were run in electrophoresis buffer at room temperature. Samples were separated for approximately 90 minutes at 100V using BioRad MiniProtean III equipment.

Once the sample proteins had been separated they were transferred onto a nitrocellulose membrane in transfer buffer, for 65 min at 105V, also using BioRad Mini Protean III equipment.

The nitrocellulose membrane was then stained with Ponceau S solution which stains all protein pink. This step is a quick check to ensure the running and

transfer steps have worked correctly as a band of protein should be clearly visible on the membrane. The Ponceau stain was washed off the membrane using water and TBS-T.

2.3.7 Blotting

The non-specific binding on the membranes was blocked using blocking buffer, gently agitated for 1 hour at room temperature. The lower half of the membrane, which contained the low molecular weight proteins, was then incubated with 1:500 dilution of anti-cyclophilin B. Cyclophilin B is used as a non-variant control protein to ensure slight differences in protein loading can be corrected for (Sinha *et al.* 2005). The upper half of each membrane was incubated with antibodies to ERK, pERK, Dok-1, PPARγ and pPPARγ at the dilutions shown in Table 2.1 and Table 2.2. All antibodies were diluted in antibody buffer. The membranes and antibody solutions were incubated overnight at 4°C in 50 ml centrifuge tubes on a roller.

Table 2.1 Details of primary and secondary antibodies used with ECL

	Cyclophilin	ERK	pERK
Approx. weight (kDa)	21	42/44	42/44
Primary antibody	Abcam ab3565 1:500	Cell Signaling #9107 1:250	Cell Signaling #9106 1:250
Secondary antibody	Dako Swine Anti- Rabbit P0217 1:2000	Dako Goat Anti- Mouse P0447 1:2000	Dako Goat Anti- Mouse P0447 1:2000
Film exposure time	10 min	1 hr	1 hr

The following day the excess antibody was removed with 9 washes in TBS-T over a one hour period, at room temperature. For secondary antibodies to be used in conjunction with ECL (Table 2.1), these were added, again in antibody buffer, and incubated at room temperature for one hour, while shaking. These secondary antibodies were conjugated with horse radish peroxidase (HRP) which catalyses the enhanced chemical luminescence (ECL) reagents in a light emitting reaction. Following secondary antibody incubation the membranes were again washed 9 times over one hour, with a final wash in water. The membranes were then incubated with the ECL reagents (Amersham, UK), as per manufacturer's directions, and exposed to autoradiography film (Kodak, UK).

Table 2.2 Details of primary and secondary antibodies used with Odyssey system

	Cyclophilin	ERK	pERK
Approx. weight (kDa)	21	42/44	42/44
Primary antibody	Abcam ab3565 1:500	Cell Signaling #9107 1:250	Cell Signaling #4370 1:250
Secondary Antibody	Odyssey 680 Anti-Rabbit 1:10000	Odyssey 680 Anti Mouse 1:10000	Odyssey 800 Anti Rabbit 1:10000

For secondary antibodies to be used in conjunction with the Odyssey system (Table 2.2), these were added, again in antibody buffer, and incubated in the dark, at room temperature for one hour, while shaking. Following secondary

antibody incubation the membranes were again washed 9 times over one hour. with a final wash in water. Protein bands were then visualised using the Odyssey scanner and software.

2.3.8 Determination of Density of Protein Bands

The density of the protein bands visualised by ECL was determined using a densitometer (BioRad, UK) and the manufacturer's software, Quantity One. The density of the protein bands visualised by the Odyssey system was determined using Odyssey software. To account for variations between gels, an internal control, constituting small volumes of each sample pooled together, was loaded onto each gel, and the cyclophilin B bands normalised to this, before being used to normalise all other protein bands. All densities were then expressed as a percentage of the mean vehicle control density for that sample group.

2.3.9 Statistical Analysis

Data were analysed with a one way ANOVA and Tukey post hoc test, using GraphPad Prism, version 4.02 (GraphPad Software Inc).

2.4 GTPγS Binding Assay

2.4.1 Materials

Human Embryonic Kidney (HEK) cells expressing rat recombinant CB2 receptors obtained from Dr Mary E Abood (Temple University, Philadelphia) were grown to confluence by Mauro Dionisi. [35S]GTPγ was purchased from GE Life Sciences (Bucks, UK).

2.4.2 Solutions

Assay buffer: 50 mM Tris-HCl, 9 mM $MgCl_2$, 0.2 mM EGTA, 150 mM NaCl, pH 7.4.

Wash buffer: 50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.4.

2.4.3 Method

HEK cells were grown to confluence and harvested using PBS containing EDTA. Cells were centrifuged at 25,000 rpm for 10 min at 4°C to separate all cell organelle membranes. The pellet was then resuspended in assay buffer, homogenised, and centrifuged as previously. The final pellet was then resuspended in assay buffer, homogenised, and diluted to a concentration of 2 μg/μl with assay buffer.

10 μg of homogenised HEK cell membranes were incubated in assay buffer, containing 0.1% BSA with GDP 100 μM, [^{35}S]GTPγ S 0.05 nM, and catechin, epicatechin or EGCG at 10 μM and 1 μM HU210, a CB_1 agonist in siliconised

glass tubes. The total assay volume was 0.5 ml, which was incubated for 90 min at 30°C in a shaking water bath. The reaction was terminated by the addition of 2 ml ice-cold wash buffer, followed by rapid filtration under vacuum through Whatman GF/C glass-fibre filters, washing with 2 x 3ml ice-cold wash buffer. Filters were transferred to 5 ml scintillation vials. Bound radioactivity was determined by liquid scintillation spectrophotometry after extraction in 3 ml scintillant overnight at room temperature. Non-specific binding was determined using 10 μM GTPγS. Basal binding was assayed in the absence of flavanol and in the presence of GDP. The stimulation by flavanol was defined as a percentage increase above basal levels (i.e., [dpm (flavanol)-dpm (no flavanol)] x 100).

2.5 2-Deoxyglucose Uptake Assay

2.5.1 Materials

[^{3}H] 2-DOG was purchased from GE Life Sciences (Bucks, UK).

2.5.2 Solutions

Reaction Buffer: 138 mM NaCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$, 4.8 mM KCL, 0.2% w/v BSA, 50 mM HEPES pH 7.4.

2.5.3 Method

3T3-L1 cells were seeded at 6 x 10^4 cells/well in 12 well plates, induced to differentiate once confluent, and maintained in medium 4. 24 hours prior to the start of the assay, medium was replaced with 12.5 mM glucose DMEM, followed by overnight serum starvation in 12.5 mM glucose medium. Cells were washed three times with PBS at 37°C and incubated with 1 ml reaction buffer (section 2.2.5) for 45 minutes. Cells were treated with 250 µl cytochalasin B for 10 minutes or EGCG/epicatechin for 30 min, 6 hr and 24 hr. 500 µl insulin (0.1 µM) was added or not, for 10 minutes, followed by the addition of 250µl of 27.8kBq [^{3}H] 2-DOG and 1.5mM 2-DOG for 10 min. Cells were then washed with ice cold PBS containing 10mM glucose (3x) and solubilised in 500µl 0.5M NaOH and 0.1% SDS. Cell lysate was transferred to scintillation vials and 5ml liquid scintillant was added. Samples were assayed for 2-DOG uptake as disintegrations/min/well using a Rackbeta liquid scintillation counter (LKB Instruments, Maryland, USA).

2.6 Gene Expression Studies

2.6.1 Materials

RNeasy Mini Total RNA Purification Kits were supplied by Qiagen (West Sussex, UK). Reagents for Reverse Transcription of RNA to cDNA were supplied by Invitrogen (Paisley, UK). TaqMan reagents were supplied by

Applied Biosystems (CA, USA). Primers and probes were supplied by Eurofins MWG GmBH (Ebersberg, Germany).

2.6.2 RNA and Protein Extraction

Cells were collected in 1 ml TriReagent and protein and RNA were simultaneously extracted (Sambrook *et al.* 2001).

2.6.3 Purification of RNA

Total RNA was further purified using the RNeasy Mini Total RNA Purification Kit according to the manufacturer's protocol, including an on column DNase digestion. Total RNA was quantified using absorption at 260 nm using a NanoDrop ® ND-1000 spectrophotometer. Total RNA was stored at -80°C until required.

2.6.4 Reverse Transcription of Total RNA to cDNA

The total RNA extracted from 3T3-L1 cells in Section 2.3.4 was reverse transcribed to cDNA using the reverse transcription reaction. Total RNA (500ng) was made up to a volume of 15 µl with DEPC treated water and 1.5 µl random primers (250 ng/µl) and 1.5 µl dNTP mix (10 mM) were added to make a total volume of 18 µl. This was heated in a thermal cycler (Primus-96, MWG Biotech) and heated to 65°C for 5 min and kept on ice. Following a

brief centrifugation, a 10.5 µl master mix was prepared and added to each sample, consisting of 6 µl 5X First Strand Buffer, 3 µl 0.1M DTT and 1.5 µl Recombinant RNasin Ribonuclease Inhibitor (1 U/µl). The mixture was incubated at 37°C for 2 min and 1.5 µl M-MLV (Moloney Murine Leukaemia Virus) Reverse Transcriptase (200 U/µl) added and mixed by pipetting gently. The mixture was then incubated at 37°C for a further 50 min and the reaction inactivated by heating at 70°C for 15 min. To remove RNA complementary to the cDNA, 1.5 µl *E.Coli* RNase H (2 U/µl) was added, and the reaction incubated at 37°C for 20 min. Following this, the mixture was diluted 1:4 with HPLC grade autoclaved water and stored at -20°C until required.

2.6.5 Analysis of mRNA Expression by TaqMan® Quantitative Real Time PCR

TaqMan reagents use a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles, and works as follows (see Figure 2.2).

Step 1 – an oligonucleotide probe is constructed with a fluorescent reporter dye bound to the 5' end and a quencher at the 3' end. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye by fluorescence resonance energy transfer (FRET) through space.

Step 2 – if the target is present, the probe anneals between primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase during extension.

Cleavage of the probe separates the reporter dye from the quencher, increasing the reporter dye signal, and also removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process.

Step 3- more reporter dye molecules are cleaved from their respective probes with each cycle, resulting in an increase in fluorescent intensity proportional to the quantity of amplicon produced. The higher the starting copy number of the nucleic acid target, the earlier a significant increase in fluorescence is observed.

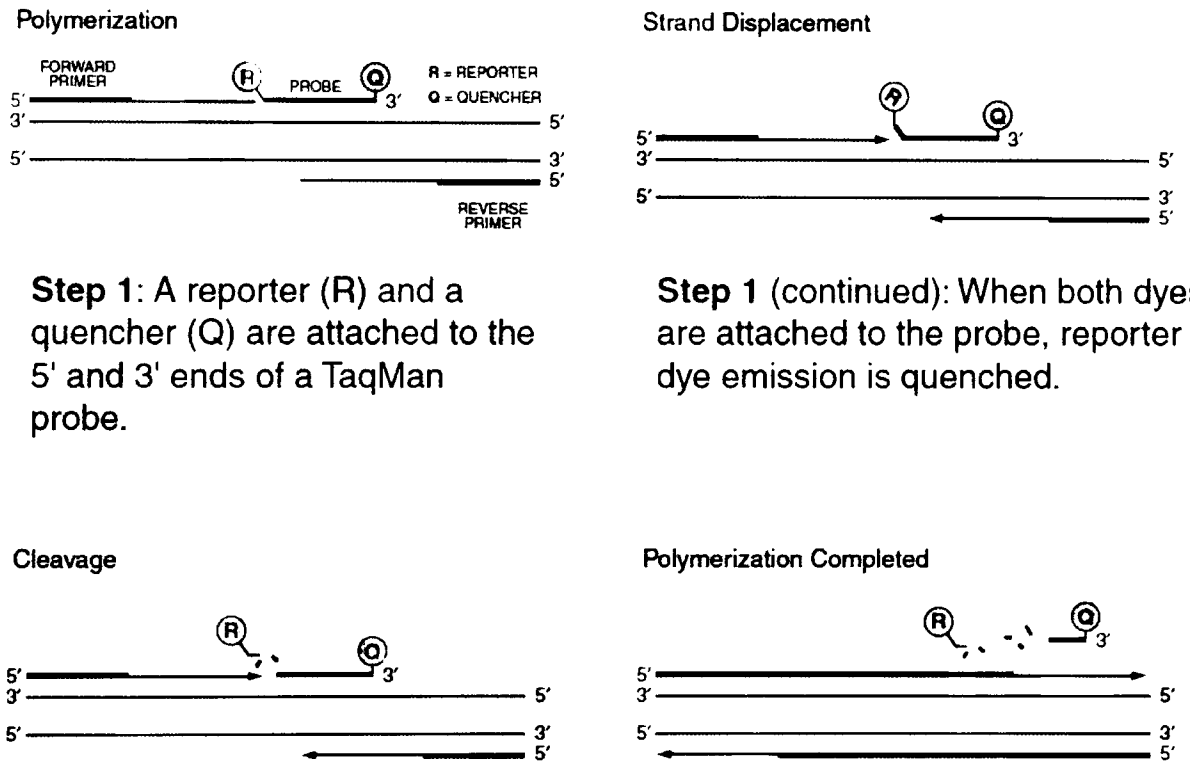


Figure 2.2 Use of fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles.
(From Relative Quantification: Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System: Getting Started Guide: Rev D (Part #: 4347824))

The probes used here for real-time PCR are hydrolysis probes and have a fluorescent reporter (FAM) in close proximity to a quencher (TAMRA), which prevents the fluorescent reporter from emitting fluorescent signals. As the PCR reaction progresses, the 5' nuclease activity of the polymerase cleaves the hydrolysis probe, releasing the fluorescent reporter from its quencher, resulting in an increase in fluorescence.

PCR amplification is characterised by three phases; lag, log-linear and plateau phases. In the lag phase, the increasing number of products remains below background fluorescence levels, until a critical point is reached where PCR amplification reaches an exponential phase (log-linear) when 10^{11}-10^{12} PCR product molecules are present. The reaction terminates when enzymes or other reaction components become limiting, also known as plateau phase.

The greater the amount of the target gene present, the fewer the number of cycles required to reach the log amplification phase for a particular gene.

Relative concentrations of mRNA can be determined by real-time PCR from standards prepared by serial dilutions of cDNA, which can also be used to determine PCR amplification efficiency. The most concentrated standard was prepared by combining 10 µl aliquots from each cDNA reaction together and standards were prepared by serial dilutions (4, 16, 64, 256:1 v/v) of the concentrated standard, which was given a concentration value of 1.

2.6.6 Primer and Probe Design for Real-time PCR

Primers and probes for PPARγ, adiponectin, resistin and leptin were designed using Primer Express® Software, version 3 (Applied Biosystems) Where possible, primer and probe sets were designed to cross intron-exon boundaries of the gene of interest to minimise risk of amplification from chromosomal DNA. Specificity of primers and probes were analysed (BLAST N) to ensure the gene of interest would be the primary target for amplification. Primers and probes were diluted to a 10 μM working concentration and stored at -20°C (see Table 2.3 for details). TaqMan ® Gene Expression Assays were ordered for adipsin (complement factor D), FABP4, PEPCK, SREBF1, eukaryotic 18S RNA and beta actin, details of which can be found in Table 2.4

Table 2.3 Oligonucleotide sequences of primers and probes used in RT-PCR. Primers and probes were designed using Primer Express® Software, version 3, blasted using http://www.ncbi.nih.gov and synthesised by Eurofins MWG GmbH.

Gene		Sequences (5'→3')	Amplicon size (bp)	GenBank Accession No.
PPARγ	FW:	CCCTGGCAAAGCATTTGTAT	225	NM_001127330
	REV:	GAAACTGGCACCCTTGAAAA		
	PROBE:	GGGCGATCTTGACAGGAAAGACAA		
Adiponectin	FW:	TGGATCTGACGACACCAAAA	157	NM_009605
	REV:	ATCCAACCTGCACAAGTTCC		
	PROBE:	CCAGTCATGCCGAAGATGACGTTA		
Resistin	FW:	TCATTTCCCCTCCTTTTCCT	210	NM_022984
	REV:	CAAGACTGCTGTGCCTTCTG		
	PROBE:	TCTTCCTTGTCCCTGAACTGCTGG		
Leptin	FW:	TGACACCAAAACCCTCATCA	213	NM_008493
	REV:	TCATTGGCTATCTGCAGCAC		
	PROBE:	CACTGGCTTGGACTTCATTCCTGG		

Table 2.4 TaqMan Inventoried Gene Expression Assays (Applied Biosystems)

Gene	Assay ID	Amplicon size (bp)	GenBank Accession No.
FABP4	Mm00445878_m1	74	NM_024406
Adipsin (complement factor D)	Mm00442664_m1	102	NM_013459
PEPCK	Mm01247058_m1	61	NM_011044
SREBF1	Mm01138344_m1	80	NM_011480
Beta-actin	Mm00607939_s1	115	NM_007393
Eukaryotic 18S ribosomal RNA	Hs99999901_s1	187	X03205

2.6.7 Real-time PCR Assay

A master mix for the target gene was made up for the number of wells required (see Table 2.5)

Table 2.5 PCR Master Mix components

	Volume per well (µl)
TaqMan 2X Universal PCR Master Mix	13
Forward Primer	0.75
Reverse Primer	0.75
Probe	0.5
HPLC Water	5
TOTAL	20 µl per well

AmpErase ® Uracil-N-Glycosylase (UNG) and AmpliTaq Gold ® DNA Polymerase are components of the TaqMan Universal PCR master mix. AmpErase UNG treatment can prevent the reamplification of carryover-PCR products by removing any uracil incorporated into single- or double-stranded DNA. AmpliTaq Gold DNA Polymerase ensures a robust reaction and dramatically reduces the amount of non-specific product formation.

20 µl master mix and 5 µl cDNA standard or unknown sample was added to each well, (all samples and standards were assayed in triplicate), sealed with optical transparent film and cycled as outlined in Table 2.6.

Table 2.6 TaqMan cycle details

Temperature	Time		Purpose
50°C	2 min	HOLD	AmpErase® UNG Activation
95°C	10 min	HOLD	AmpliTaq Gold® DNA Polymerase Activation
95°C	15 sec	40 CYCLES	Melt
60°C	1 min		Anneal/Extend

The important parameter for quantification is the threshold cycle, or the Ct value. The Ct value is the cycle at which the fluorescent signal is first recorded as statistically significant above baseline and will always occur during the exponential phase of amplification of PCR product. The standard curve was generated by plotting the Ct values against the logarithm of the initial cDNA dilutions. Data from TaqMan was only used when the slope of the standard curve was -3.2 to -3.6 (~ 100% efficiency) and when the Ct value for the triplicate readings for an individual sample were less than 0.5 Ct apart.

β-actin and 18S ribosomal RNA were used as the non-variant normalising genes and all gene expression data were normalised to β-actin levels.

Adipokines:

investigation of adiponectin and resistin release from 3T3-L1 adipocytes

3 Adipokines

3.1 Introduction

Adipose tissue is responsible for storage of excess energy as fat, and is able to release this as fatty acids and glycerol in periods of fasting and starvation. Adipose tissue is now being regarded as an endocrine organ, which is able to secrete a range of bioactive polypeptides, known as adipokines. These include adiponectin, resistin, leptin, adipsin, visfatin, interleukin-6 (IL-6), tumour necrosis factor-α (TNFα), serum amyloid A (SAA), plasminogen activator inhibitor-1 (PAI-1), angiotensinogen, vaspin and omentin.

The 3T3-L1 adipocyte cell model has been reported to secrete a number of adipokines into their media. This occurs under basal conditions and can also be regulated by the addition of insulin, growth factors and other agents (Hu *et al.* 1996; Kim *et al.* 2001). The first aim of this study was to set up a reliable cell culture system in which the release of the adipokines adiponectin and resistin from mature 3T3-L1 adipocytes could be measured, and secondly, investigate the release of these adipokines under the control of flavanols.

3.2 Effect of tissue culture plate type

While establishing 3T3-L1 cell culture conditions in six well plates in order to carry out these studies, disappointingly low differentiation efficiency was observed. These cells had not lost their ability to differentiate as good differentiation was seen when cells were plated in flasks. It was therefore

decided that the differentiation of these cells in different plate types would be examined. Information regarding plate type and brand used for 3T3-L1 studies in the literature has not been reported in detail. Significant differences in differentiation were observed between plates, and since efficient differentiation of these cells is crucial for their study, it is important that conditions providing the highest level of differentiation are used.

Table 3.1 Suppliers of cell culture plates and dishes used

Brand/Supplier	Plate Description	Catalogue Number
Corning Costar ® (Corning Ltd. Sunderland, UK)	6 well, 35 mm, tissue culture treated, flat bottom, polystyrene microplates	3506
Nunclon ™ Δ (Nalge Ltd. Hereford, UK)	6 well, 35 mm, flat bottom polystyrene	140685
Orange Scientific (Orange Scientific. Belgium)	Tissue culture 6 well test-plate, 35 mm diameter, gamma sterilised, tissue culture treated.	20030009
Falcon ® (Becton, Dickinson, Falcon. Oxford, UK)	6 well, 35 mm diameter, Multiwell ™ tissue culture plate, flat-bottom with low evaporation lid, surface-modified polystyrene.	3846
Falcon ® (Becton, Dickinson, Falcon. Oxford, UK)	6 well, 35 mm diameter, Multiwell ™ tissue culture plate, flat-bottom with low evaporation lid, tissue culture treated by vacuum gas plasma.	3046
Iwaki (Iwaki, Asahi Techno Glass Corporation. Tokyo, Japan)	6 well, 35 mm diameter, TC-treated, flat bottom, polystyrene.	3810-006
Sarstedt. (Sarstedt Ltd. Leicester, UK)	Tissue culture 6-well plate, 35 mm diameter, flat-bottom, Cell+ treated.	83.1839.300
Nunclon ™ Δ (Nalge Ltd. Hereford, UK)	35 mm diameter dish, polystyrene	150318

Cells were seeded in plates and dishes (listed in Table 3.1) at 6 x 10^4 cells/35mm well and differentiated as detailed in Chapter 2. Once differentiated, medium was removed to fix and stain the cells, as detailed in Section 2.1.4.

Six 35 mm wells were seeded per plate in each plate used, alternatively cells were seeded into six 35mm dishes. Adipocytes and fibroblasts in 3 fields/35mm well were counted at x40 magnification, and differentiation efficiency calculated as mean number of adipocytes as a percentage of total cells per well ± SEM.

Cells were photographed at x40 magnification under a light microscope using a Sony Cybershot 5.1-megapixel digital camera.

3.2.1 Results

The highest differentiation efficiency was observed in cells plated in Nunclon ™Δ 35 mm dishes, and the lowest differentiation efficiency was observed in cells plated in Corning Costar ® 6 well plates. Figure 3.2 shows a photographic comparison of cell differentiation in the two plate types. The differentiation efficiency seen in Nunclon ™Δ 35 mm dishes was significantly higher than in any other plate brand used in this study ($p<0.001$) as shown in Figure 3.1.

Corning Costar ® and Nunclon ™Δ 35 mm dishes were used in 2 more independent experiments. Differentiation efficiencies 25% ± 0.84 and 35% ±

0.83 (n=6, triplicate determinants) were observed for Corning Costar ® and 67% ± 0.80 and 73% ± 1.15 (n=6, triplicate determinants) were observed for Nunclon ™Δ 35 mm dishes (see Figure 3.3).

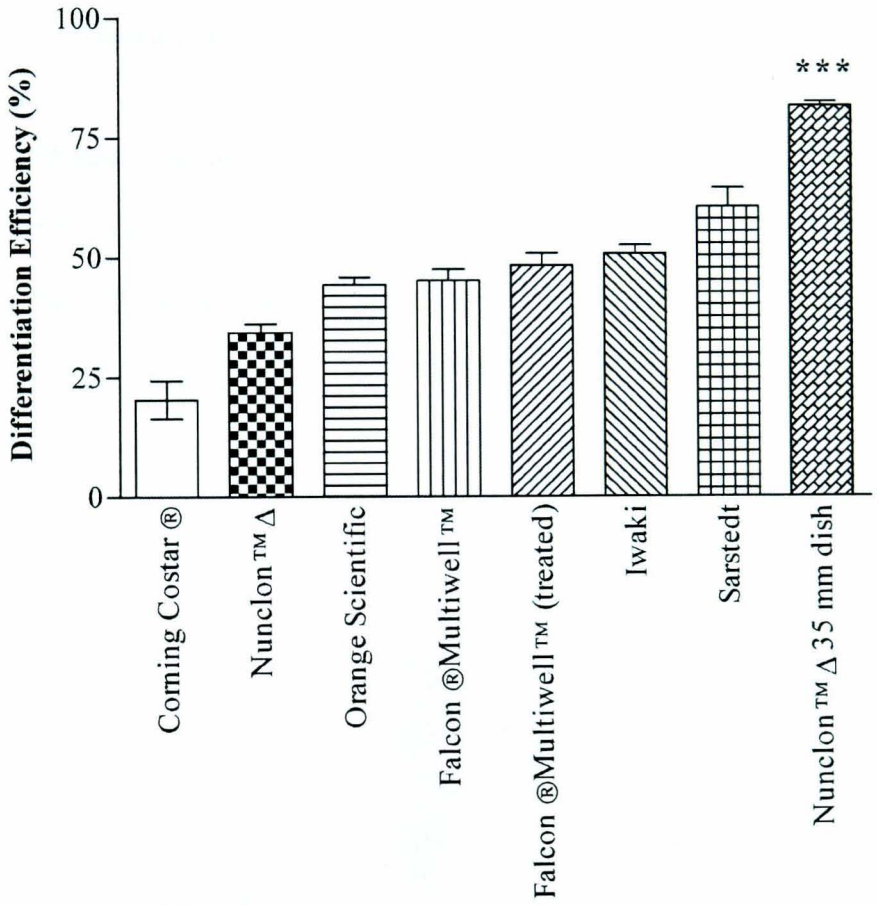


*Figure 3.1 Effect of plate type on percentage differentiation of 3T3-L1 cells. Results are expressed as percentage of differentiated 3T3-L1s ± SEM, (n=6, triplicate determinants). Statistical analysis was carried out using one-way ANOVA, and Tukey post-hoc test. *** $p<0.001$ compared to all other plate brands.*

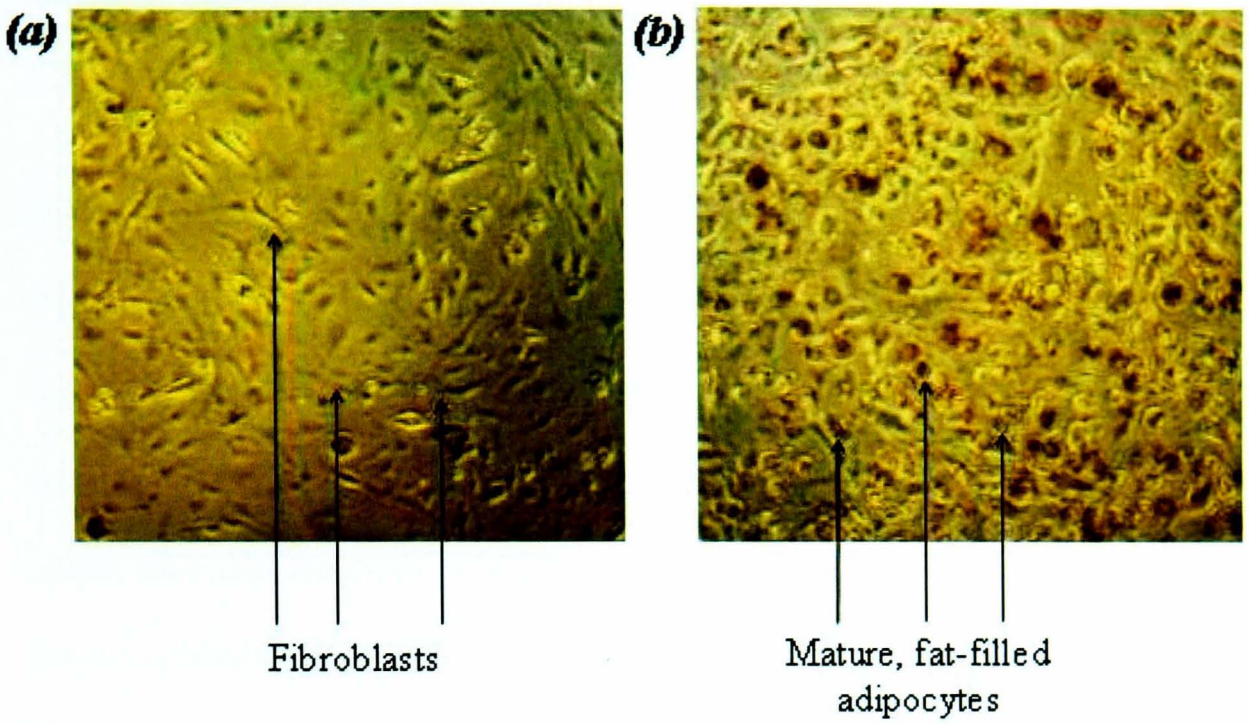


Figure 3.2 Photographs of cells plated and differentiated on (a) Corning Costar ® and (b) Nunclon ™Δ 35 mm dishes.

Cells were fixed and stained with Oil Red O. Images were taken at x40 magnification under a light microscope using a Sony Cybershot 5.1-megapixel digital camera.

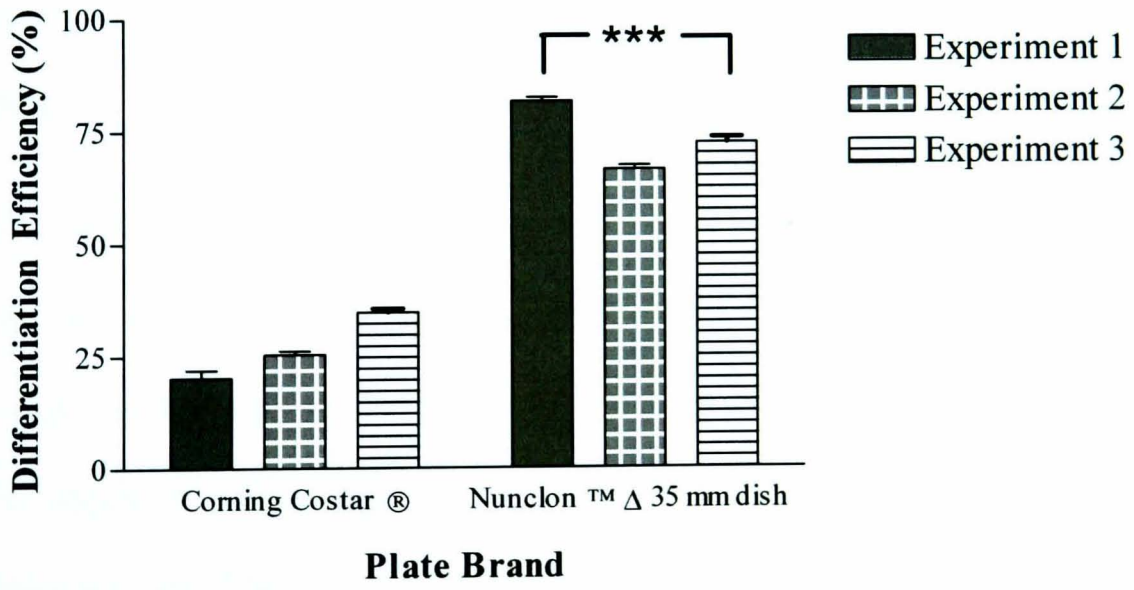


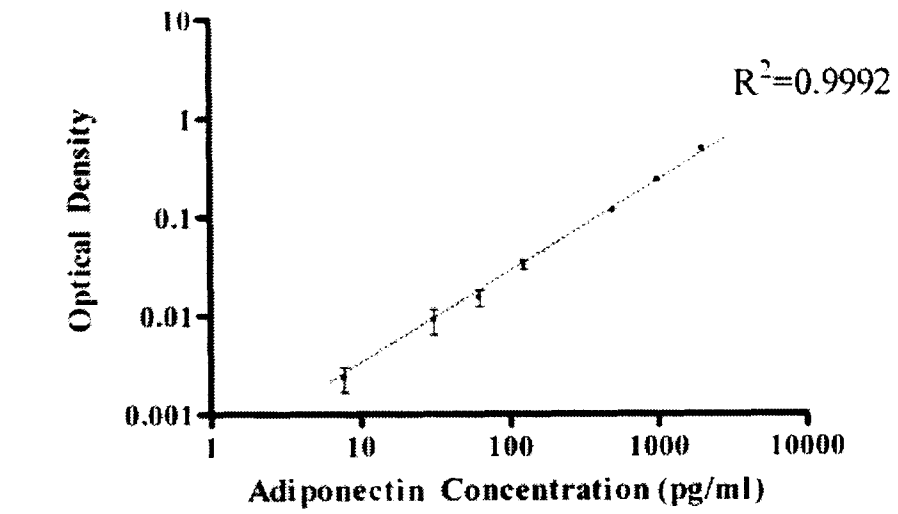
*Figure 3.3 Effect of plate type on percentage differentiation of 3T3-L1 cells. Three independent experiments using Corning Costar ® and Nunclon™ Δ 35 mm dishes. Results are expressed as percentage of differentiated 3T3-L1s ± SEM, (n=6, triplicate determinants). Statistical analysis was carried out using one-way ANOVA, and Tukey post-hoc test. *** $p<0.001$ compared to Corning Costar ®.*

3.3 Measurement of Adipokines

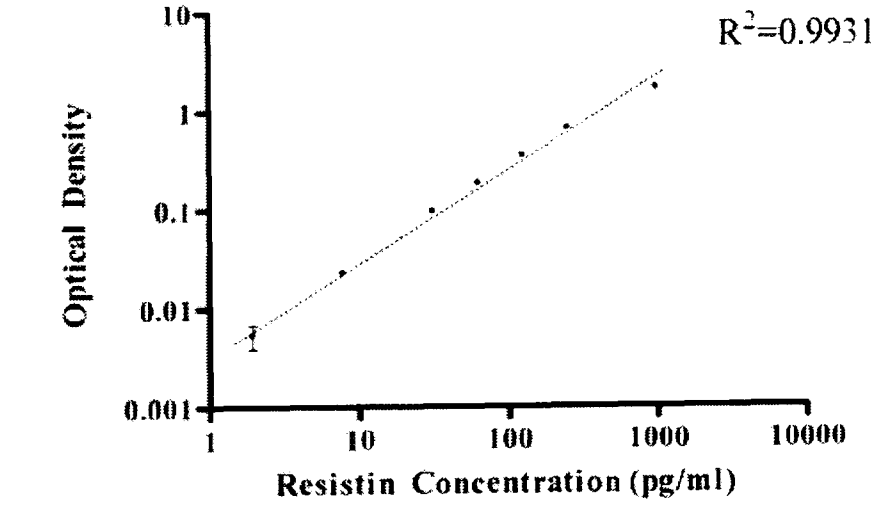
Following the development of a system in which 3T3-L1 fibroblasts were able to efficiently differentiate into mature adipocytes, the next stage of the study was to find and optimise a method for measurement of the secreted adipokines adiponectin and resistin. There are a number of kits to measure adipokines which are commercially available. The mouse adiponectin and resistin Duosets (R&D Systems Europe Ltd, Abingdon, UK) were chosen on the basis that they are specifically designed for the analysis of cell culture supernatants and are complete with protocols optimised for antibody concentrations and a protein standard which is mass calibrated to eliminate variation between assay kits. Standard curves for each adipokine had to be optimised, to ensure that all samples were falling within the middle of the range. Additionally, standard curves were constructed in medium, and blank medium was run as a control, instead of the suggested 1% BSA in PBS.

For validation of each assay, standard curves with R^2 values less than 0.97 were discounted, as were samples with replicates that were more than 2 standard deviations out. Catechin, epicatechin and EGCG treated samples for each adipokine within each experiment were run at the same time, to ensure consistency and allow these samples to be compared. For each treatment and time point, 6 dishes were cultured (n=6) and duplicate determinants of each of these were assayed for adipokine release.

Figure 3.4 shows standard curves for adiponectin and resistin, resulting from use of R&D Systems' mouse adiponectin and resistin Duosets, representative of those used in these studies.



(a)



(b)

Figure 3.4 Representative standard curves for adiponectin (a) and resistin (b) from Duosets (R&D Systems) used for analysis of cell culture supernatant. Standard curves with R^2 values of less than 0.97 were discounted from studies.

3.4 Effect of Flavanols

In order to investigate the influence of dietary factors on adipokine release from 3T3-L1 adipocytes, the effects of the green tea and cocoa flavanols, catechin, epicatechin and EGCG, were investigated.

Cells were differentiated as detailed in Chapter 2, treated with varying concentrations of catechin, epicatechin or EGCG for the required time, media removed and assayed for adiponectin or resistin using mouse adiponectin and resistin duoset ELISA kits.

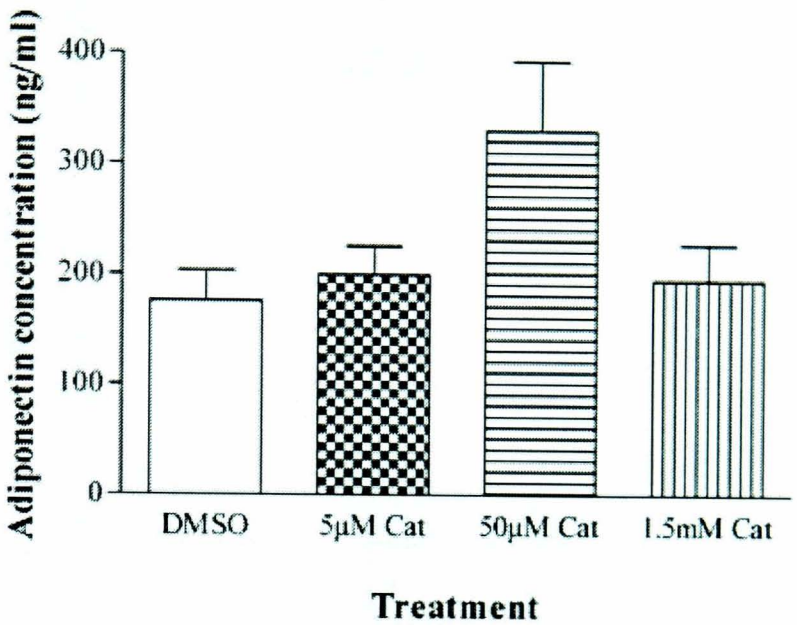
3.4.1 Preliminary Concentration Studies

In order to determine whether catechin or epicatechin had any effect on adiponectin or resistin release from 3T3-L1 adipocytes, cells were treated with 5 μM, 50 μM and 1.5 mM catechin and epicatechin.

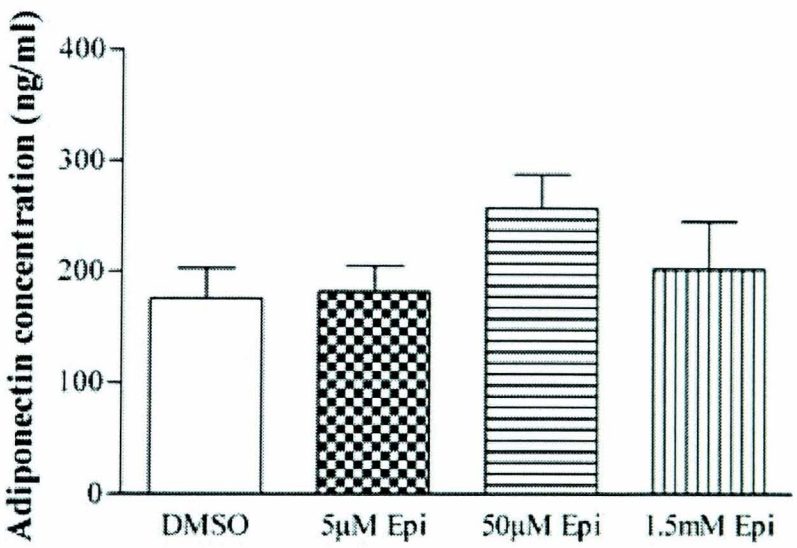
3.4.1.1 Results

Following this preliminary experiment there was evidence of a dose response effect on both adiponectin and resistin release, by both catechin and epicatechin. Both 50 μM catechin and epicatechin appeared to show a trend towards increasing adiponectin release, but this was not significant (see Figure 3.5). The highest concentrations of catechin and epicatechin, 1.5 mM, significantly decreased resistin release from 3T3-L1 adipocytes ($p<0.001$, $n=6$)

after 24 h treatment (see Figure 3.6). However, this concentration cannot be achieved physiologically, so further studies with catechin and epicatechin in the physiological range of 1-10 μM were pursued, and a time course set up.

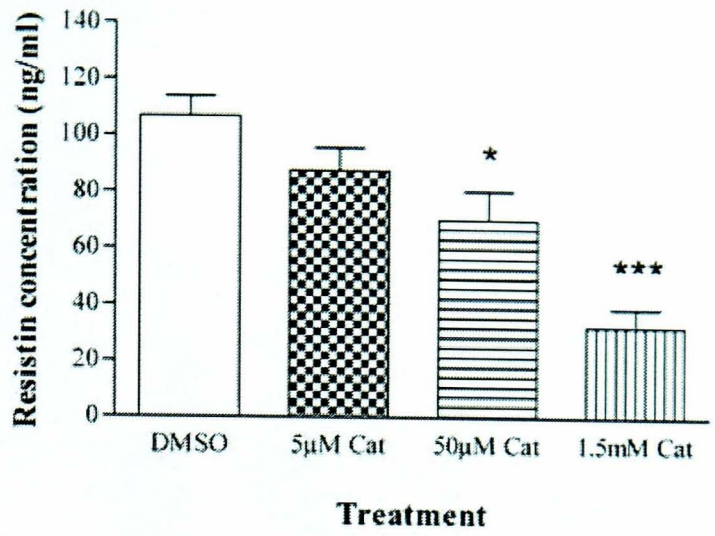


(a)

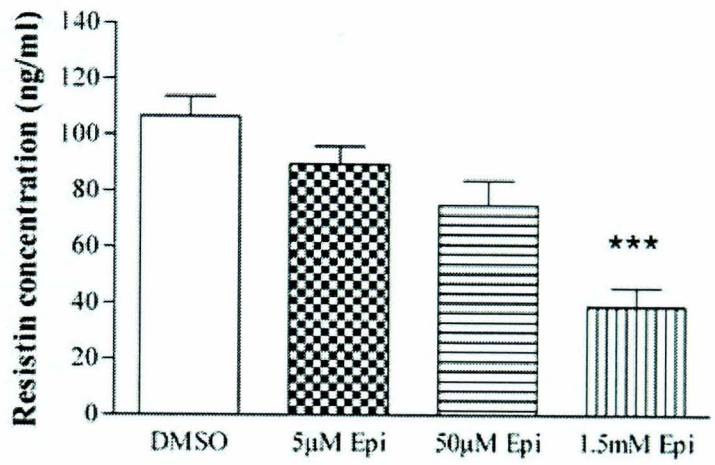


(b)

Figure 3.5 Effect of 5 μM, 50 μM and 1.5 mM catechin (a) and epicatechin (b) on adiponectin release from mature 3T3-L1 adipocytes.
Results are expressed as adiponectin concentration of medium, removed from 35 mm dishes after 24 h treatment ± SEM. (n=6, duplicate determinants). Standard Curve: R =0.9769.



(a)



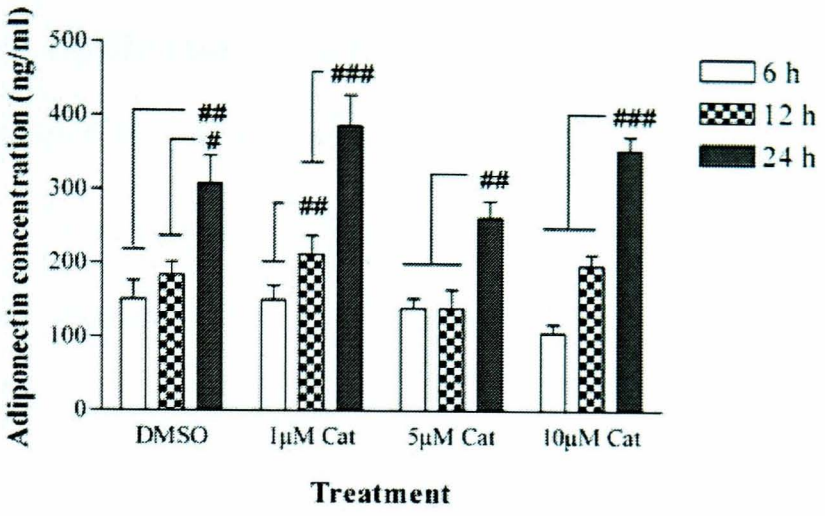
(b)

Figure 3.6 Effect of 5 μM, 50 μM and 1.5 mM catechin (a) and epicatechin (b) on resistin release from mature 3T3-L1 adipocytes.
Results are expressed as resistin concentration of medium, removed from 35 mm dishes after 24 h treatment ± SEM. (n=6, duplicate determinants).
** $p<0.05$ and *** $p<0.001$ compared to DMSO. Results analysed using one-way ANOVA, and Tukey post-hoc test. Standard Curve: $R^2=0.9764$.*

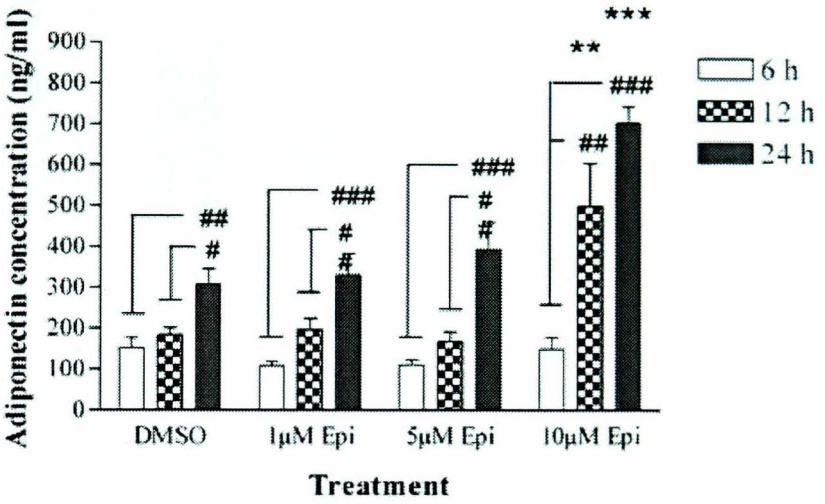
3.4.2 Concentration Range Studies

Further to the preliminary study, catechin and epicatechin in the 1-10 μM concentration range were investigated, and a treatment time course of 6, 12 and 24 h was set up. Methods were carried out as detailed in Section 2.

3.4.2.1 Results



(a)



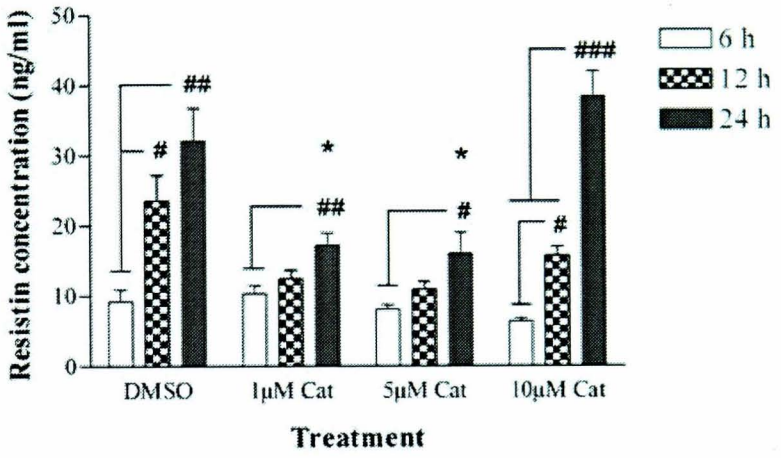
(b)

Figure 3.7 Effect of 1, 5 and 10 μM catechin (a) and epicatechin (b) on adiponectin release from mature 3T3-L1s.

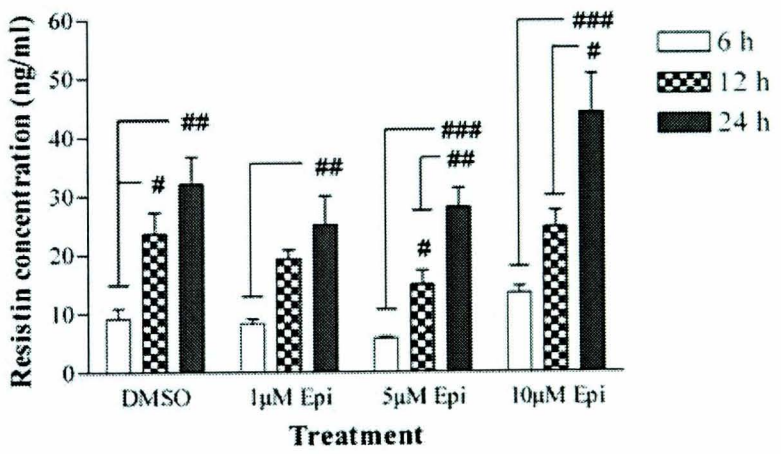
*Results are expressed as adiponectin concentration of medium, removed from 35 mm dishes after 6, 12 and 24 h ± SEM. (n=6, duplicate determinants). Standard Curve: R^2 =0.9788. ###p<0.001, ##p<0.01, #p<0.05 compared to indicated treatments. ***p<0.001 and **p<0.01 compared to DMSO control at same time point. Results analysed using one-way ANOVA, and Tukey post-hoc test.*

There appears to be an increase in basal levels of adiponectin and resistin over time, as a result of DMSO treatment, and this remains apparent when catechin

or epicatechin treatments are added. Significant changes in adipokine release are not seen after 6 or 12 h treatment with either catechin or epicatechin. 10 μM epicatechin significantly increased adiponectin release from 3T3-L1 adipocytes compared to DMSO control after 12 h ($p<0.01$, $n=6$) and 24 h ($p<0.001$, $n=6$). There appears to be a dose response effect with epicatechin however catechin appears to exert no significant effect on adiponectin release from 3T3-L1 adipocytes (see Figure 3.7).



(a)



(b)

Figure 3.8 Effect of 1, 5 and 10 μM catechin (a) and epicatechin (b) on resistin release from mature 3T3-L1s.

*Results are expressed as resistin concentration of medium, removed from 35 mm dishes after 6, 12 and 24 h ± SEM. (n=6, duplicate determinants). Standard Curve: $R^2=0.9881$. ###$p<0.001$, ##$p<0.01$, #$p<0.05$ compared to indicated treatments. *$p<0.05$ compared to DMSO control at same time point. Results analysed using one-way ANOVA, and Tukey post-hoc test.*

The release of adiponectin as a result of 5 μM catechin treatment for 24 h appears to be comparable with the levels seen in the previous experiment (see Figure 3.5 (a), approximately 200 ng adiponectin released and Figure 3.7 (a), approximately 25 ng adiponectin released at 24 hours)

Catechin at 1 and 5 μM significantly decreased resistin release compared to DMSO after 24 h ($p<0.05$, $n=6$). 10 μM catechin significantly increased resistin release compared to 1 and 5 μM catechin after 24 h ($p<0.05$, $n=6$). 10 μM epicatechin significantly increased resistin release compared to 1 μM epicatechin treatment for 24 h ($p<0.05$, $n=6$), but this was not significantly different to DMSO.

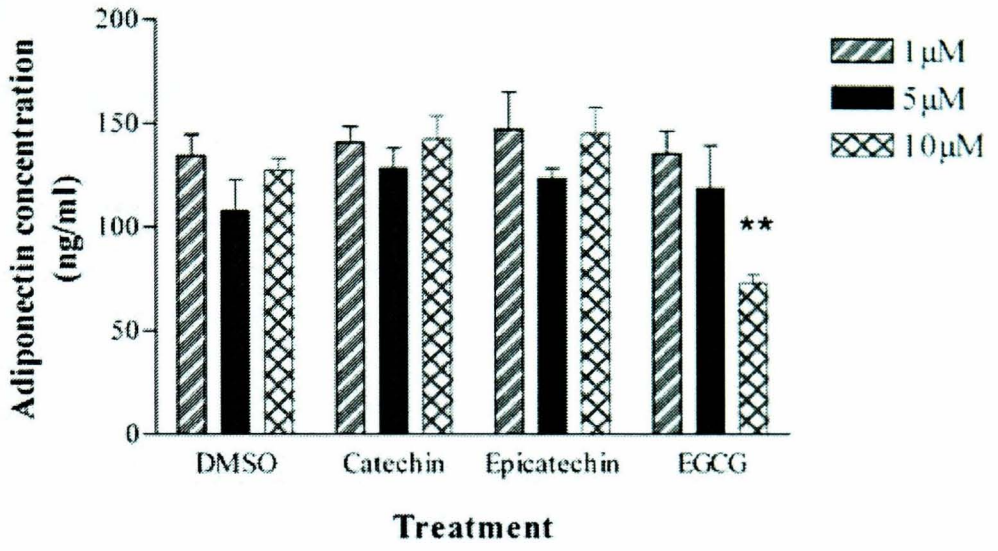
3.4.3 Effect of EGCG

At higher (10 μM) concentrations, epicatechin appears to be a more potent regulator of adiponectin release than catechin (compare Figure 3.7 (b) with Figure 3.7 (a)), but effects with both flavanols on resistin release, are comparable. It has been reported that EGCG is a more potent green tea flavanol due to the gallyl and galloyl groups which allow conformational flexibility and a large number of hydroxyl groups which may be important for hydrogen bonding (Hung *et al.* 2005). Therefore effects of greater magnitude on adipokine release may be expected.

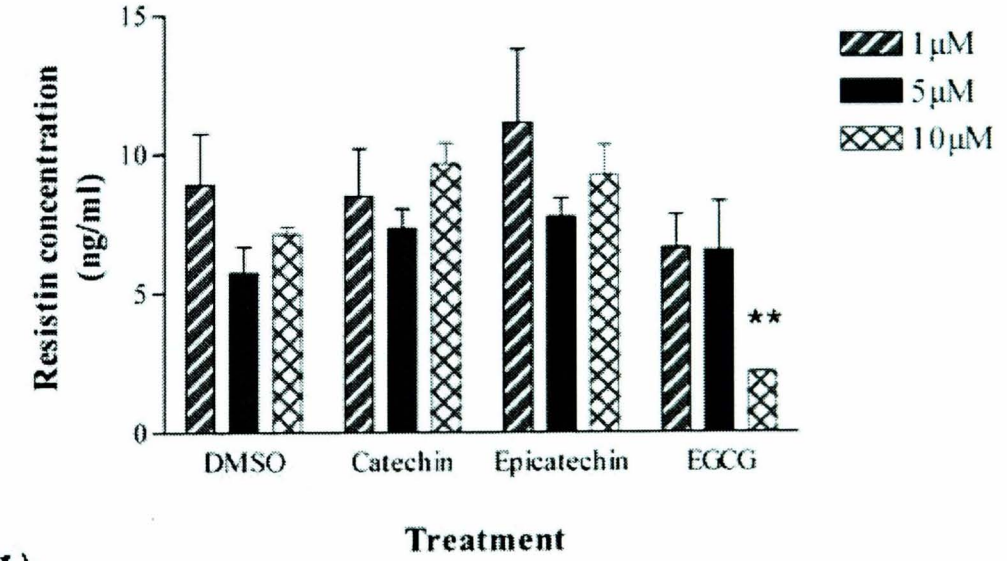
As there was no significant effect of either catechin or epicatechin on adipokine release after 6 or 12 h, a 24 h the dose response for 1, 5 and 10 μM was repeated at the 24 h time point, and EGCG was included in this next study.

3.4.3.1 Results

The effect of 10 μM epicatechin on adiponectin release after the 24 h treatment period was not seen to be significantly higher than the DMSO control, as seen in the previous study (see Figure 3.7 (b)).



(a)



(b)

Figure 3.9 Effect of 24 h treatment on adiponectin (a) and resistin (b) release from mature 3T3-L1 adipocytes using 1, 5 and 10 μM catechin, epicatechin and EGCG

Results are expressed as adiponectin or resistin concentration of medium, removed from 35 mm dishes ± SEM. (n=6, duplicate determinants). Standard

Curve: R^2*=0.9928.* ***p<0.01 compared to DMSO control at same time point. Results analysed using one-way ANOVA, and Tukey post-hoc test.*

10 μM catechin did not produce significantly higher levels of resistin release than 1 μM catechin treatment (Figure 3.9 (b)), contrary to previous observations (see Figure 3.8 (a)).

EGCG, at 10 μM, significantly decreased both adiponectin and resistin release compared to the DMSO control ($p<0.01$, $n=6$)

Decreased secretion rates were observed in the control state, 123.27±6.65 ng/ml compared to 308.00±37.61 ng/ml previously observed for adiponectin, and 7.26±0.75 ng/ml compared to 32.08±4.71 ng/ml previously determined for resistin. Therefore it may have been difficult to see any suppression of secretion with the addition of flavanols. EGCG may be a more potent agent; consequently it is able to reduce adiponectin and resistin in the under-secreting state.

3.5 Effect of Medium Glucose Concentration

Methods for 3T3-L1 differentiation and maintenance in the literature state that cells should be grown, differentiated and maintained in DMEM with a glucose concentration of 25 mM, substantially higher than the physiological glucose concentration of 5.5 mM. 25 mM glucose provides enough glucose to the cells to allow the medium to be replaced every 48 h, however, this makes it difficult to draw comparisons between the 3T3-L1 adipocyte model and a physiological situation. As a 5.5 mM medium glucose concentration would be too low for

the cells to survive for more than a few hours, it was decided that a concentration of 12.5 mM would be tested. This glucose concentration is closer to the upper physiological concentration limit (11mmol/l) and would allow the medium to be replaced every 24 h.

3.5.1 Comparison with Rosiglitazone

In order to investigate whether medium glucose concentration affects the cells' response to rosiglitazone and their ability to secrete adipokines into the medium, 3T3-L1 cells were grown and differentiated in Nunclon dishes as previously described. Mature 3T3-L1 adipocytes were then treated with 10 μM rosiglitazone for 24 h in either 12.5 mM or 25 mM glucose DMEM. Media were collected and assayed as previously described.

3.5.1.1 Results

Rosiglitazone significantly increased adiponectin release compared to the DMSO control ($p<0.01$ for 12.5 mM glucose medium and $p<0.05$ for 25 mM glucose medium, $n=6$). There was no significant difference in adiponectin release between cells treated in 12.5 mM and 25 mM glucose media in both the untreated and rosiglitazone treated state (see Figure 3.10).

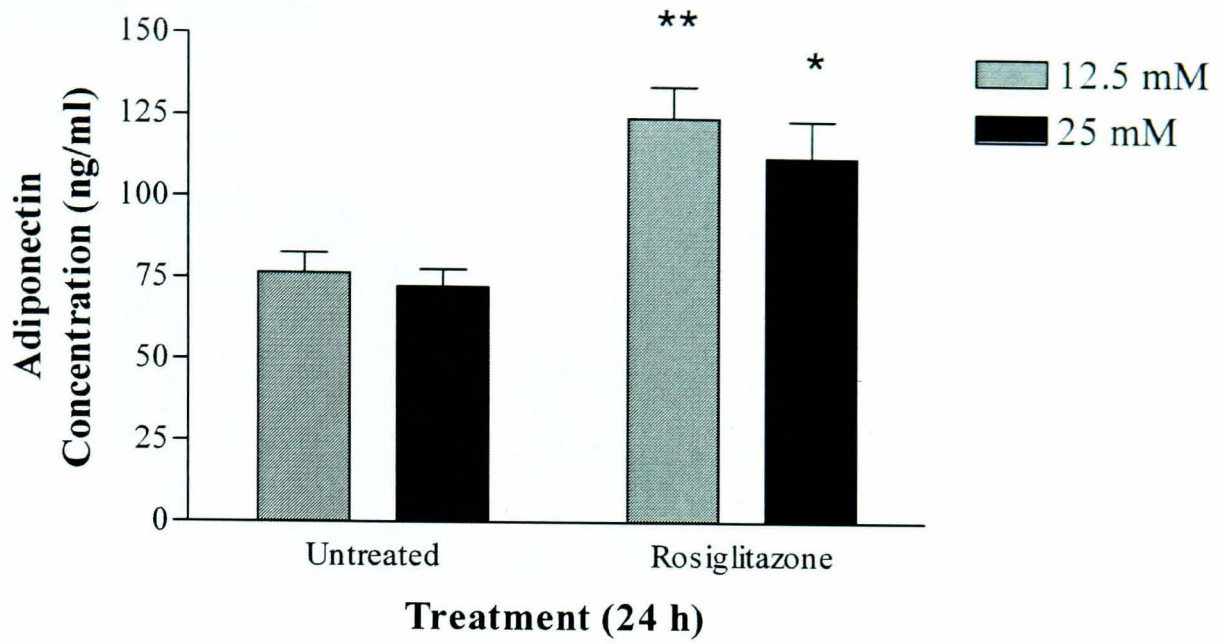


Figure 3.10 Adiponectin release from mature 3T3-L1 adipocytes grown on Nunclon 35 mm dishes, treated with 10 μM rosiglitazone for 24 h in 12.5 mM or 25mM glucose DMEM.

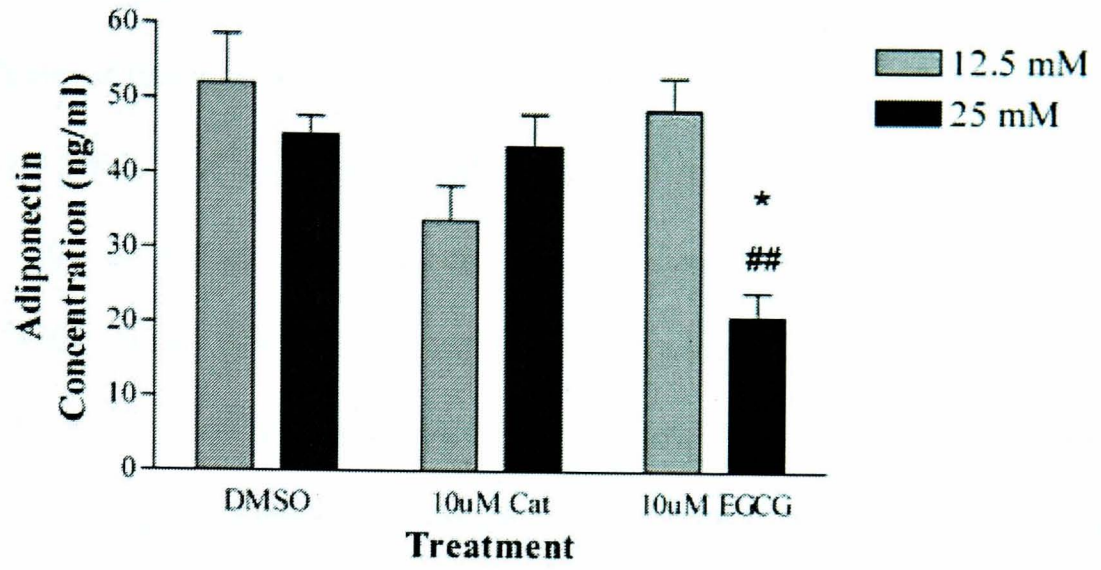
*Results are expressed as adiponectin concentration of medium, removed from 35 mm dishes ±SEM (n=3, duplicate determinants). Standard Curve: R^2=0.9940. Results analysed using one-way ANOVA, and Tukey post-hoc test. *p<0.05 and **p<0.01 compared to untreated.*

3.5.2 Effect of Catechin and EGCG on Adipokine Release

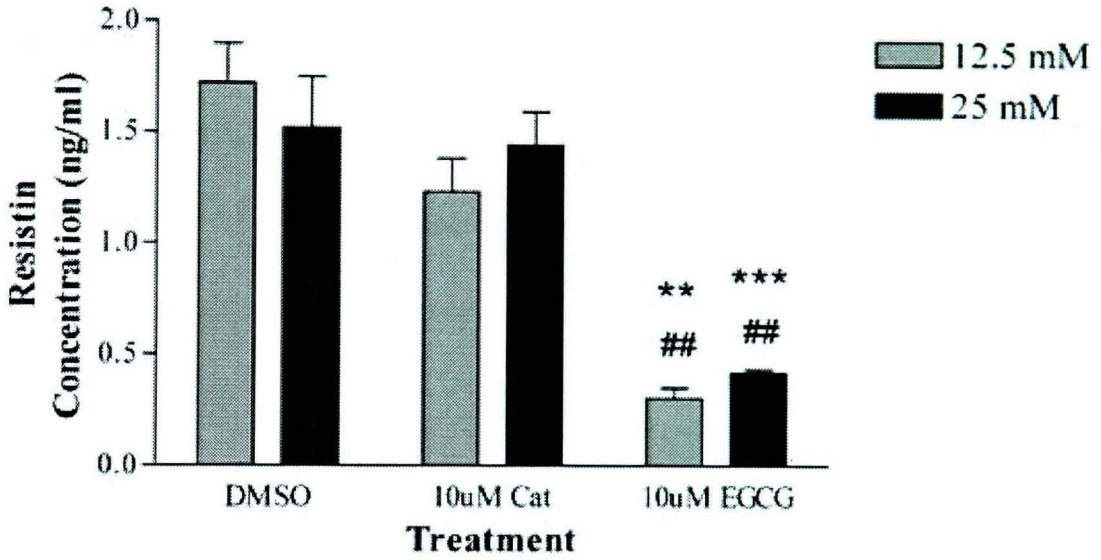
According to the previous study there did not appear to be a difference between adiponectin release from 3T3-L1 adipocytes treated with rosiglitazone in 12.5 mM and 25 mM glucose media. It was also important to check whether medium glucose levels had an effect on the ability of flavanols to mediate adipokine release.

3T3-L1 cells were grown and differentiated in Nunclon dishes as previously described. Mature 3T3-L1 adipocytes were then treated with 10 μM catechin or EGCG for 24 h in either 12.5 mM or 25 mM glucose DMEM. Media were collected and assayed as previously described.

3.5.2.1 Results



(a)



(b)

Figure 3.11 Release of adiponectin (a) and resistin (b) from mature 3T3-L1 adipocytes grown on Nunclon 35 mm dishes, treated with 10 μM Catechin and EGCG for 24 h in 12.5 mM or 25mM glucose DMEM.

*Results as adiponectin or resistin concentration of medium, removed from 35 mm dishes±SEM (n=3, duplicate determinants). Standard Curve: R^2=0.9888 Results analysed using one-way ANOVA, and Tukey post-hoc test. *, ** and *** are $p<0.05$, $p<0.01$ and $p<0.001$ respectively, compared to DMSO control in same concentration of medium glucose. ## $p<0.01$ compared to 10 μM catechin in same concentration of medium glucose.*

There is no significant difference between adiponectin or resistin release from cells treated with DMSO in 12.5 and 25 mM glucose media. There is also no significant difference between release of these adipokines when cells are treated with 10 μM catechin at either medium glucose concentration. With EGCG, however, adiponectin release was significantly decreased compared to the DMSO control when both groups were treated in 25 mM glucose medium ($p<0.05$, $n=6$). Adiponectin release in the presence of 25 mM glucose medium is also significantly different to 10 μM EGCG treatment in 12.5 mM glucose medium (see Figure 3.11 (a)).

With regard to resistin release, 10 μM EGCG significantly decreased this in the presence of both 12.5 and 25 mM glucose media compared to the DMSO control ($p<0.01$, $n=6$). There is no significant difference in release of resistin from the 3T3-L1 adipocytes between the two medium glucose concentrations (see Figure 3.11 (b)).

3.6 Discussion

The absence of a clear molecular marker for adipogenesis, along with the occurrence of adipose tissue in multiple, dispersed sites around the body has made it difficult to isolate and study preadipocytes. Most adipose tissue studies are carried out using 3T3-L1 and 3T3-F442A adipocytes. These cells are morphologically indistinguishable from fibroblasts but are already committed to the adipocyte lineage. When treated with pro-differentiative agents they undergo differentiation over a 4-6 day period to form mature fat

cells. This is thought to represent a valid model of preadipocyte differentiation in vivo, shown via transplantation studies. When 3T3-F442A preadipocytes were injected subcutaneously into Balb-C athymic mice, normal fat pads developed at the site of injection within 5 weeks (Green and Kehinde 1975). Additionally, evidence shows that the fully differentiated adipocytes mimic the metabolism of adipocytes isolated from adipose tissue. Preadipocytes in culture also develop the proteins necessary for lipolysis of triacylglycerol, uptake and intracellular translocation of fatty acids, and regulation of these processes by lipogenic and lipolytic hormones. (MacDougald and Lane 1995).

If mature, differentiated adipocytes are to be studied, it is important that the optimum conditions are used for their differentiation. The results shown here illustrate the wide variation in differentiation efficiencies as a consequence of using different plate types. The variation observed could be explained by the different tissue culture coatings applied during manufacture; however, a significant difference was seen between the Nunclon ^{TM} Δ 6 well plate cluster and Nunclon ^{TM} Δ 35 mm dishes, which have been treated in the same way, have the same diameter and therefore the same surface area for cells to attach. The variation in differentiation may therefore be dependent on the airflow to the cells and media. It is unknown whether this is a pH dependent effect. Following this, all experiments were carried out using 3T3-L1s grown and differentiated on Nunclon 35mm dishes.

Adipokines were assayed using the adiponectin and resistin Duosets from R&D Systems as these assays were optimised for use with cell culture supernatant. The relative concentrations of adiponectin produced were comparable to those

in the literature using this same system (Li *et al.* 2007). Treatment of 3T3-L1 adipocytes with 10 μM rosiglitazone for 24 h, the same as this study, increased adiponectin release almost 2 fold, a similar increase to that seen in Figure 3.10. The actual amounts detected are 10 fold lower in the referenced paper; 7 and 13 ng/ml, for control and rosiglitazone, respectively, compared to 75 and 120 ng/ml in Figure 3.10. No indication of tissue culture dish size or medium volume is given in the referenced paper.

There did, however, appear to be a progressive decrease in secretion of both adipokines from the 3T3-L1 adipocytes. The reasons for the decreased secretion rates are unknown; the 3T3-L1 adipocytes were not used beyond 8 passages to prevent them from losing their function. It is possible that the assay had reduced sensitivity, however, all assay reagents were stored according to the manufacturer's instructions for a maximum of 6 months.

The higher concentrations of catechin and epicatechin used in the preliminary study appeared to increase adiponectin release, and significantly decreased resistin release. These concentrations were not pursued though, as they were too high to represent any levels obtainable physiologically. As further studies were carried out with EGCG, construction of a dose-response curve may have provided a firmer base on which to design further experiments. It has been calculated that a cup of green tea, comprising 2.5 g of green tea leaves/200 ml of water, may contain 90 mg of EGCG (Wu and Wei 2002). A pharmacokinetic study in humans showed that a bulk dose of 100 mg EGCG resulted in a maximum plasma concentration of 189.52 ng/ml (0.4 μM) and a

bulk dose of 1600 mg EGCG was required to produce a maximum plasma concentration of 2911.35 ng/ml (6.35 μM) free EGCG (Ullmann *et al.* 2003).

Effects on adipokine release in 3T3-L1s, reported in the literature, appear to be flavonoid specific. Catechin, when incubated with 3T3-L1 adipocytes at 50 μM for 24 h increased adiponectin release to over 200% of the control, however the same dose and duration of epicatechin 3-gallate, quercetin and kaempferol suppressed adiponectin release to 79%, 54% and 42% of the control, respectively. EGCG and epicatechin, however, had no effect on adiponectin release (Cho *et al.* 2007). Feeding studies, on the other hand, provide evidence that EGCG can increase circulating levels of adiponectin (Potenza *et al.* 2007; Shimada *et al.* 2007).

There is evidence that EGCG suppresses resistin mRNA and protein expression in a dose and time dependent manner in 3T3-L1 adipocytes (Liu *et al.* 2006). 20 μM EGCG treatment led to a 35% suppression of resistin mRNA and 100 μM led to 50% suppression of resistin mRNA 3 h after treatment. Intracellular resistin protein decreased with 100 μM treatment for 3 h to 75% of the control, however there was no significant effect on resistin protein release. Interestingly, suppression of resistin mRNA was similar with EGCG, epicatechin gallate and epicatechin, but little suppression was seen with epigallocatechin. The referenced results use flavanols at much higher concentrations than this study, concentrations which are too high to be achieved by consumption of green tea.

The studies in this chapter, investigating the effects of the flavanols catechin, epicatechin and EGCG, have been carried out in medium containing 25 mM

glucose. The generally accepted protocol for 3T3-L1 growth, maintenance and differentiation suggests that this medium glucose concentration be maintained throughout the culture of these cells, and indeed, the majority of studies which use the 3T3-L1 adipocyte system use this medium glucose concentration throughout their various treatments. Physiological glucose concentration is 5.5 mM, so in order that the studies in this chapter are as relevant and transferable to a physiological situation, they should be carried out as close to physiological conditions as possible. Maintenance of cells under this lower, physiological glucose level requires that the medium be replaced at least every 12 h, such that the cells have enough glucose. This is difficult to do and also results in complications when carrying out 24 h treatments which require media collection and analysis. For this reason, a "compromise" concentration of 12.5 mM glucose containing media was decided upon, so that the cells were able to remain 24 h in the same media without feeding. This meant that the average glucose concentration was about 9 mM over the incubation period. Investigating the effects of catechin and EGCG in the two different glucose concentrations showed that in the case of resistin, there was no difference in the release, it was suppressed under both conditions. Adiponectin, however, appears to be more sensitive to glucose conditions, as treatment with EGCG in the lower glucose concentration had no effect on adiponectin release. Treatment with EGCG in the higher, 25 mM glucose containing media resulted in significantly suppressed adiponectin release as compared to the control and EGCG treatment in the lower glucose containing media. Interestingly, treatment with catechin, under both media glucose concentrations, had no effect on either adiponectin or resistin release from the 3T3-L1 adipocytes.

The results for catechin are somewhat contradictory, however, as a study earlier in the chapter showed that 10 μM catechin, in the 25 mM glucose medium, significantly increased resistin release. As medium glucose concentration affects the mRNA and secretion levels of adiponectin (Park *et al.* 2004; Huypens *et al.* 2005) and resistin (Shojima *et al.* 2002), a possible explanation for this inconsistency may be due to the fact that the 3T3-L1 adipocytes were being maintained in supraphysiological levels of glucose.

Adiponectin plays a significant role in linking obesity and insulin resistance, obesity related increments in adipocyte cell size are accompanied by reduced secretion and low circulating levels, which then promotes increased cellular lipid content and insulin resistance (Yamauchi *et al.* 2001), additionally, serum adiponectin levels increase with weight loss (Arita *et al.* 1999). Adiponectin gene expression is regulated by several extracellular signalling factors, including insulin, TNFα and β-adrenergic agonists (Havel 2004). PPARγ has been shown to induce adiponectin promoter activity though an unidentified element responsive to PPARγ (Maeda *et al.* 2001). TZD agonists of PPARγ increase adiponectin expression and circulating levels in rodents and plasma adiponectin levels in non-diabetic subjects and in patients with Type 2 diabetes. C/EBPα has been reported to regulate adiponectin gene transcription through an intronic enhancer (Qiao *et al.* 2005). SREBP-1c binds to the adiponectin promoter and mediates the insulin dependent adiponectin expression (Seo *et al.* 2004). Kruppel like factor (KLF)-7 has been reported to inhibit adiponectin gene expression in adipocytes (Kawamura *et al.* 2006).

Resistin, in mice, is expressed almost exclusively in white adipose tissue and released into the blood. In rodent models of obesity and diabetes, serum resistin levels are elevated, and administration of resistin to wild type mice worsened glucose homeostasis and insulin sensitivity and in 3T3-L1 adipocytes suppressed insulin stimulated glucose uptake. This effect was prevented by an anti-resistin antibody (Steppan *et al.* 2001). This suggests that resistin impairs insulin sensitivity and may contribute to the development of insulin resistance or diabetes in obese rodents. In both humans and mice, serum resistin protein levels are positively associated with adiposity, however, resistin mRNA in humans does not correlate with BMI (Savage *et al.* 2001). In humans, resistin expression in adipocytes is almost undetectable, and is expressed at the highest level in bone marrow (Patel *et al.* 2003). This may help to explain why murine studies suggest that resistin regulates insulin action but the conclusions from human studies are unclear, making it difficult to translate murine studies to humans. TZDs, by activating PPARγ, cause decreased resistin expression and decreased insulin resistance (Wolf 2004). Treatment of mice with TZDs lowered resistin protein levels, similar to the effects observed *in vitro*, using 3T3-L1 cells (Steppan *et al.* 2001).

The mechanisms of adiponectin and resistin regulation have not been fully elucidated as yet, however they are both altered by TZD treatment and therefore are under the control of PPARγ phosphorylation. PPARγ is a substrate of ERK1/2 and phosphorylation is believed to decrease its transcriptional activity and inhibits adipocyte differentiation (Camp and Tafuri 1997), this can also result in increased adiponectin release and suppression of

resistin release in adipocytes, shown recently by Sharma and Staels (Sharma and Staels 2007).

The studies in this chapter show, that while adiponectin release can be altered by EGCG, this only occurs at glucose levels which are much higher than physiological levels and therefore cannot be translated to a physiological environment. Resistin release can be altered at both supraphysiological and closer to physiological levels in 3T3-L1 adipocytes. It is possible that EGCG could affect the expression and/or secretion of resistin in other tissues in humans, however, as 3T3-L1s are murine derived cells, again, it is difficult to draw comparisons to human physiology.

Signalling:

investigation into the ERK pathway

4 Signalling

4.1 Introduction

Changes in adipokine levels are either due to changes in levels of secretion or synthesis. In order to determine the mechanism by which adipokine levels were being altered, the ERK1/2 MAPK pathway, summarised in Figure 1.2, was investigated. There are many known targets of the ERK1/2 pathway, transcription factors such as AP-1, NF-κB, C/EBPβ and PPARγ. There is evidence that PPARγ can regulate both the gene expression and protein levels of adiponectin (Maeda *et al.* 2001; Havel 2004) and resistin (Steppan *et al.* 2001; Wolf 2004). Additionally, C/EBPα has been reported to regulate adiponectin gene transcription through an intronic enhancer (Qiao *et al.* 2005). C/EBPα is indirectly regulated by ERK1/2 via C/EBPβ (Park *et al.* 2004).

PPARγ and C/EBPα both play important roles in adipogenesis. There is a cooperative action between these two transcription factors, where a positive feedback loop induces the expression of each factor. This drives the expression of genes that are necessary for the generation and maintenance of the adipocyte phenotype.

There is evidence to suggest that flavanols are able to alter the phosphorylation status of ERK1/2. Studies in preadipocytes (Hung *et al.* 2005), HUVECs (Chae *et al.* 2007) and human keratinocytes (Huang *et al.* 2007) demonstrate the effect of EGCG in decreasing ERK1/2 phosphorylation. Studies in primary cortical neurons (Spencer *et al.* 2003; Schroeter *et al.* 2007; Vauzour *et al.* 2007) provide evidence that flavonoids are able to alter the phosphorylation

status of ERK1/2, and this is not necessarily in the same direction. It is therefore difficult to predict the effect that green tea catechins might have on the ERK1/2 phosphorylation status in 3T3-L1 adipocytes, because although they act to decrease phosphorylation in preadipocytes, the same may not occur in adipocytes as these two cell types are very different.

Following investigation of the ERK1/2 signalling cascade, potential mechanisms of upstream activation were explored.

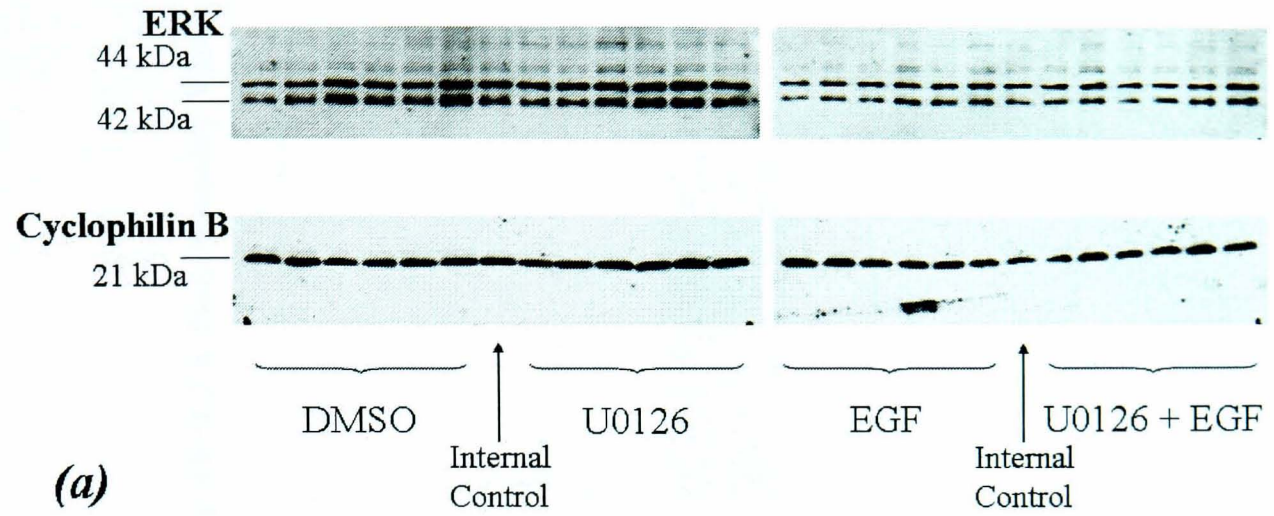
4.2 Phosphorylation of ERK1/2 using controls

Prior to investigating the effects of epicatechin and EGCG on ERK1/2 phosphorylation, it was important to ensure that the phosphorylation status of the 3T3-L1 system could be altered. U0126 is a chemically synthesised organic compound that inhibits the kinase activity of MEK1/2 and therefore inhibits phosphorylation of ERK1/2. EGF is known to be a potent stimulator of ERK1/2 phosphorylation in 3T3-L1 adipocytes (Wiese *et al.* 1995), and was therefore used as a positive control.

Cells were differentiated as detailed in Section 2, treated with 10 mM U0126 for 15 min, with and without 10 nM EGF for 10 min, with six 35 mm Nunclon dishes per treatment. Samples were prepared in lysis buffer (Section 2.3.3) and protein was determined by the Lowry method (Section 2.3.5.1). Proteins were separated by Western blotting (Section 2.3.6-7) and incubated with anti cyclophilin B, ERK1/2 and pERK1/2 antibodies as detailed in Section 2, Table 2.1. Protein bands were visualised using ECL.

To account for variations between gels, an internal control, constituting small volumes of each sample pooled together, was loaded onto each gel, and the cyclophilin B bands normalised to this, before being used to normalise all other protein bands. All densities were then expressed as a percentage of the mean vehicle control density for that sample group.

U0126 significantly diminished ($p<0.01$, $n=6$) and EGF significantly stimulated ($p<0.001$, $n=6$) ERK1/2 phosphorylation as expected. U0126 was also able to significantly reduce the EGF stimulated ERK1/2 phosphorylation ($p<0.001$, $n=6$) (see Figure 4.1 (b) and (d)). Levels of ERK1/2 remained relatively unchanged with treatment (see Figure 4.1 (a) and (c) and Figure 4.2).



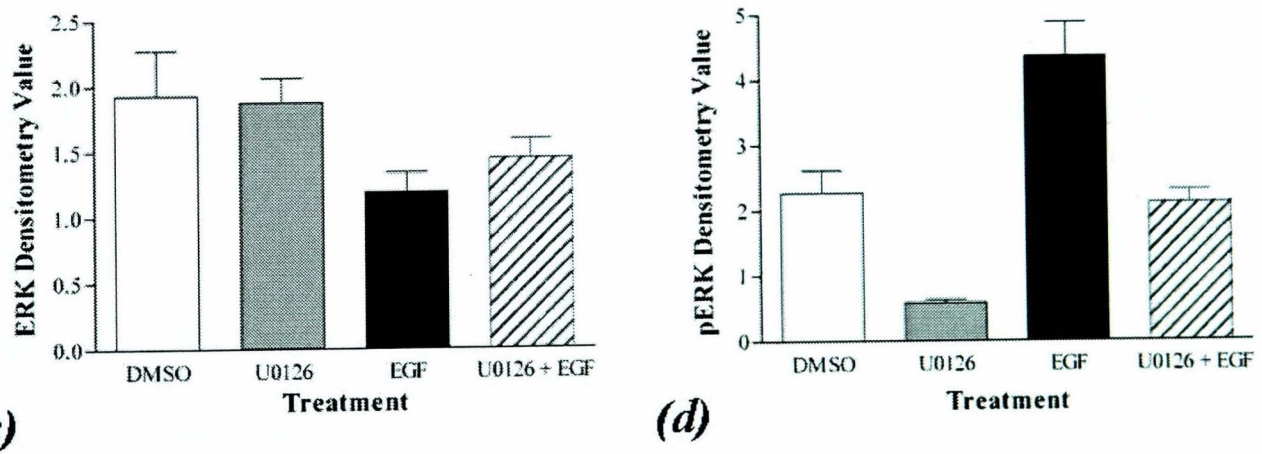


Figure 4.1 Effect on ERK1/2 and pERK1/2 following 10mM U0126 (15 min) treatment with and without 10nM EGF (10 min) of mature 3T3-L1 cells in 12.5 mM glucose DMEM

Cell lysate was subjected to SDS-PAGE and Western blotting with anti-ERK1/2 (a), p-ERK1/2 (b) and cyclophilin B antibodies. Densitometry of ERK1/2 (c) and pERK1/2 (d) blots, normalised to internal controls and cyclophilin B levels, expressed relative to DMSO

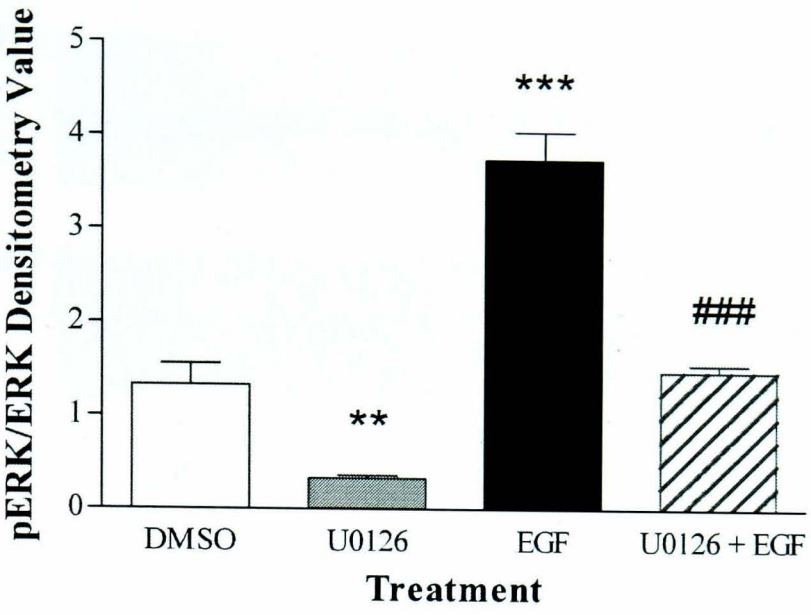


Figure 4.2 Change in pERK1/2 levels relative to ERK1/2 following treatment with 10 mM U0126 (15 min) with and without 10 nM EGF (10 min) of mature 3T3-L1 adipocytes in 12.5 mM glucose DMEM.

*Results analysed using one-way ANOVA, and Tukey post-hoc test. ** $p<0.01$ compared to control, *** $p<0.001$ compared to control, ### $p<0.001$ compared to EGF alone.*

4.3 ERK1/2 phosphorylation following 1 h flavanol treatment.

It was shown in studies on adipokine release (Chapter 3), that EGCG had the most potent effect on both resistin and adiponectin release. It was thus decided that 3T3-L1 adipocytes should be treated with 10 μM EGCG and subsequent phosphorylation of ERK1/2 should be measured. Epicatechin also appeared to have a more potent effect on both resistin and adiponectin release, compared to catechin, so epicatechin treatment was also used to investigate ERK1/2 phosphorylation.

Cells were differentiated as detailed in Section 2, treated with 10 μM epicatechin or EGCG for one hour and further processed as described earlier.

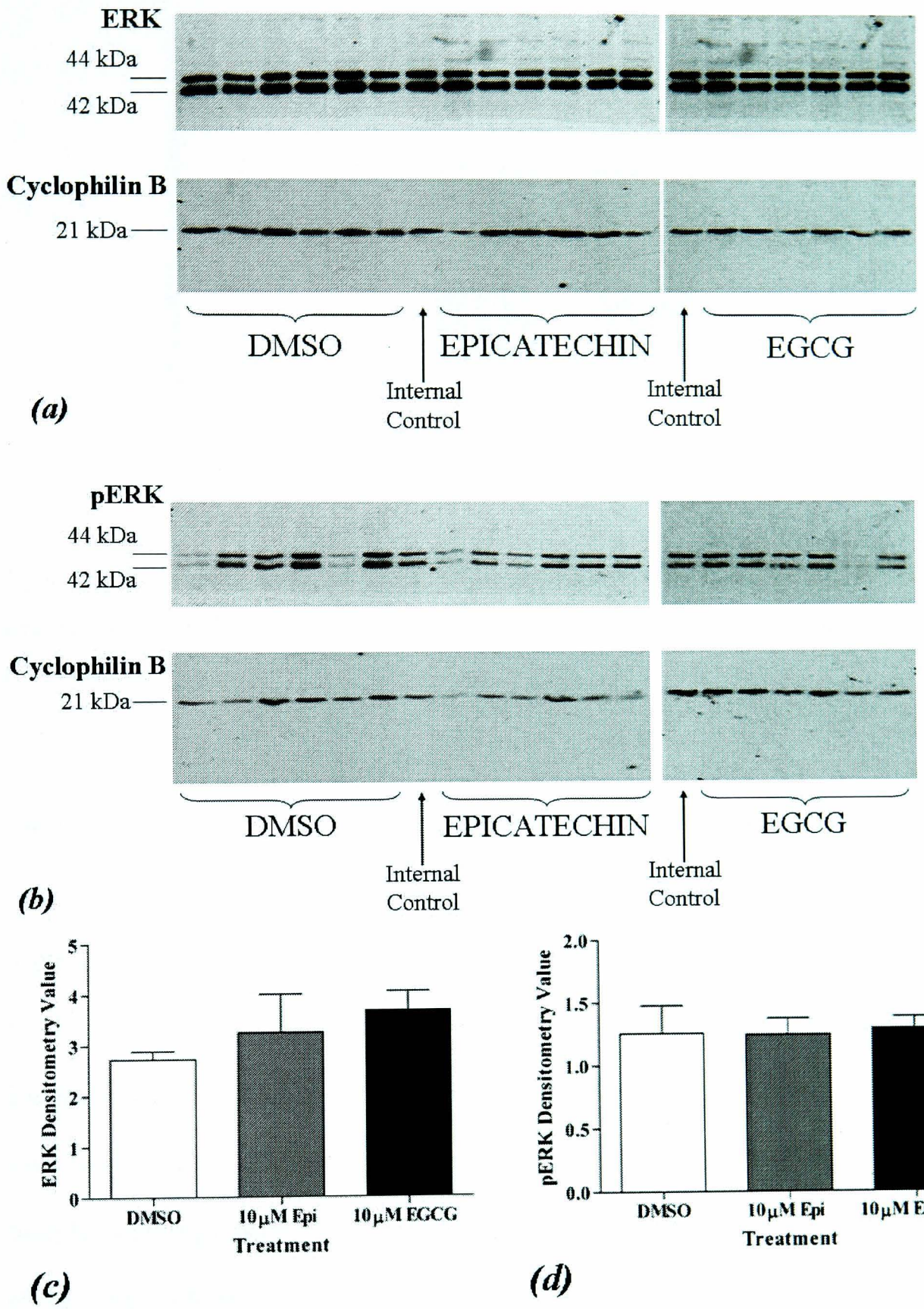


Figure 4.3 Effect on ERK1/2 and pERK1/2 following 1hr treatment of mature 3T3-L1s with 10 μM epicatechin and EGCG in 12.5 mM glucose DMEM. Cell lysates were subjected to SDS-PAGE and Western blotting with anti-ERK1/2 (a), pERK1/2 (b) and cyclophilin B antibodies. Densitometry of ERK1/2 (c) and pERK1/2 (d) blots, normalised to internal controls and cyclophilin B levels, expressed relative to DMSO control.

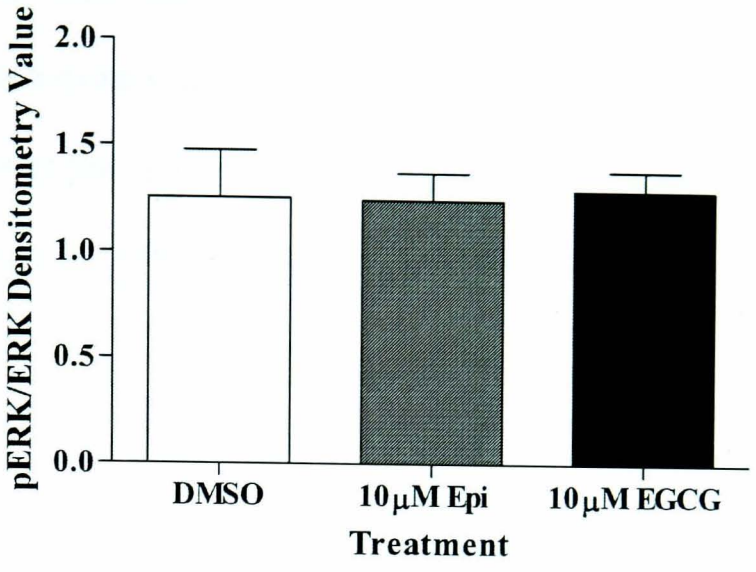


Figure 4.4 Change in pERK1/2 levels relative to ERK1/2 following 10 μM epicatechin and EGCG treatment of mature 3T3-L1 cells in 12.5 mM glucose DMEM.

There were no detectable changes in ERK1/2 phosphorylation after 1 hr treatment with 10 μM epicatechin or EGCG (see Figure 4.3 and Figure 4.4). It is possible that this may be too long and the phosphorylation event had passed, so the treatment time was decreased to 30 min and a dose response with epicatechin and EGCG was set up. There appeared to be a high degree of variability between the lanes of Figure 4.3 (b). As protein concentrations of samples were equalized before loading, this may be due to inconsistent transfer of the samples from the gel to the nitrocellulose membrane.

4.4 ERK1/2 phosphorylation following 30 min flavanol treatment

As one hour may have been too long a treatment period, and the phosphorylation event may have passed, this was reduced to 30 min in a further attempt to detect phosphorylation. Research by Prusty *et al* (2002) shows that phosphorylation of ERK1/2 can be detected as early as 5 min (Prusty *et al.* 2002). Additionally a dose response of epicatechin and EGCG was set up with 0.1, 1, 10 and 50 μM epicatechin or EGCG. Samples were prepared and processed as previously stated.

Epicatechin at 0.1 and 1 μM had no effect on ERK1/2 phosphorylation after 30 min treatment (results not shown). 10 μM epicatechin significantly increased ERK1/2 phosphorylation after 30 min exposure ($p<0.001$, $n=6$), (4-fold higher than the vehicle control). 50 μM epicatechin showed increased phosphorylation but this was not significant (see Figure 4.5 (b) and (d)). Levels of ERK1/2 remained relatively stable during treatment (see Figure 4.5 (a) and (c) and Figure 4.7).

EGCG at 0.1 μM, when incubated with 3T3-L1 cells for 30 min significantly increased ERK1/2 phosphorylation ($p<0.01$, $n=6$) (see Figure 4.6 (b) and (d)), 2.8-fold higher than the vehicle control. 10 and 50 μM EGCG had no effect on ERK1/2 phosphorylation after 30 min (results not shown). Levels of ERK1/2 remained constant throughout the treatment (see Figure 4.6 (a) and (c) and Figure 4.8)

Following this study, the Odyssey system became available and subsequent Western blots were carried out using this as it is a more sensitive system.

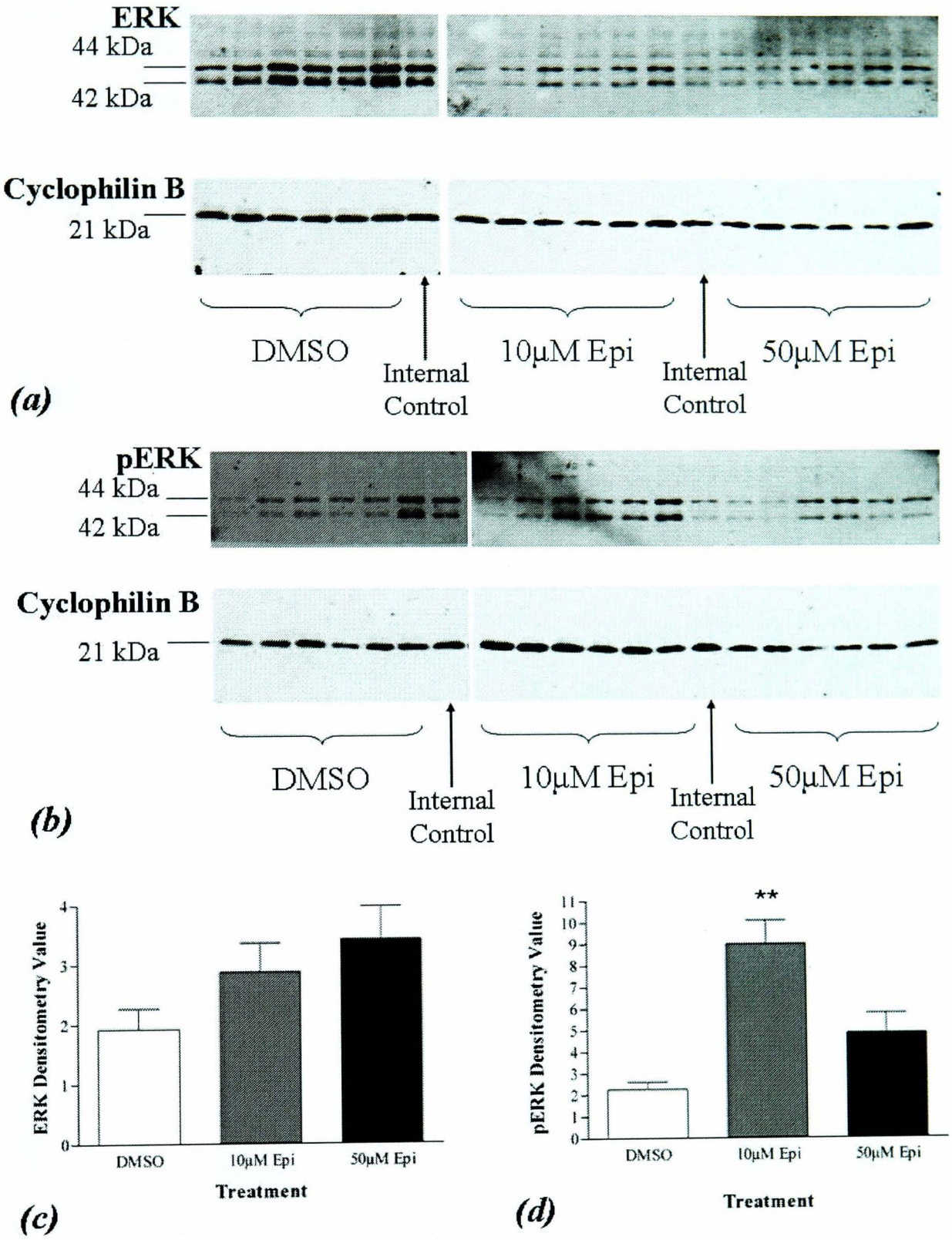


Figure 4.5 Effect on ERK1/2 and pERK1/2 following 30 minute treatment of mature 3T3-L1 cells with 10 and 50 μM epicatechin in 12.5 mM glucose DMEM

*Cell lysate was subjected to SDS-PAGE and Western blotting with anti-ERK1/2 (a), p-ERK1/2 (b) and cyclophilin B antibodies. Densitometry of ERK1/2 (c) and pERK1/2 (d) blots, normalised to internal controls and cyclophilin B levels, expressed relative to DMSO control. Results analysed using one-way ANOVA, and Tukey post-hoc test. **$p<0.001$ compared to DMSO control.*

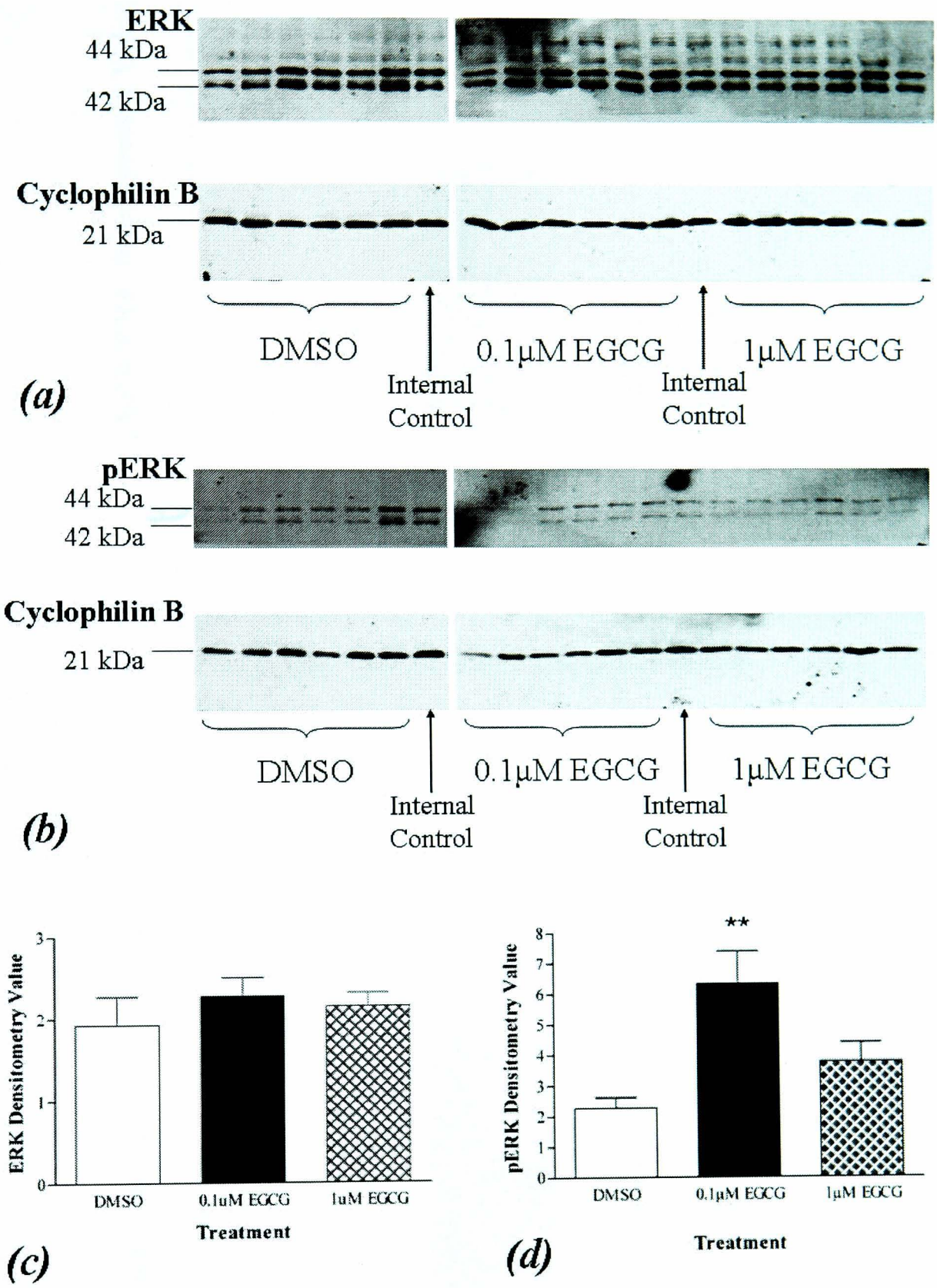


*Figure 4.6 Effect on ERK1/2 and pERK1/2 following 30 minute treatment of mature 3T3-L1s with 0.1 and 1 μM EGCG in 12.5 mM glucose DMEM. Cell lysate was subjected to SDS-PAGE and Western blotting with anti-ERK1/2 (a), p-ERK1/2 (b) and cyclophilin B antibodies. Densitometry of ERK1/2 (c) and pERK1/2 (d) blots, normalised to internal controls and cyclophilin B levels, expressed relative to DMSO control. Results analysed using one-way ANOVA, and Tukey post-hoc test. **$p<0.001$ compared to DMSO control.*

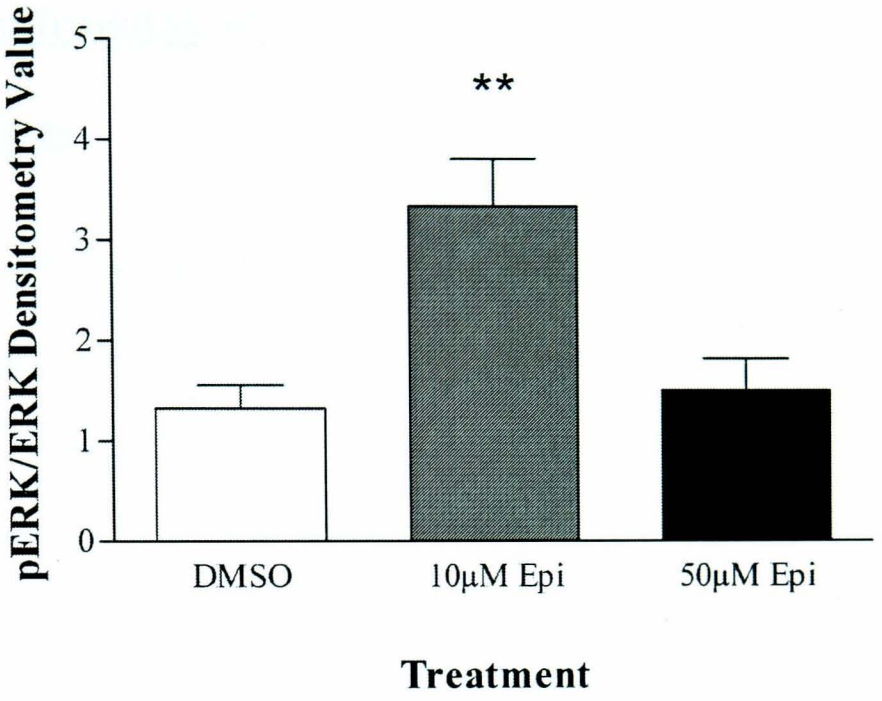


Figure 4.7 Change in pERK1/2 levels relative to ERK1/2 following treatment of mature 3T3-L1 adipocytes with 10 and 50 μM epicatechin for 30 min in 12.5 mM glucose DMEM.

*Results analysed using one-way ANOVA, and Tukey post-hoc test. **p<0.001 compared to DMSO control.*

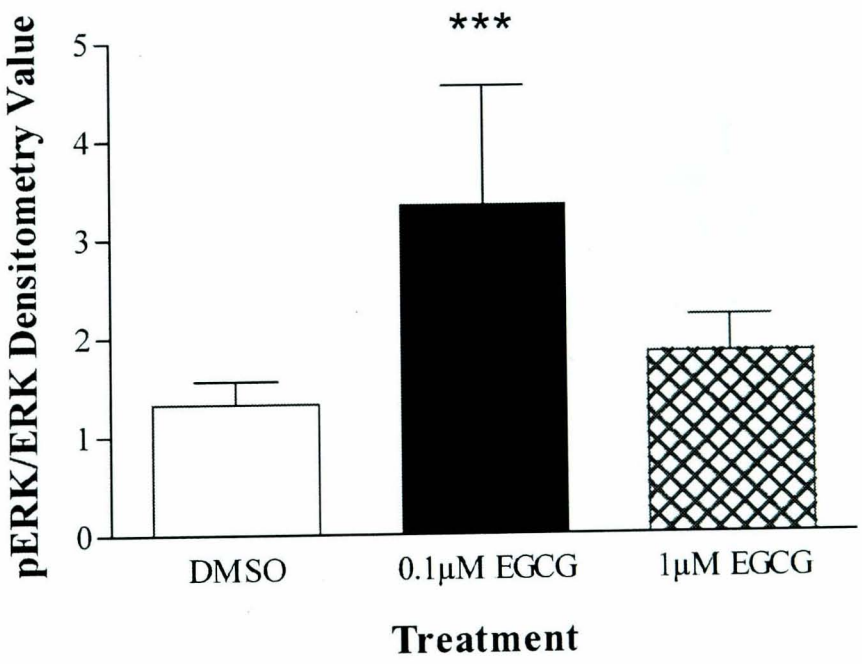


Figure 4.8 Change in pERK1/2 levels relative to ERK1/2 following treatment of mature 3T3-L1 adipocytes for 30 min with 0.1 and 1 μM epicatechin in 12.5 mM glucose DMEM.

*Results analysed using one-way ANOVA, and Tukey post-hoc test. ***p<0.01 compared to DMSO control*

4.5 Confirmation of ERK1/2 phosphorylation, use of Odyssey system

Prior to investigating the downstream effects of ERK1/2 activation, a further confirmation treatment was carried out, using the concentrations of epicatechin and EGCG that produced the maximal ERK1/2 phosphorylation response. Additionally it was important to ensure that the same results were produced with the Odyssey system as with ECL reagents.

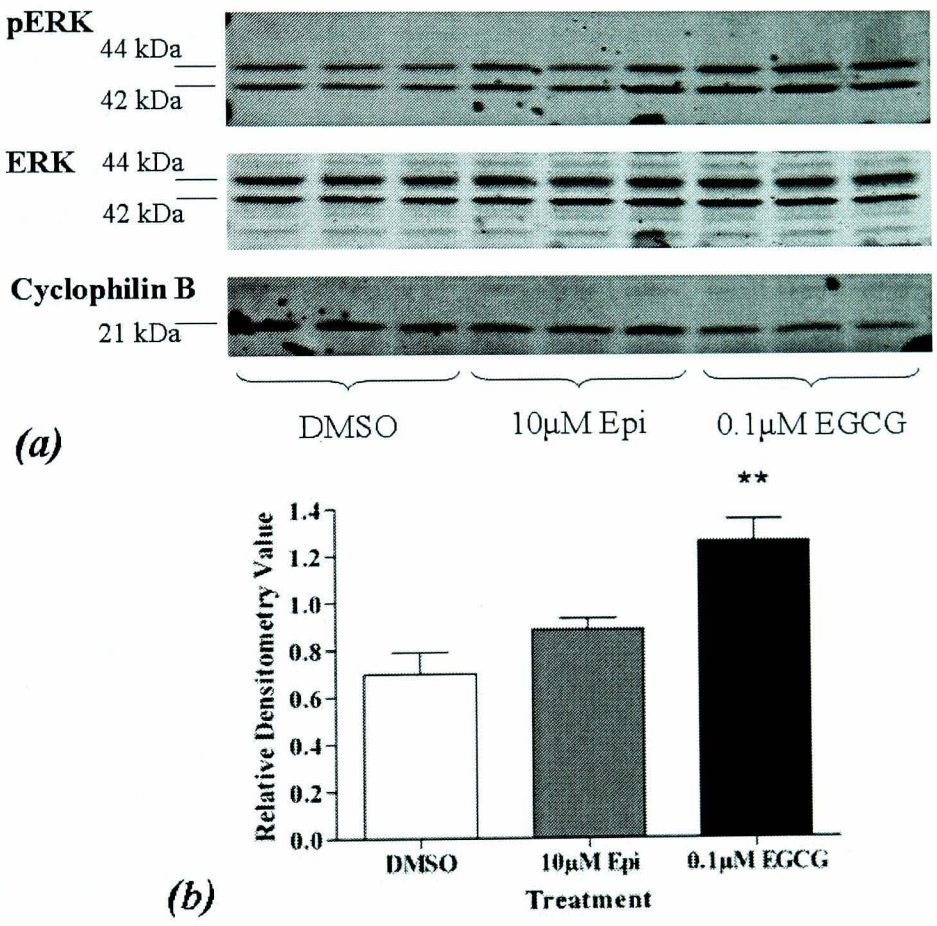


Figure 4.9 Effect on ERK1/2 and pERK1/2 following 30 minute treatment of mature 3T3-L1 cells with 10 μM epicatechin or 0.1 μM EGCG in 12.5 mM glucose DMEM.

(a) Cell lysate was subjected to SDS-PAGE and Western blotting with anti-ERK1/2, anti p-ERK1/2 and cyclophilin B antibodies; and Odyssey secondary antibodies. (b) Graphical representation of pERK1/2 levels relative to ERK1/2 levels, normalised to cyclophilin B levels, measured by densitometry. Results

analysed using one-way ANOVA, and Tukey post-hoc test. $^{**}p<0.01$ *compared to DMSO control.*

Cells were differentiated as in Section 2, treated with 10 μM epicatechin or 0.1 μM EGCG for 30 min and samples were prepared and processed as described earlier, with the exception of the use of Odyssey secondary antibodies as detailed in Section 2, Table 2.2. Protein bands were visualised and their density determined with the Odyssey scanner and software.

0.1 μM EGCG significantly increased ERK1/2 phosphorylation after 30 min (see Figure 4.9) ($p<0.01$, $n=3$), confirming the results seen in Figure 4.5 to Figure 4.8. This increase, however, was not of the same magnitude, only 1.8-fold as compared to 2.5-fold as seen previously. 30 min treatment with 10 μM epicatechin increased ERK1/2 phosphorylation 1.3-fold, but this was not significant and not supportive of the previous experiment.

4.6 GTPγS Binding Assay

Having observed that epicatechin and EGCG were able to stimulate phosphorylation of ERK1/2, the next step was to try and understand the mechanism of this stimulation by investigating processes upstream of the ERK1/2 signalling pathway. There are 3 main pathways which converge at the MEK pathway. These are the cAMP pathway, PLC pathway and Ras, all of which are downstream of GPCR activation. The GTPγS binding assay measures the level of G protein activation following agonist occupation of a G protein-coupled receptor.

Cell membranes from human embryonic kidney (HEK) cells expressing rat recombinant CB2 receptors were incubated with [^{35}S] GTPγS and 10 μM catechin, epicatechin or EGCG and 1 μM HU210 (CB1 agonist) and processed as detailed in Section 2.4.

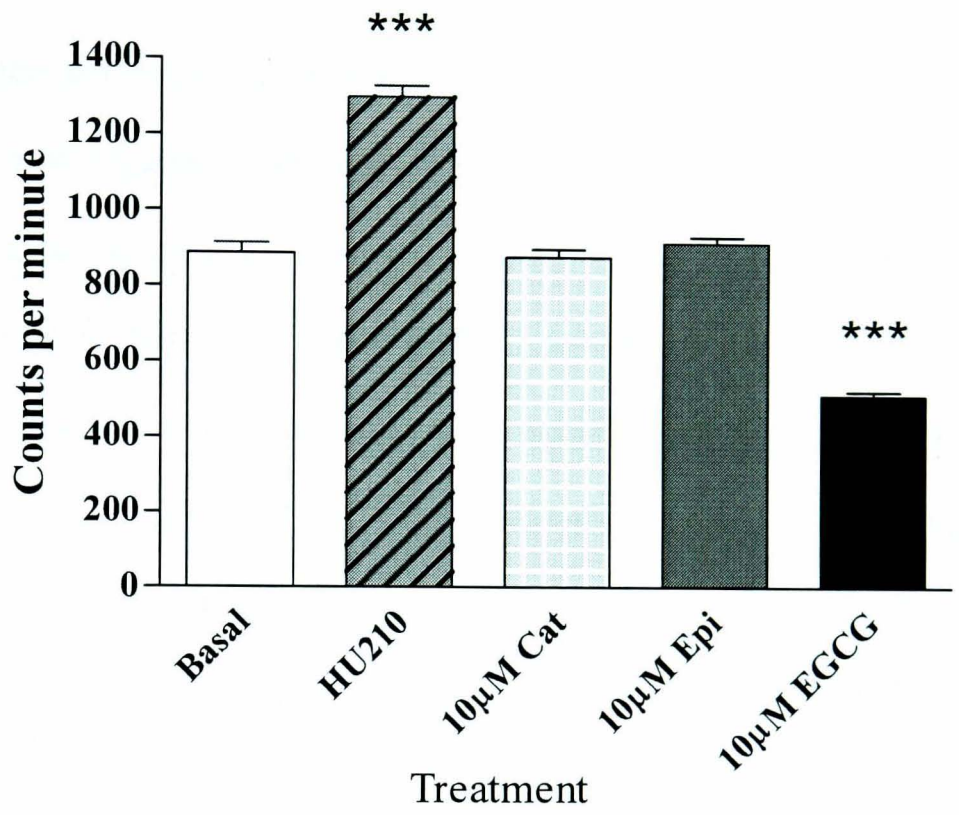


Figure 4.10 GTPγS binding in 10 μg HEK membrane preparations in the presence of 10 μM catechin, epicatechin and EGCG for 90 min.
*Results are shown as counts per minute per preparation ±SEM (n=4). Results analysed using one-way ANOVA, and Tukey post-hoc test. *** p<0.001 compared to basal and all other treatments*

10 μM catechin and epicatechin had no effect on GTPγS binding, and 10 μM EGCG appeared to negatively regulate this process in HEK cells, by significantly decreasing GTPγS binding compared to basal, catechin and epicatechin treatment ($p<0.001$, $n=4$) (see Figure 4.10).

4.7 Discussion

The aim of this chapter was to investigate the potential mechanisms by which the flavanols EGCG and epicatechin alter secretion of the adipokines, adiponectin and resistin, in 3T3-L1 adipocytes.

This chapter firstly demonstrates that the 3T3-L1 adipocyte model performs as expected with regards to alteration of the ERK1/2 phosphorylation status by the known inhibitor U0126 and known stimulator, EGF. Secondly, EGCG and epicatechin were both able to alter ERK1/2 phosphorylation by increasing it to levels above those seen with the vehicle control. Both 0.1 μM EGCG and 10 μM epicatechin increased ERK1/2 phosphorylation by 2.5-fold compared to the vehicle control, levels comparable to those produced by 10 nM EGF treatment (2.8-fold higher than the vehicle control). A beneficial progression from this study would be to try and establish whether EGCG and epicatechin are acting through the same mechanism as EGF by investigating whether there is any additive effect of EGCG or epicatechin with EGF, or indeed, whether this is the maximum achievable ERK1/2 phosphorylation by treating with the optimum concentrations of both EGCG and epicatechin.

EGF, acting via the EGF receptor (a receptor tyrosine kinase) directly increases ERK1/2 phosphorylation by activating Ras in the ERK1/2 cascade. The mechanisms of flavanol action are currently unknown, the 67 kDa laminin receptor may be playing a role in this as it has been suggested as an "EGCG receptor" and has been discovered in cancer cells (Tachibana *et al.* 2004) nerve cells, hepatocytes and muscle cells (Mecham 1991). There is also some

evidence demonstrating that the 67 kDa laminin receptor signalling pathway involves the ERK1/2 pathway. Laminins have been identified in adipocytes (Niimi *et al.* 1997; Burton *et al.* 2004), but as yet, there is no evidence of this 67 kDa laminin receptor in adipocytes. A specific plasma membrane binding site for polyphenols has been recently described in rat brain (Han *et al.* 2006), suggesting that they may act through a steroid like receptor in neurons.

EGCG at 50 μM has been shown to decrease ERK1/2 phosphorylation in 3T3-L1 preadipocytes (Hung *et al.* 2005). This chapter demonstrates that EGCG is able to increase ERK1/2 phosphorylation in 3T3-L1 adipocytes. This may help to explain the variability and inconsistency seen in the results of the previous chapter. Although the 3T3-L1 adipocytes used throughout these studies were considered to be at least 90% differentiated in each case, this was only checked by looking at their morphology and it is therefore possible that between studies, dishes with differences in the percentage of differentiated adipocytes may have been used. Therefore, any conflicting data may be a result of having a mixed culture of adipocytes in various stages of differentiation. This could be confirmed by comparing ERK1/2 phosphorylation of 3T3-L1 preadipocytes and adipocytes.

The effects of epicatechin on ERK1/2 phosphorylation have been investigated in cortical neurons (Schroeter *et al.* 2007). A 15 min treatment of 100-300 nM epicatechin stimulated a robust phosphorylation of ERK1/2. At higher epicatechin concentrations, the extent of phosphorylation was much lower, returning to basal at levels greater than 1 μM. This ERK1/2 phosphorylation,

in combination with the PI3 kinase pathway, was shown to regulate CREB phosphorylation.

The concentrations of flavonoids used for *in vitro* studies are very important as some exert anti apoptotic actions at low concentration but act as pro apoptotic stimuli at higher concentrations. Quercetin and its metabolites 3'-O-methyl quercetin and 4'-O-methyl quercetin have been previously shown to inhibit ERK1/2 phosphorylation, an action underlying their potentially pro-apoptotic actions in neurons. Interestingly, lower concentrations of quercetin stimulated CREB (Spencer *et al.* 2003), which could reflect an anti apoptotic protective response, as CREB-dependent upregulation of gene expression is neuroprotective.

EGCG and epicatechin have different optimum concentrations; there was a 100 fold difference in optimum concentrations to produce the same, significant increase. It seems that in most cases, gallated catechins, especially EGCG, are more active than other catechins (Hung *et al.* 2005). EGCG contains the largest number of hydroxyl groups on its three aromatic rings among the tea catechins, and these hydroxyl groups may be important for hydrogen bonding. Also, EGCG has both gallyl and galloyl groups, which have some conformational flexibility that may also be important for interaction with other molecules.

Having established that EGCG and epicatechin were able to stimulate ERK1/2 phosphorylation, it was important to try and establish the mechanism of upstream activation of this pathway. There are several upstream components

that join the ERK1/2 pathway; GPCRs, receptor tyrosine kinases (both ligand dependent and independent (Gardner *et al.* 2003)) and integrins.

The first method used to potentially elucidate which upstream component was being activated was to determine whether the flavanols under investigation bound to a GPCR. A GTPγS binding assay was therefore established, using HEK cells, expressing rat recombinant CB2 receptors. EGCG, at 10 μM, negatively regulated GTPγS binding in HEK cells, whereas epicatechin and catechin at the same concentrations did not alter binding significantly compared to basal levels. This negative regulation suggests that EGCG could act as an antagonist at a GPCR, in this case the CB2 receptor, and this pathway may be worth pursuing. Ideally, this assay would have been transferred to the 3T3-L1 system, however, due to time restraints, this was not possible. In addition, a dose-response curve of each flavanol should have been constructed. As EGCG treatment produced greater stimulation of pERK at 0.1 μM, compared to 1 μM, investigation of GTPγS binding with 0.1 μM EGCG may have produced more information regarding the mechanism of action of EGCG in this pathway.

Measurement of cyclic AMP (cAMP) generation from ATP by adenylyl cyclase following GPCR activation was another method employed to help elucidate the mechanism of ERK1/2 activation by the flavanols. Cyclic AMP is a small molecule that has a key role in the regulation of intracellular signal transduction. cAMP is derived from ATP by the catalytic action of the signalling enzyme adenylyl cyclase. This cyclisation reaction involves the intramolecular attack of the 3'-OH group of the ribose unit on the α-phosphoryl

group of ATP to form a phosphodiester bond, this is driven by the subsequent hydrolysis of the released pyrophosphate. The activity is terminated by the hydrolysis of cAMP to 5'-AMP by specific cAMP phosphodiesterases. Receptors may be linked to the generation of cAMP by one of two routes. One group of receptors is associated with increased adenylyl cyclase activity and elevated cAMP levels mediated through a guanine nucleotide binding protein, G_s. Activation of the second group leads to inhibition of adenylyl cyclase and a reduction in cAMP generation. The mechanism of inhibition of adenylyl cyclase activity appears to be mediated through a distinct guanine nucleotide binding protein, G_i. These two G-proteins act on adenylyl cyclase either to stimulate or inhibit the conversion of ATP to cAMP.

This assay was a three step process; firstly, radioceptor binding protein was prepared from the supernatant of porcine adrenal homogenate; secondly, cAMP was generated in the cells by incubating the cells with radiolabelled cAMP in the presence of the flavanols or IBMX (a non-selective phosphodiesterase inhibitor), followed by the addition of forskolin (a non-selective adenyl cyclase activator) and ice cold concentrated hydrochloric acid to stop the reaction. Thirdly, cAMP estimation was carried out by incubating binding protein with each sample, filtering samples through Whatman filters and determining bound radioactivity through liquid scintillation spectrophotometry.

A considerable period of time was spent attempting to optimise this assay, but a functional readout of bound radioactivity in this assay could not be obtained, and therefore results could not be included.

As previously discussed, EGF stimulation of the ERK1/2 pathway requires the EGF receptor which is a tyrosine kinase. EGCG could, potentially, be an agonist for a tyrosine kinase receptor. There is evidence in cervical cells that EGCG inhibits EGF signalling by inhibiting EGF-dependent activation of the EGF receptor, which then suppresses the EGF receptor dependent activation of ERK1/2. Interestingly, downstream of ERK1/2, there was only selective inhibition of its phosphorylation of specific substrates, suggesting that EGCG acts simultaneously at multiple levels to inhibit EGF dependent signalling (Sah *et al.* 2004). In addition to inhibiting EGFR, cell-free studies demonstrated that EGCG directly inhibited ERK1/2, suggesting that EGCG acts simultaneously at multiple levels to inhibit EGF-dependent signalling.

As previously mentioned, the ERK1/2 pathway can be also stimulated in a ligand-independent manner via the EGF receptor by PPARα and γ ligands (Gardner *et al.* 2003), so it could be possible that the flavanols are acting in a similar manner.

ERK1/2 is not the only signalling pathway that has been implicated in EGCG and epicatechin action. The Akt (Sah *et al.* 2004; Morikawa *et al.* 2007; Pan *et al.* 2007) and PI3-kinase (Kim *et al.* 2007; Schroeter *et al.* 2007) pathways also play an important role and these are worth pursuing in future studies.

Following attempts to identify the mechanism of upstream ERK1/2 activation, attention was focussed on the potential downstream effects of ERK1/2 activation. The ERK1/2 pathway has many substrates, including NF-κB, AP-1, STAT and CREB, and activation of this pathway could have a number of potentially beneficial outputs. In order to obtain a physiologically relevant

functional readout, the link between the ERK1/2 pathway and glucose uptake was investigated and is the focus of the following chapter. Work in 3T3-L1 adipocytes showed that chronic activation of ERK1/2 leads to upregulation of GLUT1 expression and down regulation of GLUT4 expression, resulting in slightly increased basal glucose transport but decreased insulin stimulated transport (Fujishiro *et al.* 2003). However, treatment of mature 3T3-L1 adipocytes with the MEK inhibitors PD98059 and U0126 inhibited insulin mediated glucose uptake (Harmon *et al.* 2004). Investigating the effects of EGCG and epicatechin on glucose uptake in 3T3-L1 adipocytes may help to clarify this.

Glucose Uptake:

effect of epicatechin and EGCG

5 Glucose Uptake

5.1 Introduction

In order to understand the effects of EGCG and epicatechin on mature 3T3-L1 adipocytes and find a potential physiological outcome of this effect, glucose uptake was investigated. It was shown in the previous chapter that epicatechin at 10 μM and EGCG at 0.1 μM, when incubated with 3T3-L1 adipocytes resulted in an increase in ERK1/2 phosphorylation. Therefore, epicatechin at concentrations of 1, 10 and 50 μM and EGCG at concentrations of 0.1, 1 and 10 μM were used to treat 3T3-L1 adipocytes and their effect on 2-deoxyglucose (2DG) uptake was measured as an index of glucose uptake. Increased ERK1/2 phosphorylation was seen after 30 min treatment; therefore 2-deoxyglucose uptake was measured after this time to see if any effect was a direct result of ERK1/2 phosphorylation. Additionally, 3T3-L1 adipocytes were incubated with epicatechin and EGCG for 6 and 24 h to allow for the phosphorylation signal to be propagated.

5.2 Preliminary assays

Prior to investigating the effects of EGCG and epicatechin on 2-deoxyglucose uptake, it was important to establish whether the glucose uptake system could be altered in the 3T3-L1 adipocytes. For this reason, firstly an insulin dose response was carried out, and the effect of a known inhibitor of glucose

transport, cytochalasin B (Ebstensen and Plagemann 1972; Kletzien *et al.* 1972; Mizel and Wilson 1972; Lachaal *et al.* 1996), was investigated.

5.2.1 Insulin dose response

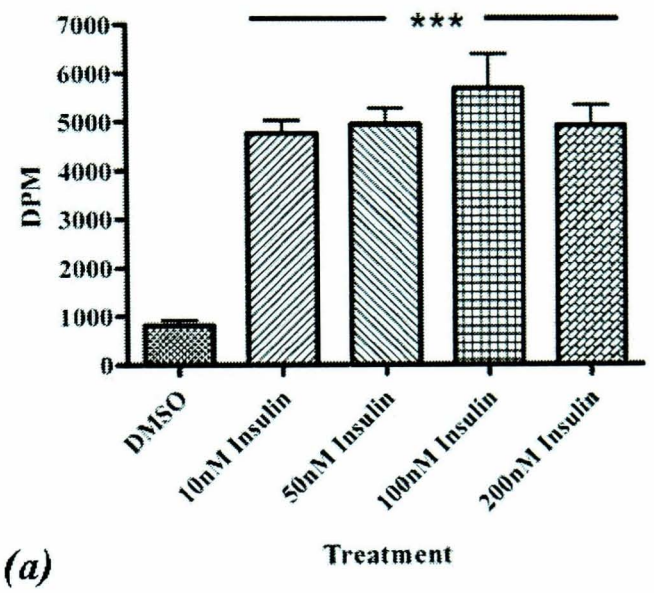
Mature 3T3-L1 adipocytes, differentiated as detailed in Section 2.3.1, were treated with 0.01% DMSO, 10, 50, 100 and 200 nM insulin for 10 min, followed by 27.8 kBq [^{3}H] 2-DG and 1.5 mM 2-DG for 10 min. Assay information is detailed in section 2.3.5.

All insulin concentrations used significantly increased glucose uptake compared to DMSO control (Figure 5.1a) ($p<0.001$). Although a dose response trend was apparent with 10 nM insulin resulting in the lowest level of glucose uptake and 100 nM the highest there was no significant difference between the concentrations. It was therefore decided that 10 nM insulin would be used in subsequent experiments with EGCG and epicatechin.

5.2.2 Effect of Cytochalasin B

Mature 3T3-L1 adipocytes, differentiated as detailed in section 2.3.1 were pre-treated with 20 μM cytochalasin B for 10 min, followed by 10 min with and without 100 nM insulin, for 10 min. 27.8 kBq [^{3}H] 2-DOG and 1.5 mM 2-DOG was added for 10 min, and uptake of 2-deoxyglucose was measured.

Cytochalasin B significantly decreased basal and insulin mediated glucose uptake ($p<0.001$, Figure 5.1b). As the glucose uptake system was responding as expected, the effect of epicatechin and EGCG was investigated.



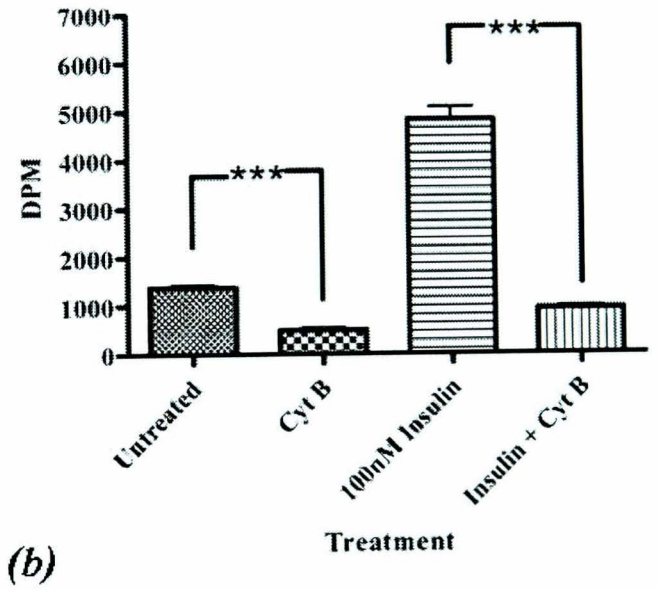


Figure 5.1 2-Deoxyglucose uptake by mature 3T3-L1 adipocytes in 12 well plates, in response to varying insulin concentrations (a) and using cytochalasin B as an inhibitor (b).

*Adipocytes were treated with 10, 50, 100 and 200 nM insulin for 10 min (a) or 10 min treatment with 20 μM cytochalasin B, with and without 100 nM insulin for 10 min (b). Uptake is measured as disintegrations per minute (DPM) per well. Data were analysed using a one-way ANOVA, *** $p<0.001$ ($n=6$).*

5.3 Effect of Epicatechin and EGCG

Mature 3T3-L1 adipocytes, differentiated as outlined in Section 2.3.1 were treated with 1, 10 and 50 μM epicatechin and 0.1, 1 and 10 μM EGCG for 30 min, 6 h and 24 h. 10 nM insulin was added, or not, for 10 min, followed by 27.8kBq [^{3}H] 2-DG and 1.5 mM 2-DG for 10 min, and uptake of 2-deoxyglucose was measured.

Neither epicatechin nor EGCG had any effect on either basal or insulin mediated glucose uptake at any concentration or time point investigated (Figure 5.2 and Figure 5.3)

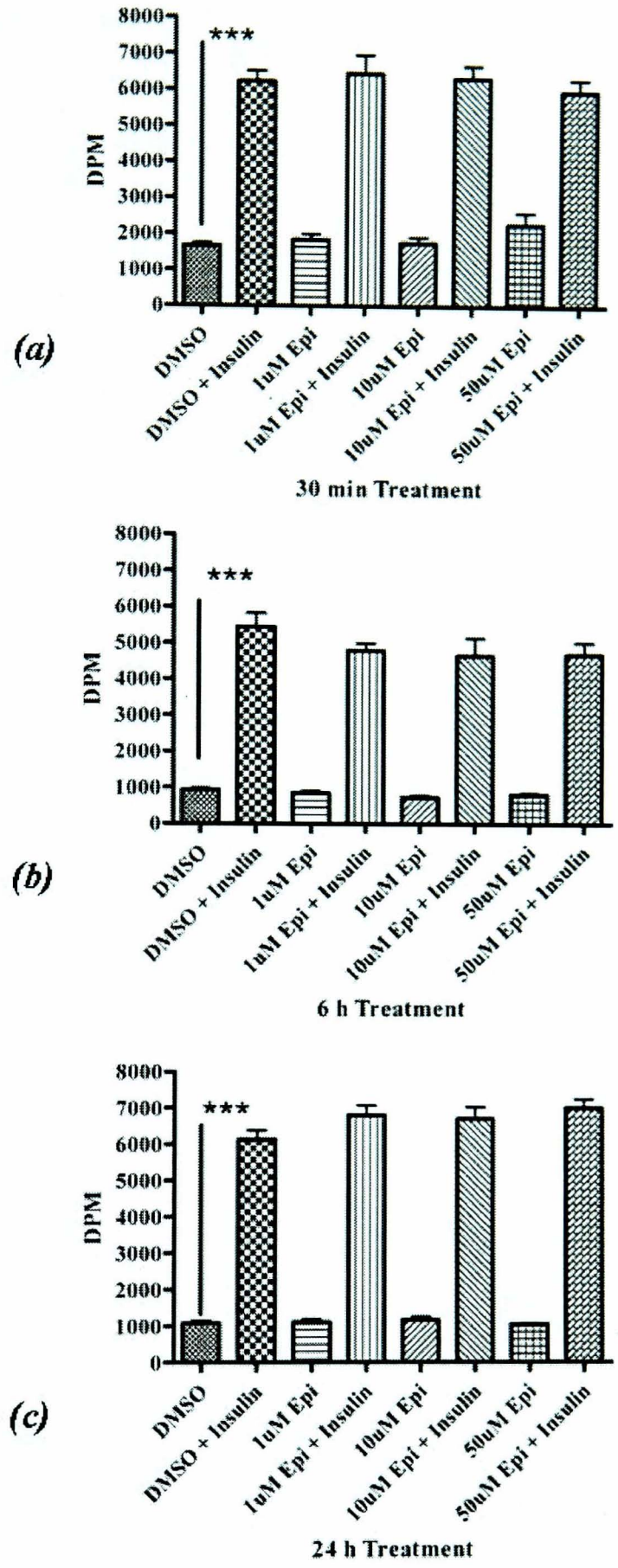


Figure 5.2 2-Deoxyglucose uptake by mature 3T3-L1 adipocytes in 12 well plates, following 30 min (a), 6h (b) and 24h (c) treatment with either 1, 10 or 50 μM epicatechin with and without 10 nM insulin for 10 min.

*Uptake is measured as disintegrations per minute (DPM) per well. Data were analysed using a one-way ANOVA, *** $p<0.001$ (n=6).*

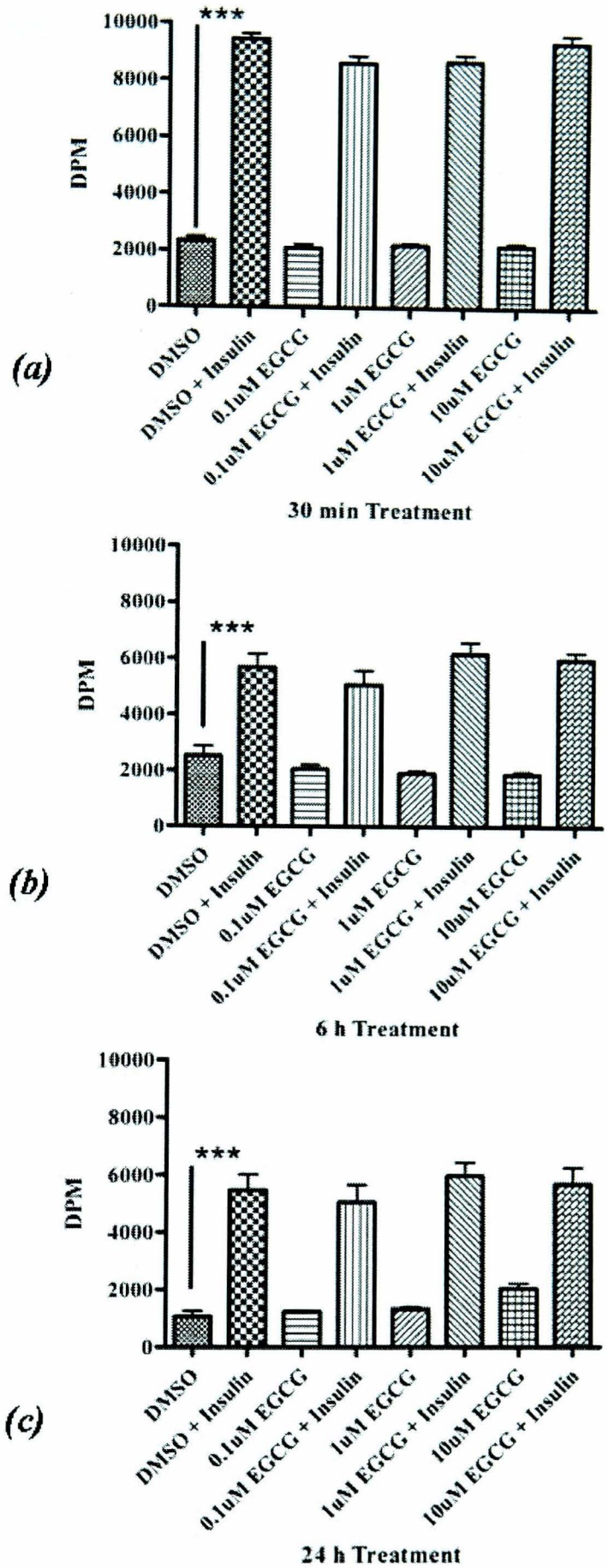


Figure 5.3 2-Deoxyglucose uptake by mature 3T3-L1 adipocytes in 12 well plates, following 30 min (a), 6 h (b) and 24 h (c) treatment with either 0.1, 1 or 10 μM EGCG with and without 10 nM insulin for 10 min.
*Uptake is measured as disintegrations per minute (DPM) per well. Data were analysed using a one-way ANOVA, *** $p<0.001$ (n=6).*

5.4 Discussion

Plant polyphenols act to both increase and decrease basal and insulin stimulated glucose uptake, but their concentrations, optimum incubation times and potential modes of actions vary (detailed in Section 1.6). This study shows that neither epicatechin nor EGCG, at the times and concentrations investigated, altered basal or insulin mediated glucose uptake in mature 3T3-L1 adipocytes.

Kaempferol and quercetin, both of which have similar structures to epicatechin (see Figure 5.4) both significantly improved insulin-mediated glucose uptake in mature 3T3-L1 adipocytes when added for 3 days following differentiation (Fang *et al.* 2008). 5 and 10 μM of each compound had no effect, 20 μM of each increased glucose uptake by approximately 25% and 50 μM by approximately 30%. It is possible that had epicatechin and EGCG been added at 50 μM for 3 days there may have been an increase in glucose uptake. The concentrations and treatment periods in this study were chosen based on the hypothesis that any effect may be downstream of ERK1/2. Furthermore, the concentrations of the flavonols used in the study by Fang *et al* are far too high to be achieved physiologically. As stated previously from studies on the EGCG content of green tea and pharmacokinetic data on EGCG in humans, attainment of such high levels are unrealistic.

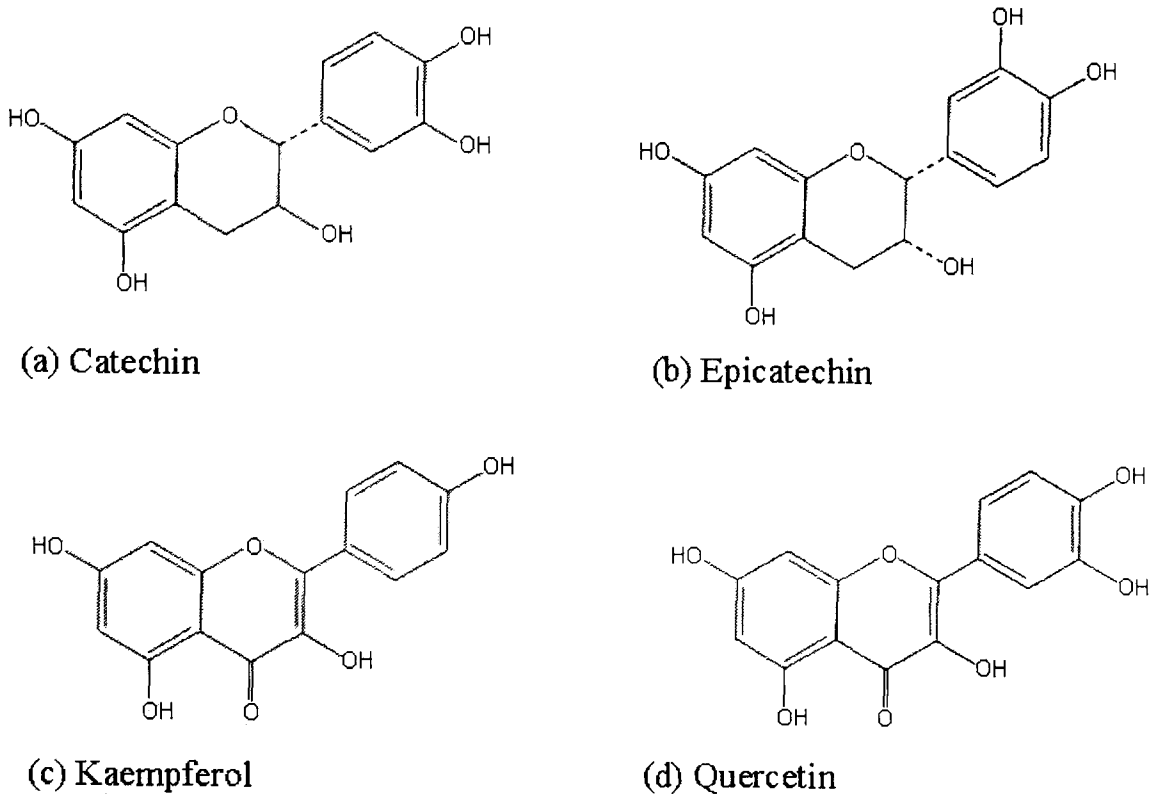


Figure 5.4 Chemical structures of catechin (a), epicatechin (b), kaempferol (c) and quercetin (d).

The basal conditions of the 3T3-L1 adipocytes used in the kaempferol and quercetin study vary significantly from those of the 3T3-L1 adipocytes used in this study. The differentiation protocol used by Fang *et al.* involves 48 h with 1.72 μM insulin, 1 μM dexamethasone and 0.5 mM IBMX in DMEM (10% FCS and supplements) and maintenance for a further 7 days with 1.72 μM insulin in DMEM (10% FCS and supplements). Treatment for 3 days is also carried out in this medium. The present differentiation protocol involved 166 nM insulin, 0.25 μM dexamethasone and 0.5 mM IBMX for 48 h and 166 nM insulin for a further 48 h, after which, cells were maintained in insulin free medium (DMEM, 10% FCS and supplements) for a further 48 h before replacing it with a lower glucose DMEM (12.5 mM). Epicatechin/EGCG treatment was carried out in this lower glucose, insulin-free medium. This

difference in conditions may be a factor which could explain the difference in the way mature 3T3-L1 adipocytes behave when treated with very similar compounds. It may also be possible that the cells used by Fang et al. were insulin resistant, having been exposed to such a high concentration of insulin for a 12 day period, encompassing differentiation, maintenance and treatment. This study by Fang et al suggests that kaempferol and quercetin are acting as weak partial agonists for PPARγ and compete for the same binding site as a traditional PPARγ agonist, rosiglitazone.

The effect of berberine, an isoquinoline alkaloid isolated from Chinese medicinal herbs such as *Coptidis rhizoma* and *Cortex phellodendri* has been studied in normal and insulin resistant 3T3-L1 adipocytes (Kim *et al.* 2007). Cells were made insulin resistant by exposure to 10 nM insulin for 12 h. This concentration is in fact 1/170 of the concentration used during the differentiation and treatment in the study by Fang et al. The study by Kim *et al.* showed that 5 μM berberine for 6 h significantly enhanced basal glucose uptake in normal and insulin resistant cells and modestly increased insulin-stimulated glucose uptake. Interestingly, this same berberine treatment increased ERK1/2 phosphorylation, and the exposure to an ERK inhibitor PD98059, significantly decreased berberine stimulated glucose uptake. Further work in this paper went on to show that berberine mediated glucose uptake was a result of enhanced GLUT1 expression, as a consequence of ERK1/2 stimulation.

There is further evidence to suggest that ERK1/2 plays a role in glucose transport (Harmon *et al.* 2004). Treatment of mature 3T3-L1 adipocytes with

the MEK inhibitors PD98059 and U0126 inhibited insulin mediated glucose uptake. As both epicatechin and EGCG had increased ERK1/2 phosphorylation at 10 μM and 0.1 μM respectively, increased glucose uptake was expected.

(-)Catechin, which is also structurally very similar to epicatechin (see Figure 5.4), increased insulin dependent glucose uptake in differentiated 3T3-L1 adipocytes (Cho *et al.* 2007). Fully differentiated 3T3-L1 adipocytes were treated with 50 μM (-) catechin for 24 h. Initially, the effect of (-) catechin and various other flavonoids, including epicatechin, EGCG, quercetin, kaempferol and other structurally related compounds on adiponectin release, was investigated. (-) Catechin increased adiponectin release to 213% of the control, interestingly; however, quercetin and kaempferol at 50 μM suppressed adiponectin release to 54% and 42% of the control, respectively, whereas epicatechin and EGCG had no effect on adiponectin release. The effect of (-)-catechin on adiponectin release was thought to be mediated by Kruppel-like factor 7, a transcription factor, the activity of which is linked to the expression of adiponectin, leptin, PPARγ, C/EBPα and aP2 (Cho *et al.* 2007).

Genistein, an isoflavone derivative, inhibited both basal and insulin stimulated glucose uptake after15 min at 50 μM (Bazuine *et al.* 2005). This is thought to be a result of interference with the insulin-induced glucose uptake directly and not by inhibiting GLUT4 translocation. Myricetin and quercetin, which are both structurally similar to epicatechin (see Figure 5.4), and catechin-gallate, when incubated with isolated rat adipocytes at 0.1-100 μM also dose-dependently decreased basal and insulin stimulated methylglucose uptake

(Strobel *et al.* 2005). As this study was carried out in isolated rat adipocytes as opposed to mature 3T3-L1 adipocytes, it may not be correct to compare this to the other studies.

As compounds that are structurally related to epicatechin increase glucose uptake, it was hypothesized that epicatechin, if not EGCG would have the same result. The mechanisms of action appear to vary between the compounds and therefore it is difficult to predict how another compound may behave. It is possible that at 10 μM insulin, the glucose uptake was at the maximum response. The use of insulin at concentrations lower than 10 μM may have allowed the epicatechin or EGCG to further increase the glucose uptake response.

There is evidence to suggest that EGCG is insulinomimetic and has been seen to lower glucose production in H4IIE rat hepatoma cells (Waltner-Law *et al.* 2002). In this same study, it was reported that EGCG decreases expression of genes that control gluconeogenesis such as PEPCK and G6Pase and activates the same kinases as insulin and promotes phosphorylation of insulin signalling proteins such as IRS-1 and IR-β in H4IIE cells (Waltner-Law *et al.* 2002). The *in vivo* evidence is somewhat contradictory as green tea, when given to rats as a replacement for drinking water for 3 weeks significantly reduced glucose uptake, accompanied by a decrease in translocation of GLUT4 (Ashida *et al.* 2004). However after 12 weeks green tea significantly increased both basal and insulin mediated glucose uptake (Wu *et al.* 2004).

Taking into consideration the studies by Fang *et al*, Kim *et al* and Cho *et al* it appears that the majority of flavanols investigated to date increase glucose

uptake in 3T3-L1 adipocytes. This is through varying mechanisms, one suggests partial agonism of PPARγ and decreased adiponectin release, another through the ERK1/2 pathway, and the last through increased adiponectin release mediated by the suppression of Kruppel-like factor 7 (KLF7) protein. Evidence from *in vivo* studies using green tea is not consistent. Although in this study it is not evident that glucose uptake is altered by epicatechin or EGCG treatment, it is possible to suggest that under different conditions of concentration and time, epicatechin and EGCG could increase basal and/or insulin-mediated glucose uptake.

Gene Expression:

effect of flavanols on FABP4, leptin, resistin, adiponectin, adipsin, SREBP, PEPCK and PPARγ2 expression

6 Gene Expression

6.1 Introduction

In order to try and understand the downstream effects of flavanol stimulation of 3T3-L1 adipocytes, the expression of various genes, known to be either up or downregulated in obesity, was investigated. These genes were adipocyte fatty acid binding protein (FABP4, also known as aP2), leptin, resistin, adiponectin, adipsin/complement factor D, sterol regulatory element binding protein (SREBP) and PPARγ2. See Table 6.1 below for whether these genes are up or downregulated in obesity, the fold change, if published, and whether there is evidence of their regulation by flavanols. In addition to these genes, expression of β-actin and eukaryotic 18S rRNA were measured as non-variant control genes.

6.2 Methods

3T3-L1 adipocytes were grown and differentiated as detailed in Chapter 2. When cells were fully differentiated they were treated with DMSO, 10 μM epicatechin or 0.1 μM EGCG for 6 or 24 hours. Cells were collected in TriReagent, and processed as detailed in Section 2.6. Details of primers, probes and TaqMan cycles are described in Section 2.6. Gene expression quantities (in arbitrary units) were determined from the mean of triplicate determinants of each sample. As stated in Section 2.6.7, data from Taqman was only used if the slope of the standard curve for each plate was between –

3.2 and –3.6, indicating that the efficiency of the process is almost 100%, In addition, Ct values of triplicate readings for an individual sample that were more than 0.5 Ct apart were excluded. Target gene expression was normalised by dividing the amount of target gene by the amount of reference gene for each sample.

Table 6.1 Genes measured by TaqMan analysis of 3T3-L1 adipocytes treated with 10 μM epicatechin or 0.1 μM EGCG for 6 and 24 h.
References are (1) (Xu et al. 2006), (2) (Nadler et al. 2000), (3) (Li et al. 2002), (4) (Haluzik et al. 2004), (5) (Kao et al. 2000), (6) (Liu et al. 2006), (7) (Nagao et al. 2009), (8) (Ashida et al. 2004).

Gene	Regulation of Gene Expression	
	In Obesity	Flavanol Treatment
FABP4	Marked increase (1)	-
Leptin	3-fold increase (2)	Decrease (5)
Resistin	2.2-fold increase (3)	Decrease (6)
Adiponectin	3-fold decrease (4)	Increase (7)
Adipsin	8.3-fold decrease (2)	-
SREBP	2.7-fold decrease (2)	-
PEPCK	5.3-fold decrease (2)	-
PPARγ2	Decrease (2)	Decrease (8)

6.3 Results

6.3.1 Variation Between Housekeeping Genes

18S RNA and β-actin were used as non-variant control or reference genes. Expression of the target genes was then normalised to expression of a reference gene. Expression of these two genes was not constant, and therefore was compared to decide which should be used as the reference gene.

Figure 6.1 shows regression analysis of 18S and β-actin expression in the samples used. It is apparent that expression of these genes does not correlate, and this is further confirmed by the very poor R^2 value of 0.003.

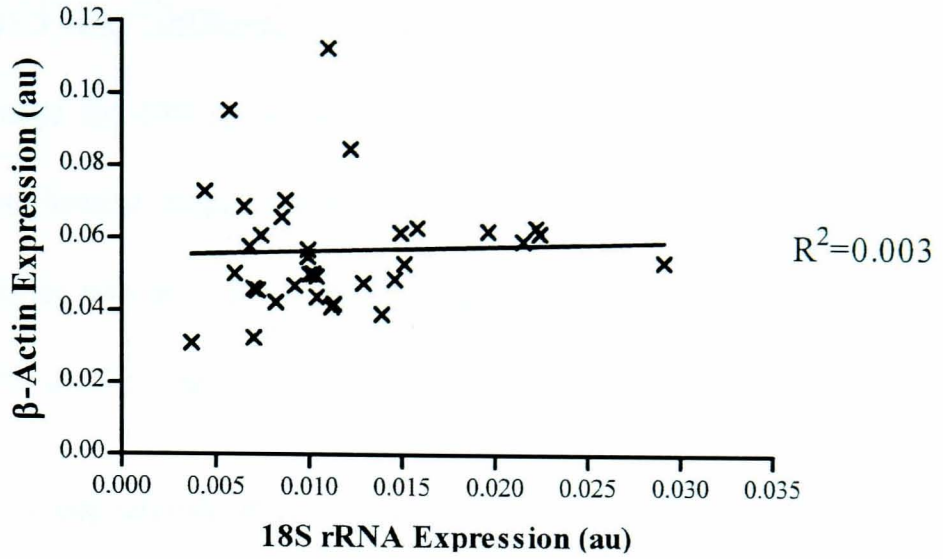


Figure 6.1 Correlation between gene expression of 18S rRNA and β-actin in samples used for TaqMan.
Results were analysed using Pearson's correlation. Measured in arbitrary units (au).

Despite considerable variation of 18S rRNA expression between treatment groups (Figure 6.2 (a)), compared to the slightly more consistent β-actin gene expression (Figure 6.2 (b)), a one-way ANOVA of 18S rRNA expression does not show any significant differences between the treatment groups.

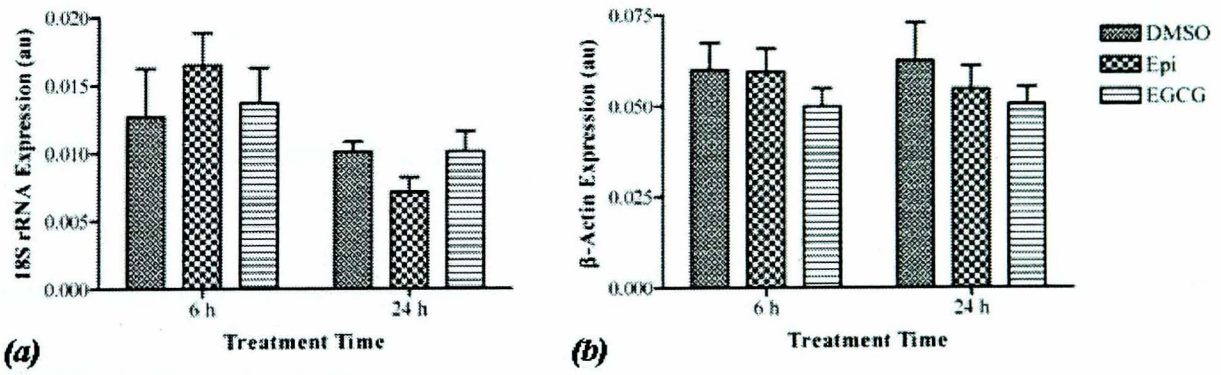


Figure 6.2 Variation in 18S rRNA (a) and β-actin (b) expression between treatment groups, measured in arbitrary units (au).

Comparisons of the data spread (see Table 6.2), calculated by dividing the range by the median of each treatment group, reveals that expression of 18S rRNA in these samples is more widely spread than that of β-actin. There is

almost a 5-fold difference between the lowest and highest value for median/range for 18S rRNA compared to only a 2 fold difference between the highest and lowest values for β-actin. Use of 18S rRNA as a reference gene for these samples would increase the variability between data, and for this reason, β-actin was used as the reference gene.

Table 6.2 Comparison of the spread of 18S rRNA and β-actin gene expression in samples used for TaqMan analysis.
Calculated by dividing the range by the median for each treatment group.

Reference Gene	Treatment	Mean Quantity	SD	Median	Range	Range/ Median
18S rRNA	6 h DMSO	0.0127	0.0087	0.0102	0.0234	2.3054
	6 h Epi	0.0165	0.0054	0.0158	0.0120	0.7595
	6 h EGCG	0.0137	0.0064	0.0132	0.0152	1.1515
	24 h DMSO	0.0101	0.0017	0.0101	0.0047	0.4677
	24 h Epi	0.0071	0.0026	0.0078	0.0063	0.8129
	24 h EGCG	0.0101	0.0036	0.0093	0.0081	0.8710
β-actin	6 h DMSO	0.0600	0.0183	0.0544	0.0515	0.9467
	6 h Epi	0.0595	0.0155	0.0607	0.0465	0.7661
	6 h EGCG	0.0499	0.0121	0.0496	0.0309	0.6236
	24 h DMSO	0.0626	0.0257	0.0536	0.0700	1.3072
	24 h Epi	0.0548	0.0157	0.0539	0.0419	0.7781
	24 h EGCG	0.0507	0.0116	0.0472	0.0295	0.6250

6.3.2 Expression of Target Genes in Samples

Data for expression of adipsin, SREBP, FABP4, PEPCK, adiponectin and PPARγ2 mRNA normalised to β-actin expression are shown in Table 6.3 and Figure 6.3. Levels of leptin and resistin mRNA in these samples were undetectable, and therefore data is not shown for these genes.

Treatment of 3T3-L1 adipocytes with 10 μM epicatechin or 0.1 μM EGCG for 6 and 24 hours does not appear to significantly alter any of the genes investigated.

Table 6.3 Amount of target genes adipsin, SREBP, FABP4, PEPCK, adiponectin and PPARγ2 mRNA, normalised to β-actin gene expression. Levels of leptin and resistin, although measured, were undetectable.(-) represents missing values. Gene expression was measured in arbitrary units (au).

Sample		Adipsin	SREBP	FABP4	PEPCK	Adiponectin	PPARγ
6 h DMSO	1	0.8205	0.6219	0.8076	0.9086	0.3592	0.4270
	2	0.9055	0.8322	0.9656	0.9267	0.7244	0.5164
	3	0.8506	0.8858	0.8156	0.7365	0.3517	0.2900
	4	1.0450	0.7922	0.9255	0.7084	0.3122	0.4406
	5	0.8518	0.6636	0.7168	0.7547	0.2710	0.3648
	6	0.7962	0.7166	0.8102	0.6672	0.3696	0.3409
6 h Epi	1	0.7475	0.7664	0.7213	0.7849	0.2983	0.3927
	2	0.8120	0.8388	0.7522	0.6984	0.2202	0.2475
	3	1.0456	1.0138	0.8840	0.8217	0.3491	0.5153
	4	0.8312	0.8489	0.8529	0.7262	0.2168	0.2650
	5	1.0065	0.6884	0.6483	0.6048	0.2156	0.2018
	6	0.7756	0.5710	0.8290	0.4859	0.3404	0.2527
6 h EGCG	1	0.8368	0.7189	0.7808	0.6554	0.2219	0.3075
	2	0.8704	0.9840	0.9013	0.8402	0.4571	0.3553
	3	0.8907	0.8058	0.7071	0.6917	0.2334	0.3275
	4	1.2130	1.0999	0.9169	0.7073	0.2211	0.2016
	5	0.6812	0.5860	0.7405	0.5914	0.2858	0.1722
	6	0.8521	0.7776	0.8300	0.6329	0.4472	0.2315
24 h DMSO	1	1.0326	1.0240	0.7480	0.9637	0.4470	0.5297
	2	0.9390	0.8934	0.6628	0.8687	0.2249	0.2377
	3	1.0422	0.8594	0.6860	0.7771	0.2931	0.3365
	4	1.2908	0.8009	0.8372	0.9792	0.2826	0.3395
	5	0.7499	0.7054	0.5426	0.6519	0.2989	0.2631
	6	0.9178	0.6966	0.6778	0.7506	0.2658	0.2669
24 h Epi	1	1.1264	0.8739	0.7419	1.0110	0.2784	0.4458
	2	0.9068	0.8695	0.7091	0.7667	0.3975	0.5070
	3	0.6588	0.6524	0.3689	0.6111	0.2023	0.2351
	4	0.8253	0.7691	0.6144	0.7139	0.2167	0.3257
	5	0.6970	0.6419	0.6929	0.6610	0.3064	0.3533
	6	0.7207	0.6853	0.8695	0.7677	0.3120	0.2505
24 h EGCG	1	0.9103	0.8354	0.6109	0.8786	0.2482	0.2831
	2	0.9656	0.9856	1.0016	1.0895	(-)	(-)
	3	0.7936	0.7247	0.6763	0.8033	0.3157	0.3962
	4	1.1482	0.8041	0.7537	0.8704	0.2417	0.2658
	5	0.8394	0.6577	0.7630	0.8443	0.3607	0.5110
	6	0.7390	0.6182	0.6604	0.7617	0.2837	0.2186

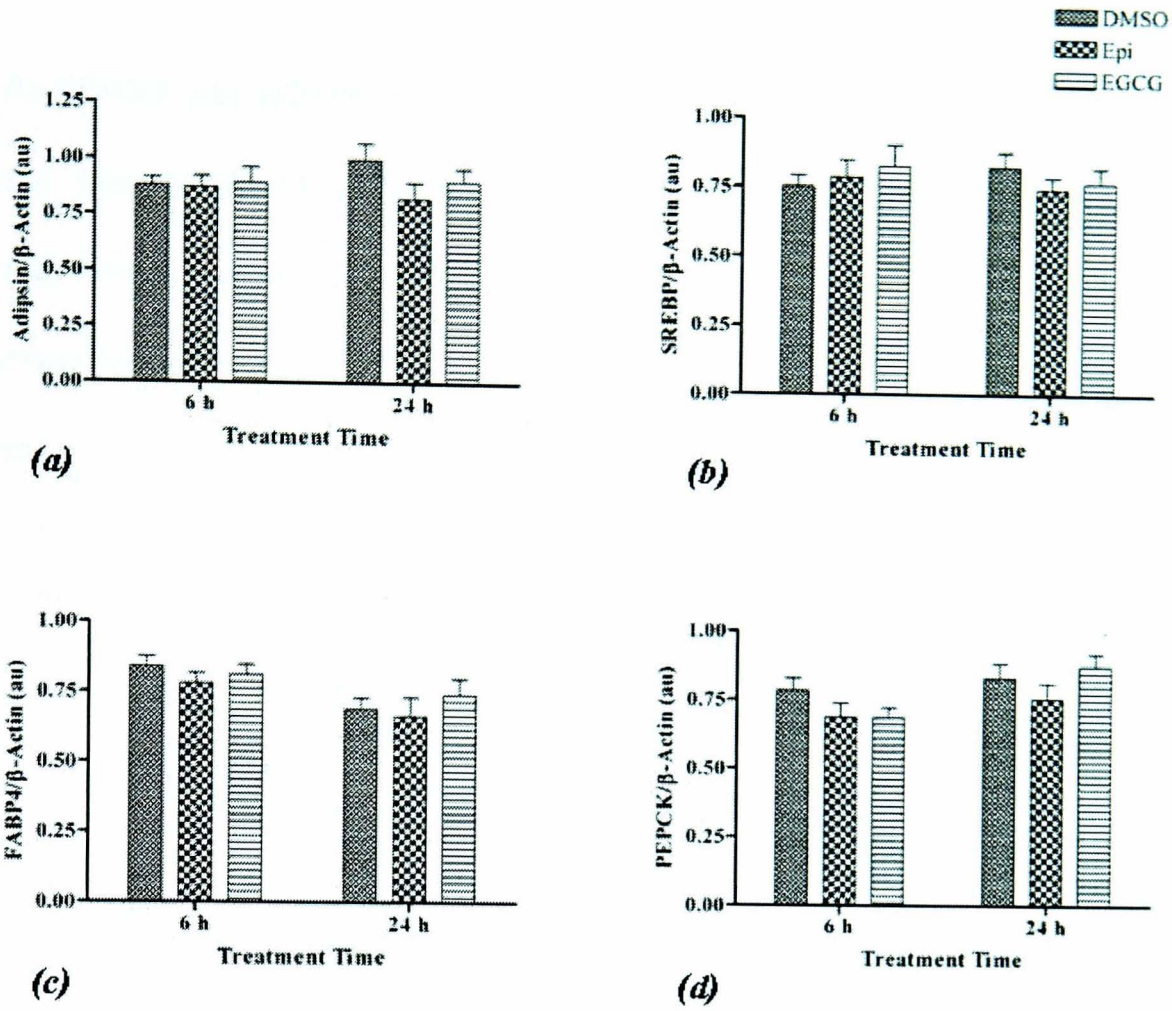


Figure 6.3 Expression of adipsin (a), SREBP (b), FABP4 (c), PEPCK (c), adiponectin (e) and PPARγ (e) relative to β-actin.
Leptin and resistin levels were undetectable in these samples. Gene expression was measured in arbitrary units (au).

6.3.3 Correlation of Target Gene Expression

As PPARγ and adiponectin are SREBP controlled genes, and FABP4, PEPCK and adiponectin are all PPARγ controlled, analysis of PPARγ or adiponectin expression compared to SREBP, and FABP4, PEPCK or adiponectin expression compared to PPARγ should show some correlation (see Figure 6.4 and Figure 6.5*)*.

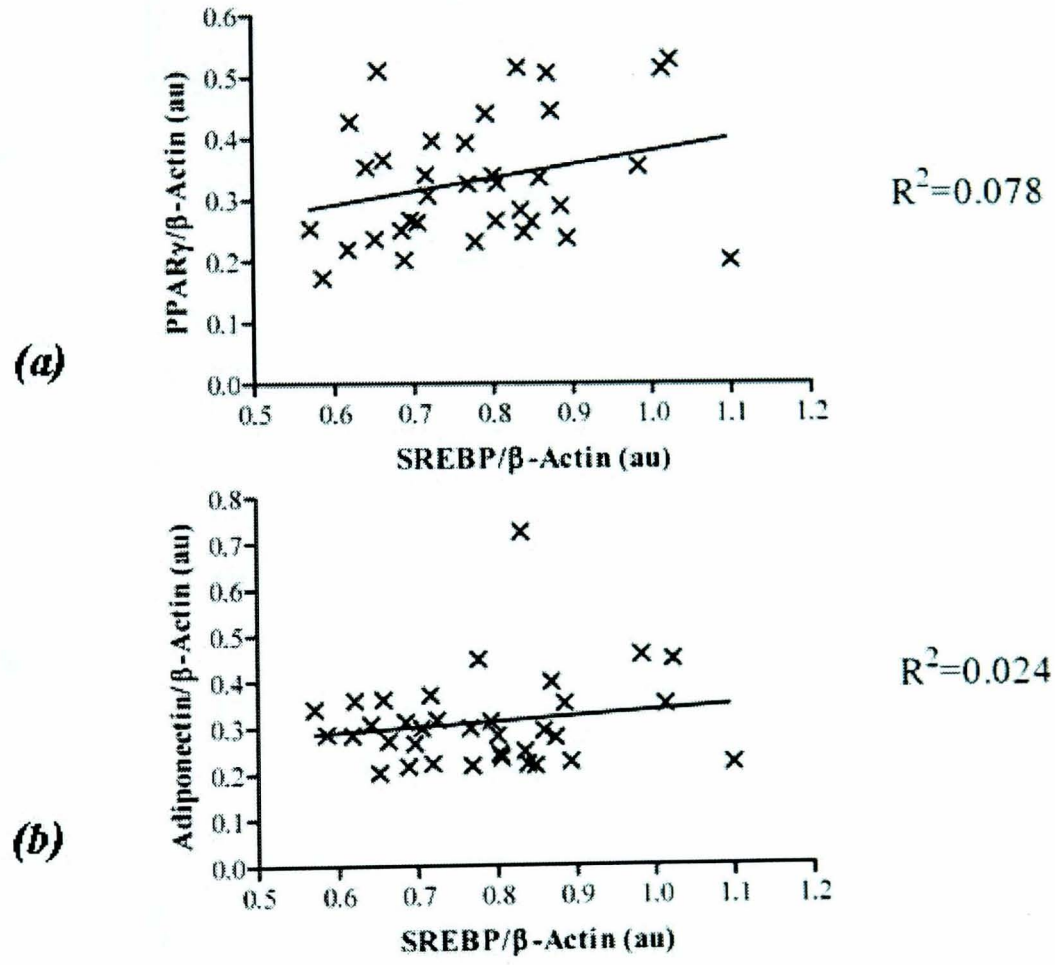


Figure 6.4 Correlation between gene expression of SREBP and PPARγ (a) and adiponectin (b) relative to β-actin.
Results were analysed using Pearson's correlation. Measured in arbitrary units (au).

The poor R^2 values of 0.078 for SREBP vs PPARγ (Figure 6.4 (a)), 0.024 for SREBP vs adiponectin (Figure 6.5 (b)) and 0.082 for PPARγ vs FABP4

(Figure 6.5 (a)) shows that there is no correlation. However, the R^2 values of 0.330 for PPARγ vs PEPCK (Figure 6.5 (b)) and 0.320 for PPARγ vs adiponectin (Figure 6.5 (c)) show significant correlation as analysis by Pearson's correlation gives p values of 0.0003 and 0.0004 respectively.

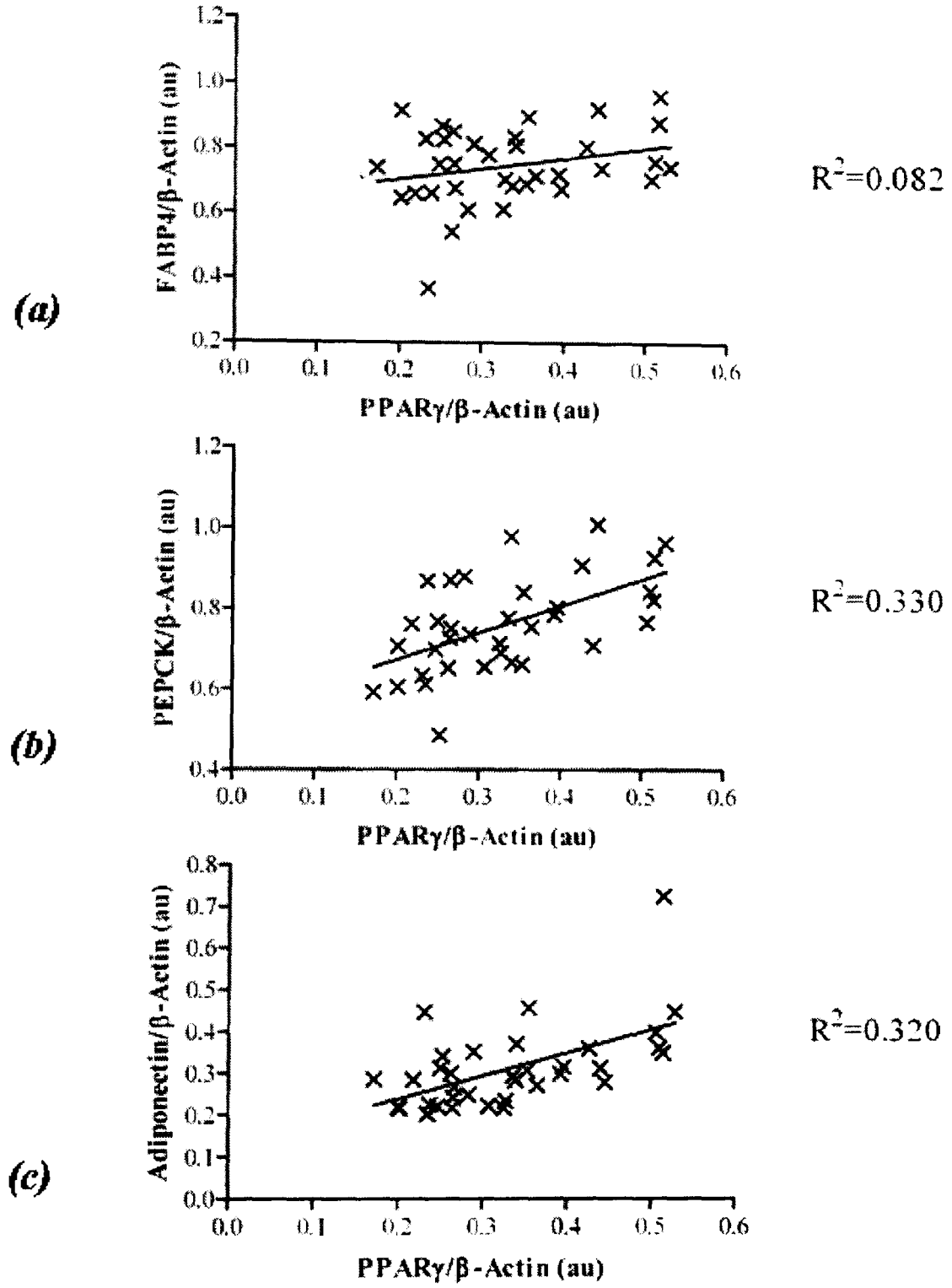


Figure 6.5 Correlation between gene expression of PPARγ and FABP4 (a), PEPCK (b) and adiponectin (c) relative to β-actin.

Results were analysed using Pearson's correlation. Expression was measured in arbitrary units (au).

6.4 Discussion

The aim of this chapter was to investigate the downstream effects of flavanol stimulation in 3T3-L1 adipocytes. Previous chapters have shown that both epicatechin and EGCG were able to alter adipokine release from 3T3-L1 adipocytes and stimulate ERK1/2 phosphorylation, but did not alter basal or insulin mediated glucose uptake.

Time points for epicatechin and EGCG treatment were chosen on the basis that ERK1/2 phosphorylation was observed at 30 minutes. 6 hours was considered to be enough time for gene expression to alter, and 24 hours would show whether any change seen was long lasting. It is possible that neither of these time points were long enough to see changes in gene expression. Concentrations of 10 μM for epicatechin and 0.1 μM for EGCG were chosen on the basis that these produced the maximum ERK1/2 phosphorylation seen in Chapter 4.

The use of eukaryotic 18S ribosomal RNA as a reference gene was unsuccessful and showed considerable variation between treatment groups, although this was not significant. β-actin expression was a lot more consistent in these samples, and was therefore used as the reference gene to which all target gene expression data was normalised. Studies carried out by others use both β-actin and eukaryotic 18S rRNA as reference genes but variability between samples and treatments is not commented on. It is possible that neither 18S rRNA or β-actin are appropriate reference genes for 3T3-L1 cells.

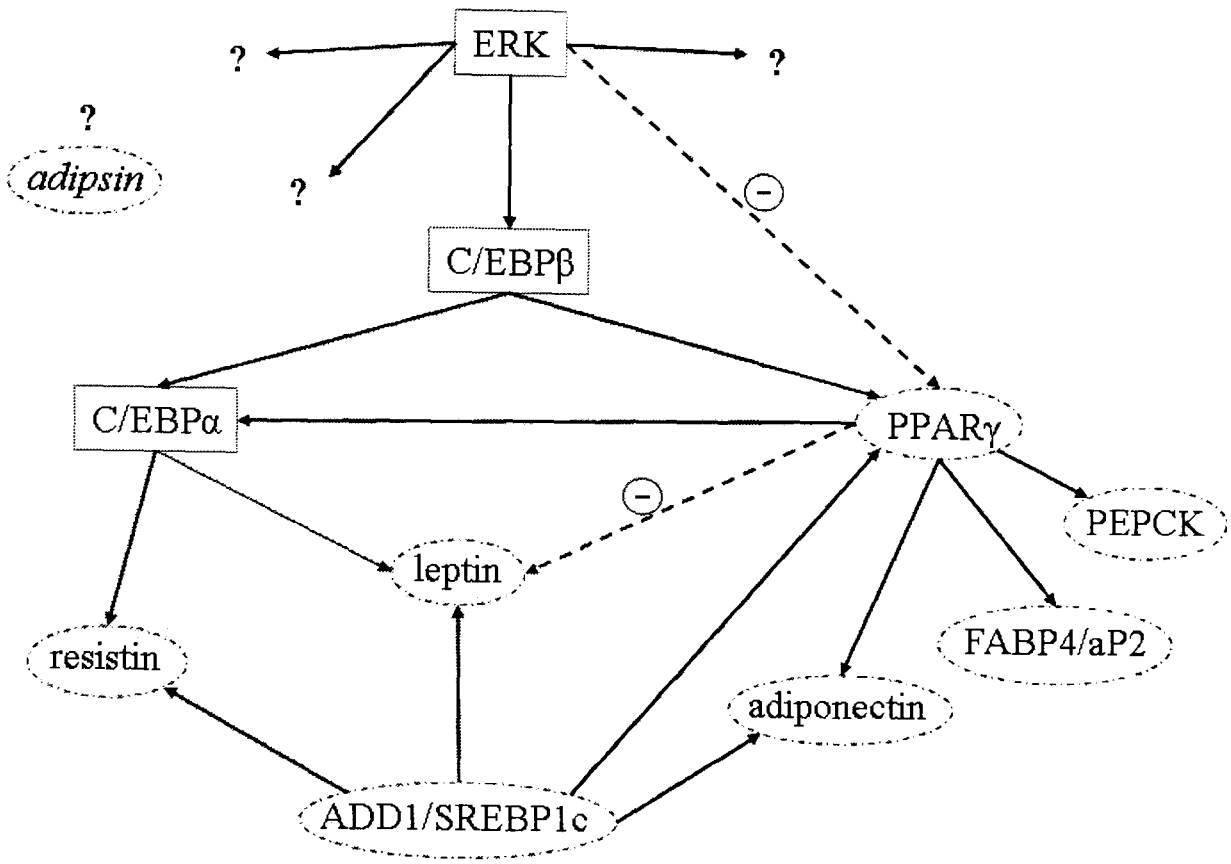


Figure 6.6 Regulation of target genes by ERK1/2, C/EBPα, C/EBPβ and PPARγ.

Adipogenesis is regulated by PPARγ and C/EBPα (Rosen et al. 2000), and the expression of these transcription factors is regulated by C/EBPβ, which is phosphorylated by ERK1/2 during adipogenesis (Park et al. 2004). Phosphorylation of PPARγ by ERK1/2 inhibits its activity (Hu et al. 1996). PPARγ directly regulates the adipogenic genes FABP4/aP2 (Spiegelman et al. 1993) and PEPCK (Tontonoz et al. 1995), and indirectly regulates the expression of adiponectin through activation of C/EBPα. ADD1/SREBP1c regulates the expression of PPARγ by controlling the production of endogenous PPARγ ligands (Kim et al. 1998) and directly activating transcription of PPARγ (Fajas et al. 1999). In differentiated adipocytes, ADD1/SREBP1c regulates adiponectin expression (Seo et al. 2004), transactivates leptin gene expression (Kim et al. 1998) and along with C/EBPα, regulates resistin gene expression (Seo et al. 2003). Leptin expression is also regulated by both C/EBPα, which activates it, and PPARγ, which down-regulates it (Hollenberg et al. 1997). No connection has currently been elucidated between adipsin expression and any of the genes or transcription factors mentioned although its expression is sharply down-regulated in obesity (Nadler et al. 2000).

The genes investigated in this chapter; FABP4/aP2, leptin, resistin, adiponectin, adipsin, ADD1/SREBP1c, PEPCK and PPARγ2, were all chosen on the basis that they were up or down-regulated in obesity. Their regulation is in some way connected to the ERK1/2 pathway and they have complex interrelationships with each other (summarised in Figure 6.6).

In this study, no significant changes in expression of these genes were observed following treatment with 10 μM epicatechin or 0.1 μM EGCG for 6 or 24 hours. Although some of these genes are up-regulated in obesity, and others down-regulated (see Table 6.1), they are all upregulated during adipogenesis (Fajas *et al.* 1998; Nadler *et al.* 2000; Rosen and Spiegelman 2000; Walczak and Tontonoz 2002). As the 3T3-L1 adipocytes used for this study were fully differentiated, adipogenesis is considered to be complete, and the genes involved in it may no longer be upregulated.

Figure 6.6 shows how the target genes investigated in this study are regulated. Resistin and leptin expression are controlled by C/EBPα, which, through C/EBPβ is activated downstream of ERK1/2. As both epicatechin and EGCG were shown to phosphorylate ERK1/2 (Chapter 4), it might be expected that they would increase the expression of resistin and leptin. Levels of leptin and resistin were undetectable in any of the samples tested, and perhaps, although the genes are expressed, their levels are far too low to be detected by this method. Detection of resistin (Patel *et al.* 2003) and leptin (Schoof *et al.* 2004) by TaqMan is reported in the literature, but only in human tissue and not in 3T3-L1 cells. Leptin expression is also negatively regulated by PPARγ, however there is evidence to suggest that PPARγ activity is inhibited by

phosphorylation by activated ERK1/2 (Hu *et al.* 1996; Adams *et al.* 1997; Camp and Tafuri 1997; Reginato *et al.* 1998; Chan *et al.* 2001). This should mean, therefore, that PPARγ should be unable to inhibit leptin expression, providing further rationale for the expected increase in leptin expression.

PPARγ also regulates the expression of adiponectin, FABP4 and PEPCK, genes which are down-regulated in obesity but up-regulated during adipogenesis. As previously mentioned, phosphorylation of PPARγ by ERK1/2 inhibits its activity (Hu *et al.* 1996; Adams *et al.* 1997; Camp and Tafuri 1997; Reginato *et al.* 1998; Chan *et al.* 2001), and therefore activation of ERK1/2 by epicatechin and EGCG should decrease expression of these genes.

ADD1/SREBP1c opposes the action of ERK1/2 by activating PPARγ, so indirectly activating expression of PEPCK and FABP4/aP2. Additionally, ADD1/SREBP1c directly up-regulates leptin, resistin and adiponectin. There is also some evidence to suggest that ERK1/2 may regulate the expression of SREBP1c in skeletal muscle (Boonsong *et al.* 2007).

FABP4, PEPCK and adiponectin are regulated by PPARγ and have PPAR response elements (PPREs), and ADD1/SREBP1c regulates PPARγ, adiponectin, leptin and resistin. It therefore follows that expression of these pairs of genes should correlate. This however was not the case for PPARγ and FABP4/aP2, ADD1/SREBP1c and PPARγ or ADD1/SREBP1c. However there did appear to be correlation between expression of PPARγ and PEPCK and PPARγ and adiponectin. As there were no significant changes in expression of these genes, it is somewhat unexpected that expression of only

two pairs of these genes should correlate, whereas the other pairs did not show significant correlation, especially as FABP4 and PEPCK show very similar patterns of expression in these samples. However, as there are no changes in gene expression, it is possible that this is just the normal range of distribution of gene expression in these samples and therefore there is not suffiecient variation to detect any correlation.

The majority of gene expression work that has been reported in the literature is the result of analysis of adipose tissue from animal models of obesity. This could mean that any changes could be caused by whole body metabolic effects that are not present in cell culture models. Another explanation is that the changes reported in these instances are the result of altered gene expression in a latent preadipocyte population, whereas this study used the 3T3-L1 adipocyte cell model and efficient differentiation of preadipocytes to adipocytes was ensured before cell culture dishes were selected for treatment.

General Discussion

7 Discussion

Green tea consumption has been believed for centuries to have significant health benefits. More recently, the effects of green tea, and more importantly, the flavanols that are its active constituents, have been investigated with regards to their effects on obesity. Intraperitoneal injection and feeding studies in rodents and feeding studies in humans, demonstrate that green tea flavanols, and more specifically EGCG, are able to prevent increases in body weight and adipose tissue mass when both lean and obese subjects are fed a high fat diet. Additionally, other factors such as decreased leptin levels and lipogenesis and increased adiponectin levels and β-oxidation, suggest that EGCG and related flavanols are able to beneficially modulate the development of obesity.

The aim of these studies was to investigate the effect of green tea derived catechins upon adipocyte metabolism. The first objective was, therefore, to establish an efficient cell culture system in which adiponectin and resistin could be measured by ELISA. Further to this, the effects of (+)-catechin, (-)-epicatechin and (-)-EGCG on adiponectin release were measured. Under high medium glucose concentrations, catechin and epicatechin appear to positively regulate adiponectin and resistin release, whereas EGCG negatively regulates this. The EGCG effect on adiponectin release is dependent on medium glucose concentration – lowering the medium glucose concentration from 25 to 12.5 mM ablated the lowering effect that EGCG had on adiponectin release. Interestingly, medium glucose concentration did not affect the extent of the EGCG-induced decrease in resistin release. Resistin levels are increased in obesity and associated insulin resistance, and the effect of EGCG in lowering

resistin levels without lowering adiponectin levels (under lower, more physiological glucose concentrations) can be paralleled to the action of TZDs, which decrease resistin expression through PPARγ and subsequently improve insulin resistance (Hartman *et al.* 2002).

Studies in the literature investigating adipokine release as a result of flavonoid treatment demonstrate that adipokine release is flavonoid specific. A study by Cho *et al* demonstrated that 50 μM catechin, epicatechin 3-gallate, quercetin and kaempferol for 24 h altered adiponectin release from 3T3-L1 adipocytes to 200%, 79%, 54% and 42% respectively of the control. In this study, EGCG and epicatechin had no effect on adiponectin release (Cho *et al.* 2007). Feeding studies, on the other hand, provide evidence that EGCG can increase circulating levels of adiponectin (Potenza *et al.* 2007; Shimada *et al.* 2007). The findings of this thesis are not in agreement with many of the studies cited; many studies in the literature have used primary cell or *in vivo* models, so it is very difficult to draw comparisons. In addition, the studies that have used the 3T3-L1 adipocyte model all use varying differentiation and maintenance conditions, which in some cases, may have resulted in the adipocytes becoming insulin resistant.

In order to try and understand the mechanisms by which the flavanols were able to alter adipokine release, the ERK1/2 MAPK signalling pathway was investigated. Epicatechin and EGCG both increased ERK1/2 phosphorylation in the 3T3-L1 system, although there was a 100-fold difference in optimum concentrations to produce the same significant effect. Evidence in the literature suggests that gallated catechins, especially EGCG, are more active

than other catechins as the gallyl and galloyl groups have conformational flexibility (Hung *et al.* 2005). This may be important for interactions with other molecules and may allow EGCG to bind with a receptor. EGCG also contains the largest number of hydroxyl groups on its three aromatic rings, and these may be important for hydrogen bonding. Having established the stimulatory effect of epicatechin and EGCG on the ERK1/2 pathway, upstream activation by way of GTPγS binding was measured. EGCG was the most potent in the GTPγS binding assay and negatively regulated this process. Although cAMP generation was investigated as another mechanism of upstream activation, a functional readout could not be obtained due to technical problems with the assay normally used for this purpose.

A study by Fujishiro *et al* showed that chronic activation of ERK1/2 in 3T3-L1 adipocytes leads to up-regulation of GLUT1 expression and down-regulation of GLUT4 expression, resulting in slightly increased basal glucose transport but decreased insulin-stimulated transport (Fujishiro *et al.* 2003). However, in a study by Harmon *et al* in which mature 3T3-L1 adipocytes were treated with the MEK inhibitors PD98059 and U0126, insulin-mediated glucose uptake was inhibited (Harmon *et al.* 2004). Therefore, glucose uptake was chosen as a potential physiological outcome downstream of ERK1/2 phosphorylation; however neither epicatechin nor EGCG were able to alter this. There is plenty of evidence to suggest that other flavonoids are able to alter glucose uptake, kaempferol, quercetin (Fang *et al.* 2008) and (-) catechin (Cho *et al.* 2007) significantly improve insulin-mediated glucose uptake in 3T3-L1 adipocytes, and berberine enhances basal glucose uptake (Kim *et al.* 2007). Genistein (Bazuine *et al.* 2005), myricetin, quercetin and catechin-gallate (Strobel *et al.*

2005) however, inhibit both basal and insulin-stimulated glucose uptake. The majority of these studies have been carried out in 3T3-L1 adipocytes so should be comparable to the results in this study, however in some cases the basal conditions of the 3T3-L1 adipocytes vary significantly to those in this study. Notably the continued high insulin levels in the study by Fang *et al* may have resulted in the 3T3-L1 adipocytes becoming insulin resistant. It is possible that under different time and concentration conditions, epicatechin and EGCG may be able to modify basal or insulin-stimulated glucose uptake.

The final investigation into the effects of flavanols was to explore the expression of various genes known to be altered as a result of obesity (Nadler *et al.* 2000; Li *et al.* 2002; Haluzik *et al.* 2004; Xu *et al.* 2006). These genes are also up-regulated during adipogenesis (Fajas *et al.* 1998; Nadler *et al.* 2000; Rosen and Spiegelman 2000; Walczak and Tontonoz 2002). The expression of the genes chosen is under complex regulation by the ERK1/2 pathway, the C/EBP transcription factors and PPARγ throughout adipogenesis. As the 3T3-L1 adipocytes used for this study were fully differentiated, adipogenesis is considered to be complete, and the genes involved in it may no longer be up-regulated. Although significant changes in expression of these genes were not determined as a result of epicatechin or EGCG treatment, there appeared to be some correlation between some pairs of genes where one regulates the other.

There is some evidence in the literature indicating that adipocyte gene expression is altered as a result of flavonoid treatment (Kao *et al.* 2000; Ashida *et al.* 2004; Liu *et al.* 2006; Nagao *et al.* 2009), however these are mostly in animal models, or also may be the result of changes in preadipocytes as

compared to mature adipocytes. Genes were chosen for investigation in this study on the basis of results from animal models, however, it is difficult to compare these studies to those carried out in animal or human models, or to extrapolate any findings to these models as the system used in these studies is an immortalised cell line, comprising only one cell type. In whole body models, compounds such as the flavanols are not able to target one cell type; they affect the whole body and can have direct and indirect effects on many tissues, which may then result in paracrine effects to other tissues in the body. Furthermore, in whole body systems, metabolism and bioavailability of these compounds is an issue, whereas this is not the case in the 3T3-L1 model used in these studies. As previously stated, evidence from pharmacokinetic data in humans suggests that attainment of high levels of EGCG in the body is unrealistic. It is therefore difficult to transfer data in immortalised cell lines or isolated primary cells to a whole body system in which such low plasma levels are achievable.

Further work that may help to understand the effects and mechanisms, if any, would firstly involve obtaining a functional readout of the cyclic AMP generation assay, in order to further elucidate how signalling upstream of the ERK1/2 pathway is occurring. Secondly, transactivation of PPARγ might yield interesting results, as although PPARγ gene expression was not altered as a result of flavanol treatment, there was correlation between the expression of PPARγ and PEPCK and PPARγ and adiponectin. Investigating all of the downstream ERK1/2 targets and the signalling pathways activated and genes regulated would help to ascertain what the downstream targets and physiological outcomes of the flavanol actions on ERK1/2 are. Only the

adipokines adiponectin and resistin were investigated as secreted factors from the 3T3-L1 adipocytes, but a more thorough investigation of all secreted factors may help to further determine the effects that flavanols have on 3T3-L1 adipocytes. This could be done both by a Luminex assay to measure known adipokines and cytokines, and by co-culture with other cell types to investigate the direct effect these potentially unknown secreted products may have on other cell types.

A thorough investigation of adipokine release, ERK1/2 signalling, glucose uptake and gene expression, under the influence of flavanol treatment, showed that although molecular changes occurred in the 3T3-L1 adipocyte system, these did not translate into functional readouts. Therefore, it appears unlikely that these have major direct effects on adipocytes function.

References

8 References

Adams, M., M. J. Reginato, D. Shao, M. A. Lazar and V. K. Chatterjee (1997). "Transcriptional activation by peroxisome proliferator-activated receptor gamma is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site." J Biol Chem **272**(8): 5128-32.

Aherne, S. A. and N. M. O'Brien (2002). "Dietary flavonols: chemistry, food content, and metabolism." Nutrition **18**(1): 75-81.

Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi and Y. Matsuzawa (1999). "Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity." Biochem Biophys Res Commun **257**(1): 79-83.

Ashida, H., T. Furuyashiki, H. Nagayasu, H. Bessho, H. Sakakibara, T. Hashimoto and K. Kanazawa (2004). "Anti-obesity actions of green tea: possible involvements in modulation of the glucose uptake system and suppression of the adipogenesis-related transcription factors." Biofactors **22**(1-4): 135-40.

Balentine, D. A., S. A. Wiseman and L. C. Bouwens (1997). "The chemistry of tea flavonoids." Crit Rev Food Sci Nutr **37**(8): 693-704.

Banerjee, R. R. and M. A. Lazar (2001). "Dimerization of resistin and resistin-like molecules is determined by a single cysteine." J Biol Chem **276**(28): 25970-3.

Banerjee, R. R., S. M. Rangwala, J. S. Shapiro, A. S. Rich, B. Rhoades, Y. Qi, J. Wang, M. W. Rajala, A. Pocai, P. E. Scherer, C. M. Steppan, R. S. Ahima, S. Obici, L. Rossetti and M. A. Lazar (2004). "Regulation of fasted blood glucose by resistin." Science **303**(5661): 1195-8.

Bazuine, M., P. J. van den Broek and J. A. Maassen (2005). "Genistein directly inhibits GLUT4-mediated glucose uptake in 3T3-L1 adipocytes." Biochem Biophys Res Commun **326**(2): 511-4.

Beecher, G. R. (2003). "Overview of dietary flavonoids: nomenclature, occurrence and intake." J Nutr **133**(10): 3248S-3254S.

Benito, M., A. Porras, A. R. Nebreda and E. Santos (1991). "Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of ras oncogenes." Science **253**(5019): 565-8.

Bokarewa, M., I. Nagaev, L. Dahlberg, U. Smith and A. Tarkowski (2005). "Resistin, an adipokine with potent proinflammatory properties." J Immunol **174**(9): 5789-95.

Boonsong, T., L. Norton, K. Chokkalingam, K. Jewell, I. Macdonald, A. Bennett and K. Tsintzas (2007). "Effect of exercise and insulin on SREBP-1c expression in human skeletal muscle: potential roles for the ERK1/2 and Akt signalling pathways." Biochem Soc Trans **35**(Pt 5): 1310-1.

Bose, M., J. D. Lambert, J. Ju, K. R. Reuhl, S. A. Shapses and C. S. Yang (2008). "The major green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice." J Nutr **138**(9): 1677-83.

Bravo, L., R. Abia, M. A. Eastwood and F. Saura-Calixto (1994). "Degradation of polyphenols (catechin and tannic acid) in the rat intestinal tract. Effect on colonic fermentation and faecal output." Br J Nutr **71**(6): 933-46.

Bryant, N. J., R. Govers and D. E. James (2002). "Regulated transport of the glucose transporter GLUT4." Nat Rev Mol Cell Biol **3**(4): 267-77.

Burton, G. R., R. Nagarajan, C. A. Peterson and R. E. McGehee, Jr. (2004). "Microarray analysis of differentiation-specific gene expression during 3T3-L1 adipogenesis." Gene **329**: 167-85.

Cabrera, C., R. Artacho and R. Gimenez (2006). "Beneficial effects of green tea--a review." J Am Coll Nutr **25**(2): 79-99.

Camp, H. S. and S. R. Tafuri (1997). "Regulation of peroxisome proliferator-activated receptor gamma activity by mitogen-activated protein kinase." J Biol Chem **272**(16): 10811-6.

Chae, Y. J., C. H. Kim, T. S. Ha, J. Hescheler, H. Y. Ahn and A. Sachinidis (2007). "Epigallocatechin-3-O-gallate inhibits the angiotensin II-induced adhesion molecule expression in human umbilical vein endothelial cell via inhibition of MAPK pathways." Cell Physiol Biochem **20**(6): 859-66.

Chan, G. K., R. A. Deckelbaum, I. Bolivar, D. Goltzman and A. C. Karaplis (2001). "PTHrP inhibits adipocyte differentiation by down-regulating PPAR gamma activity via a MAPK-dependent pathway." Endocrinology **142**(11): 4900-9.

Chen, J., L. Wang, Y. S. Boeg, B. Xia and J. Wang (2002). "Differential dimerization and association among resistin family proteins with implications for functional specificity." J Endocrinol **175**(2): 499-504.

Cho, S. Y., P. J. Park, H. J. Shin, Y. K. Kim, D. W. Shin, E. S. Shin, H. H. Lee, B. G. Lee, J. H. Baik and T. R. Lee (2007). "(-)-Catechin suppresses expression of Kruppel-like factor 7 and increases expression and secretion of adiponectin protein in 3T3-L1 cells." Am J Physiol Endocrinol Metab **292**(4): E1166-72.

Chow, H. H., Y. Cai, D. S. Alberts, I. Hakim, R. Dorr, F. Shahi, J. A. Crowell, C. S. Yang and Y. Hara (2001). "Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E." Cancer Epidemiol Biomarkers Prev **10**(1): 53-8.

Day, C. (1999). "Thiazolidinediones: a new class of antidiabetic drugs." Diabet Med **16**(3): 179-92.

Degawa-Yamauchi, M., J. E. Bovenkerk, B. E. Juliar, W. Watson, K. Kerr, R. Jones, Q. Zhu and R. V. Considine (2003). "Serum resistin (FIZZ3) protein is increased in obese humans." J Clin Endocrinol Metab **88**(11): 5452-5.

Ebstensen, R. D. and P. G. Plagemann (1972). "Cytochalasin B: inhibition of glucose and glucosamine transport." Proc Natl Acad Sci U S A **69**(6): 1430-4.

Fajas, L., J. C. Fruchart and J. Auwerx (1998). "Transcriptional control of adipogenesis." Curr Opin Cell Biol **10**(2): 165-73.

Fajas, L., K. Schoonjans, L. Gelman, J. B. Kim, J. Najib, G. Martin, J. C. Fruchart, M. Briggs, B. M. Spiegelman and J. Auwerx (1999). "Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism." Mol Cell Biol **19**(8): 5495-503.

Fang, X. K., J. Gao and D. N. Zhu (2008). "Kaempferol and quercetin isolated from Euonymus alatus improve glucose uptake of 3T3-L1 cells without adipogenesis activity." Life Sci **82**(11-12): 615-22.

Fantuzzi, G. (2005). "Adipose tissue, adipokines, and inflammation." J Allergy Clin Immunol **115**(5): 911-9.

Fingar, D. C. and M. J. Birnbaum (1994). "A role for Raf-1 in the divergent signaling pathways mediating insulin-stimulated glucose transport." J Biol Chem **269**(13): 10127-32.

Fujinami, A., H. Obayashi, K. Ohta, T. Ichimura, M. Nishimura, H. Matsui, Y. Kawahara, M. Yamazaki, M. Ogata, G. Hasegawa, N. Nakamura, T. Yoshikawa, K. Nakano and M. Ohta (2004). "Enzyme-linked immunosorbent assay for circulating human resistin: resistin concentrations in normal subjects and patients with type 2 diabetes." Clin Chim Acta **339**(1-2): 57-63.

Fujishiro, M., Y. Gotoh, H. Katagiri, H. Sakoda, T. Ogihara, M. Anai, Y. Onishi, H. Ono, M. Abe, N. Shojima, Y. Fukushima, M. Kikuchi, Y. Oka and T. Asano (2003). "Three mitogen-activated protein kinases

inhibit insulin signaling by different mechanisms in 3T3-L1 adipocytes." Mol Endocrinol **17**(3): 487-97.

Furuyashiki, T., H. Nagayasu, Y. Aoki, H. Bessho, T. Hashimoto, K. Kanazawa and H. Ashida (2004). "Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells." Biosci Biotechnol Biochem **68**(11): 2353-9.

Gabriely, I., X. H. Ma, X. M. Yang, G. Atzmon, M. W. Rajala, A. H. Berg, P. Scherer, L. Rossetti and N. Barzilai (2002). "Removal of visceral fat prevents insulin resistance and glucose intolerance of aging: an adipokine-mediated process?" Diabetes **51**(10): 2951-8.

Gardner, O. S., B. J. Dewar, H. S. Earp, J. M. Samet and L. M. Graves (2003). "Dependence of peroxisome proliferator-activated receptor ligand-induced mitogen-activated protein kinase signaling on epidermal growth factor receptor transactivation." J Biol Chem **278**(47): 46261-9.

Gilmore, T. D. (2006). "Introduction to NF-kappaB: players, pathways, perspectives." Oncogene **25**(51): 6680-4.

Givant-Horwitz, V., B. Davidson and R. Reich (2004). "Laminin-induced signaling in tumor cells: the role of the M(r) 67,000 laminin receptor." Cancer Res **64**(10): 3572-9.

Goldstein, B. J. (2002). "Insulin resistance as the core defect in type 2 diabetes mellitus." Am J Cardiol **90**(5A): 3G-10G.

Green, H. and O. Kehinde (1975). "An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion." Cell **5**(1): 19-27.

Green, H. and O. Kehinde (1979). "Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line." J Cell Physiol **101**(1): 169-71.

Green, H. and M. Meuth (1974). "An established pre-adipose cell line and its differentiation in culture." Cell **3**(2): 127-33.

Guerre-Millo, M. (2004). "Adipose tissue and adipokines: for better or worse." Diabetes Metab **30**(1): 13-9.

Haluzik, M., J. Parizkova and M. M. Haluzik (2004). "Adiponectin and its role in the obesity-induced insulin resistance and related complications." Physiol Res **53**(2): 123-9.

Han, Y. S., S. Bastianetto, Y. Dumont and R. Quirion (2006). "Specific plasma membrane binding sites for polyphenols, including resveratrol, in the rat brain." J Pharmacol Exp Ther **318**(1): 238-45.

Harmon, A. W., D. S. Paul and Y. M. Patel (2004). "MEK inhibitors impair insulin-stimulated glucose uptake in 3T3-L1 adipocytes." Am J Physiol Endocrinol Metab **287**(4): E758-66.

Hartman, H. B., X. Hu, K. X. Tyler, C. K. Dalal and M. A. Lazar (2002). "Mechanisms regulating adipocyte expression of resistin." J Biol Chem **277**(22): 19754-61.

Hasegawa, N., N. Yamda and M. Mori (2003). "Powdered green tea has antilipogenic effect on Zucker rats fed a high-fat diet." Phytother Res **17**(5): 477-80.

Hauge, C. and M. Frodin (2006). "RSK and MSK in MAP kinase signalling." J Cell Sci **119**(Pt 15): 3021-3.

Haugen, F., A. Jorgensen, C. A. Drevon and P. Trayhurn (2001). "Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes." FEBS Lett **507**(1): 105-8.

Havel, P. J. (2004). "Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism." Diabetes **53 Suppl 1**: S143-51.

Hollenberg, A. N., V. S. Susulic, J. P. Madura, B. Zhang, D. E. Moller, P. Tontonoz, P. Sarraf, B. M. Spiegelman and B. B. Lowell (1997). "Functional antagonism between CCAAT/Enhancer binding protein-alpha and peroxisome proliferator-activated receptor-gamma on the leptin promoter." J Biol Chem **272**(8): 5283-90.

Hu, E., J. B. Kim, P. Sarraf and B. M. Spiegelman (1996). "Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARgamma." Science **274**(5295): 2100-3.

Hu, E., P. Liang and B. M. Spiegelman (1996). "AdipoQ is a novel adipose-specific gene dysregulated in obesity." J Biol Chem **271**(18): 10697-703.

Huang, C. C., W. B. Wu, J. Y. Fang, H. S. Chiang, S. K. Chen, B. H. Chen, Y. T. Chen and C. F. Hung (2007). "(-)-Epicatechin-3-gallate, a green tea polyphenol is a potent agent against UVB-induced damage in HaCaT keratinocytes." Molecules **12**(8): 1845-58.

Huang, S. and M. P. Czech (2007). "The GLUT4 glucose transporter." Cell Metab **5**(4): 237-52.

Hung, P. F., B. T. Wu, H. C. Chen, Y. H. Chen, C. L. Chen, M. H. Wu, H. C. Liu, M. J. Lee and Y. H. Kao (2005). "Antimitogenic effect of green tea (-)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the ERK and Cdk2 pathways." Am J Physiol Cell Physiol **288**(5): C1094-108.

Huypens, P., E. Quartier, D. Pipeleers and M. Van de Casteele (2005). "Metformin reduces adiponectin protein expression and release in 3T3-L1 adipocytes involving activation of AMP activated protein kinase." Eur J Pharmacol **518**(2-3): 90-5.

Janke, J., S. Engeli, K. Gorzelniak, F. C. Luft and A. M. Sharma (2002). "Resistin gene expression in human adipocytes is not related to insulin resistance." Obes Res **10**(1): 1-5.

Kadowaki, T., T. Yamauchi, N. Kubota, K. Hara, K. Ueki and K. Tobe (2006). "Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome." J Clin Invest **116**(7): 1784-92.

Kahn, B. B. (1996). "Lilly lecture 1995. Glucose transport: pivotal step in insulin action." Diabetes **45**(11): 1644-54.

Kao, Y. H., H. H. Chang, M. J. Lee and C. L. Chen (2006). "Tea, obesity, and diabetes." Mol Nutr Food Res **50**(2): 188-210.

Kao, Y. H., R. A. Hiipakka and S. Liao (2000). "Modulation of endocrine systems and food intake by green tea epigallocatechin gallate." Endocrinology **141**(3): 980-7.

Karin, M., Z. Liu and E. Zandi (1997). "AP-1 function and regulation." Curr Opin Cell Biol **9**(2): 240-6.

Kawamura, Y., Y. Tanaka, R. Kawamori and S. Maeda (2006). "Overexpression of Kruppel-like factor 7 regulates adipocytokine gene expressions in human adipocytes and inhibits glucose-induced insulin secretion in pancreatic beta-cell line." Mol Endocrinol **20**(4): 844-56.

Kershaw, E. E. and J. S. Flier (2004). "Adipose tissue as an endocrine organ." J Clin Endocrinol Metab **89**(6): 2548-56.

Khan, N. and H. Mukhtar (2007). "Tea polyphenols for health promotion." Life Sci **81**(7): 519-33.

Kim, J. A., G. Formoso, Y. Li, M. A. Potenza, F. L. Marasciulo, M. Montagnani and M. J. Quon (2007). "Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn." J Biol Chem **282**(18): 13736-45.

Kim, J. B., P. Sarraf, M. Wright, K. M. Yao, E. Mueller, G. Solanes, B. B. Lowell and B. M. Spiegelman (1998). "Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1." J Clin Invest **101**(1): 1-9.

Kim, J. B., H. M. Wright, M. Wright and B. M. Spiegelman (1998). "ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand." Proc Natl Acad Sci U S A **95**(8): 4333-7.

Kim, K. H., K. Lee, Y. S. Moon and H. S. Sul (2001). "A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation." J Biol Chem **276**(14): 11252-6.

Kim, S. H., E. J. Shin, E. D. Kim, T. Bayaraa, S. C. Frost and C. K. Hyun (2007). "Berberine activates GLUT1-mediated glucose uptake in 3T3-L1 adipocytes." Biol Pharm Bull **30**(11): 2120-5.

Klaus, S., S. Pultz, C. Thone-Reineke and S. Wolfram (2005). "Epigallocatechin gallate attenuates diet-induced obesity in mice by decreasing energy absorption and increasing fat oxidation." Int J Obes (Lond) **29**(6): 615-23.

Kletzien, R. F., J. F. Perdue and A. Springer (1972). "Cytochalasin A and B. Inhibition of sugar uptake in cultured cells." J Biol Chem **247**(9): 2964-6.

Kubota, N., Y. Terauchi, H. Miki, H. Tamemoto, T. Yamauchi, K. Komeda, S. Satoh, R. Nakano, C. Ishii, T. Sugiyama, K. Eto, Y. Tsubamoto, A. Okuno, K. Murakami, H. Sekihara, G. Hasegawa, M. Naito, Y. Toyoshima, S. Tanaka, K. Shiota, T. Kitamura, T. Fujita, O. Ezaki, S. Aizawa, T. Kadowaki and et al. (1999). "PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance." Mol Cell **4**(4): 597-609.

Lachaal, M., A. L. Rampal, W. Lee, Y. Shi and C. Y. Jung (1996). "GLUT1 transmembrane glucose pathway. Affinity labeling with a transportable D-glucose diazirine." J Biol Chem **271**(9): 5225-30.

Li, J., X. Yu, W. Pan and R. H. Unger (2002). "Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity." Am J Physiol Endocrinol Metab **282**(6): E1334-41.

Li, Y. Y., H. S. Wu, L. Tang, C. R. Feng, J. H. Yu, Y. Li, Y. S. Yang, B. Yang and Q. J. He (2007). "The potential insulin sensitizing and glucose lowering effects of a novel indole derivative in vitro and in vivo." Pharmacol Res **56**(4): 335-43.

Lin, J., M. A. Della-Fera and C. A. Baile (2005). "Green tea polyphenol epigallocatechin gallate inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes." Obes Res **13**(6): 982-90.

Liu, H. S., Y. H. Chen, P. F. Hung and Y. H. Kao (2006). "Inhibitory effect of green tea (-)-epigallocatechin gallate on resistin gene expression in 3T3-L1 adipocytes depends on the ERK pathway." Am J Physiol Endocrinol Metab **290**(2): E273-81.

Lyon, C. J., R. E. Law and W. A. Hsueh (2003). "Minireview: adiposity, inflammation, and atherogenesis." Endocrinology **144**(6): 2195-200.

MacDougald, O. A. and M. D. Lane (1995). "Transcriptional regulation of gene expression during adipocyte differentiation." Annu Rev Biochem **64**: 345-73.

Maeda, N., M. Takahashi, T. Funahashi, S. Kihara, H. Nishizawa, K. Kishida, H. Nagaretani, M. Matsuda, R. Komuro, N. Ouchi, H. Kuriyama, K. Hotta, T. Nakamura, I. Shimomura and Y. Matsuzawa (2001). "PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein." Diabetes **50**(9): 2094-9.

McTernan, P. G., F. M. Fisher, G. Valsamakis, R. Chetty, A. Harte, C. L. McTernan, P. M. Clark, S. A. Smith, A. H. Barnett and S. Kumar (2003). "Resistin and type 2 diabetes: regulation of resistin expression by insulin and rosiglitazone and the effects of recombinant resistin on lipid and glucose metabolism in human differentiated adipocytes." J Clin Endocrinol Metab **88**(12): 6098-106.

Mecham, R. P. (1991). "Receptors for laminin on mammalian cells." Faseb J **5**(11): 2538-46.

Miles, P. D., Y. Barak, W. He, R. M. Evans and J. M. Olefsky (2000). "Improved insulin-sensitivity in mice heterozygous for PPAR-gamma deficiency." J Clin Invest **105**(3): 287-92.

Mizel, S. B. and L. Wilson (1972). "Inhibition of the transport of several hexoses in mammalian cells by cytochalasin B." J Biol Chem **247**(12): 4102-5.

Mora, S. and J. E. Pessin (2002). "An adipocentric view of signaling and intracellular trafficking." Diabetes Metab Res Rev **18**(5): 345-56.

Morikawa, K., C. Ikeda, M. Nonaka, S. Pei, M. Mochizuki, A. Mori and S. Yamada (2007). "Epigallocatechin gallate-induced apoptosis does not affect adipocyte conversion of preadipocytes." Cell Biol Int **31**(11): 1379-87.

Morrison, R. F. and S. R. Farmer (1999). "Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis." J Biol Chem **274**(24): 17088-97.

Murase, T., S. Haramizu, A. Shimotoyodome, A. Nagasawa and I. Tokimitsu (2005). "Green tea extract improves endurance capacity and increases muscle lipid oxidation in mice." Am J Physiol Regul Integr Comp Physiol **288**(3): R708-15.

Murase, T., S. Haramizu, A. Shimotoyodome, I. Tokimitsu and T. Hase (2006). "Green tea extract improves running endurance in mice by stimulating lipid utilization during exercise." Am J Physiol Regul Integr Comp Physiol **290**(6): R1550-6.

Nadler, S. T., J. P. Stoehr, K. L. Schueler, G. Tanimoto, B. S. Yandell and A. D. Attie (2000). "The expression of adipogenic genes is decreased in obesity and diabetes mellitus." Proc Natl Acad Sci U S A **97**(21): 11371-6.

Nagaev, I. and U. Smith (2001). "Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle." Biochem Biophys Res Commun **285**(2): 561-4.

Nagao, T., T. Hase and I. Tokimitsu (2007). "A green tea extract high in catechins reduces body fat and cardiovascular risks in humans." Obesity (Silver Spring) **15**(6): 1473-83.

Nagao, T., S. Meguro, T. Hase, K. Otsuka, M. Komikado, I. Tokimitsu, T. Yamamoto and K. Yamamoto (2009). "A Catechin-rich Beverage Improves Obesity and Blood Glucose Control in Patients With Type 2 Diabetes." Obesity (Silver Spring) **17**(2): 310-7.

Nedergaard, J., T. Bengtsson and B. Cannon (2007). "Unexpected evidence for active brown adipose tissue in adult humans." Am J Physiol Endocrinol Metab **293**(2): E444-52.

Niimi, T., C. Kumagai, M. Okano and Y. Kitagawa (1997). "Differentiation-dependent expression of laminin-8 (alpha 4 beta 1 gamma 1) mRNAs in mouse 3T3-L1 adipocytes." Matrix Biol **16**(4): 223-30.

Novikoff, A. B., P. M. Novikoff, O. M. Rosen and C. S. Rubin (1980). "Organelle relationships in cultured 3T3-L1 preadipocytes." J Cell Biol **87**(1): 180-96.

Ntambi, J. M. and K. Young-Cheul (2000). "Adipocyte differentiation and gene expression." J Nutr **130**(12): 3122S-3126S.

Pan, M. H., C. C. Lin, J. K. Lin and W. J. Chen (2007). "Tea polyphenol (-)-epigallocatechin 3-gallate suppresses heregulin-beta1-induced fatty acid synthase expression in human breast cancer cells by inhibiting phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase cascade signaling." J Agric Food Chem **55**(13): 5030-7.

Park, B. H., L. Qiang and S. R. Farmer (2004). "Phosphorylation of C/EBPbeta at a consensus extracellular signal-regulated kinase/glycogen synthase kinase 3 site is required for the induction of adiponectin gene expression during the differentiation of mouse fibroblasts into adipocytes." Mol Cell Biol **24**(19): 8671-80.

Park, S. K., S. Y. Oh, M. Y. Lee, S. Yoon, K. S. Kim and J. W. Kim (2004). "CCAAT/enhancer binding protein and nuclear factor-Y regulate adiponectin gene expression in adipose tissue." Diabetes **53**(11): 2757-66.

Patel, L., A. C. Buckels, I. J. Kinghorn, P. R. Murdock, J. D. Holbrook, C. Plumpton, C. H. Macphee and S. A. Smith (2003). "Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators." Biochem Biophys Res Commun **300**(2): 472-6.

Potenza, M. A., F. L. Marasciulo, M. Tarquinio, E. Tiravanti, G. Colantuono, A. Federici, J. A. Kim, M. J. Quon and M. Montagnani (2007). "EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR." Am J Physiol Endocrinol Metab **292**(5): E1378-87.

Prusty, D., B. H. Park, K. E. Davis and S. R. Farmer (2002). "Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes." J Biol Chem **277**(48): 46226-32.

Qi, M. and E. A. Elion (2005). "MAP kinase pathways." J Cell Sci **118**(Pt 16): 3569-72.

Qiao, L., P. S. Maclean, J. Schaack, D. J. Orlicky, C. Darimont, M. Pagliassotti, J. E. Friedman and J. Shao (2005). "C/EBPalpha regulates human adiponectin gene transcription through an intronic enhancer." Diabetes **54**(6): 1744-54.

Ray, L. B. and T. W. Sturgill (1987). "Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro." Proc Natl Acad Sci U S A **84**(6): 1502-6.

Reginato, M. J., S. L. Krakow, S. T. Bailey and M. A. Lazar (1998). "Prostaglandins promote and block adipogenesis through opposing effects on peroxisome proliferator-activated receptor gamma." J Biol Chem **273**(4): 1855-8.

Reginato, M. J. and M. A. Lazar (1999). "Mechanisms by which Thiazolidinediones Enhance Insulin Action." Trends Endocrinol Metab **10**(1): 9-13.

Reusch, J. E., L. A. Colton and D. J. Klemm (2000). "CREB activation induces adipogenesis in 3T3-L1 cells." Mol Cell Biol **20**(3): 1008-20.

Rosen, E. D. and B. M. Spiegelman (2000). "Molecular regulation of adipogenesis." Annu Rev Cell Dev Biol **16**: 145-71.

Rosen, E. D., C. J. Walkey, P. Puigserver and B. M. Spiegelman (2000). "Transcriptional regulation of adipogenesis." Genes Dev **14**(11): 1293-307.

Sah, J. F., S. Balasubramanian, R. L. Eckert and E. A. Rorke (2004). "Epigallocatechin-3-gallate inhibits epidermal growth factor receptor

signaling pathway. Evidence for direct inhibition of ERK1/2 and AKT kinases." J Biol Chem **279**(13): 12755-62.

Sambrook, J., D. W. Russell and Cold Spring Harbor Laboratory. (2001). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory.

Savage, D. B., C. P. Sewter, E. S. Klenk, D. G. Segal, A. Vidal-Puig, R. V. Considine and S. O'Rahilly (2001). "Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans." Diabetes **50**(10): 2199-202.

Scherer, P. E., S. Williams, M. Fogliano, G. Baldini and H. F. Lodish (1995). "A novel serum protein similar to C1q, produced exclusively in adipocytes." J Biol Chem **270**(45): 26746-9.

Schoof, E., A. Stuppy, F. Harig, R. Carbon, T. Horbach, W. Stohr, W. Rascher and J. Dotsch (2004). "Comparison of leptin gene expression in different adipose tissues in children and adults." Eur J Endocrinol **150**(4): 579-84.

Schroeter, H., P. Bahia, J. P. Spencer, O. Sheppard, M. Rattray, E. Cadenas, C. Rice-Evans and R. J. Williams (2007). "(-)Epicatechin stimulates ERK-dependent cyclic AMP response element activity and up-regulates GluR2 in cortical neurons." J Neurochem **101**(6): 1596-606.

Seo, J. B., H. M. Moon, M. J. Noh, Y. S. Lee, H. W. Jeong, E. J. Yoo, W. S. Kim, J. Park, B. S. Youn, J. W. Kim, S. D. Park and J. B. Kim (2004). "Adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element-binding protein 1c regulates mouse adiponectin expression." J Biol Chem **279**(21): 22108-17.

Seo, J. B., M. J. Noh, E. J. Yoo, S. Y. Park, J. Park, I. K. Lee, S. D. Park and J. B. Kim (2003). "Functional characterization of the human resistin promoter with adipocyte determination- and differentiation-dependent factor 1/sterol regulatory element binding protein 1c and CCAAT enhancer binding protein-alpha." Mol Endocrinol **17**(8): 1522-33.

Sharma, A. M. and B. Staels (2007). "Review: Peroxisome proliferator-activated receptor gamma and adipose tissue--understanding obesity-related changes in regulation of lipid and glucose metabolism." J Clin Endocrinol Metab **92**(2): 386-95.

Shimada, M., K. Mochizuki, N. Sakurai and T. Goda (2007). "Dietary supplementation with epigallocatechin gallate elevates levels of circulating adiponectin in non-obese type-2 diabetic Goto-Kakizaki rats." Biosci Biotechnol Biochem **71**(8): 2079-82.

Shojima, N., H. Sakoda, T. Ogihara, M. Fujishiro, H. Katagiri, M. Anai, Y. Onishi, H. Ono, K. Inukai, M. Abe, Y. Fukushima, M. Kikuchi, Y. Oka

and T. Asano (2002). "Humoral regulation of resistin expression in 3T3-L1 and mouse adipose cells." Diabetes **51**(6): 1737-44.

Sinha, A., D. V. Faller and G. V. Denis (2005). "Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A." Biochem J **387**(Pt 1): 257-69.

Smith, P. J., L. S. Wise, R. Berkowitz, C. Wan and C. S. Rubin (1988). "Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes." J Biol Chem **263**(19): 9402-8.

Spencer, J. P., C. Rice-Evans and R. J. Williams (2003). "Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability." J Biol Chem **278**(37): 34783-93.

Spiegelman, B. M., L. Choy, G. S. Hotamisligil, R. A. Graves and P. Tontonoz (1993). "Regulation of adipocyte gene expression in differentiation and syndromes of obesity/diabetes." J Biol Chem **268**(10): 6823-6.

Spiegelman, B. M. and H. Green (1981). "Cyclic AMP-mediated control of lipogenic enzyme synthesis during adipose differentiation of 3T3 cells." Cell **24**(2): 503-10.

Steppan, C. M., S. T. Bailey, S. Bhat, E. J. Brown, R. R. Banerjee, C. M. Wright, H. R. Patel, R. S. Ahima and M. A. Lazar (2001). "The hormone resistin links obesity to diabetes." Nature **409**(6818): 307-12.

Steppan, C. M., E. J. Brown, C. M. Wright, S. Bhat, R. R. Banerjee, C. Y. Dai, G. H. Enders, D. G. Silberg, X. Wen, G. D. Wu and M. A. Lazar (2001). "A family of tissue-specific resistin-like molecules." Proc Natl Acad Sci U S A **98**(2): 502-6.

Strobel, P., C. Allard, T. Perez-Acle, R. Calderon, R. Aldunate and F. Leighton (2005). "Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes." Biochem J **386**(Pt 3): 471-8.

Tachibana, H., K. Koga, Y. Fujimura and K. Yamada (2004). "A receptor for green tea polyphenol EGCG." Nat Struct Mol Biol **11**(4): 380-1.

Telfer, J. F., J. Urquhart and D. H. Crouch (2005). "Suppression of MEK/ERK signalling by Myc: role of Bin-1." Cell Signal **17**(6): 701-8.

Tong, Q. and G. S. Hotamisligil (2001). "Molecular mechanisms of adipocyte differentiation." Rev Endocr Metab Disord **2**(4): 349-55.

Tontonoz, P., E. Hu, J. Devine, E. G. Beale and B. M. Spiegelman (1995). "PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene." Mol Cell Biol **15**(1): 351-7.

Ullmann, U., J. Haller, J. P. Decourt, N. Girault, J. Girault, A. S. Richard-Caudron, B. Pineau and P. Weber (2003). "A single ascending dose

study of epigallocatechin gallate in healthy volunteers." J Int Med Res **31**(2): 88-101.

Vauzour, D., K. Vafeiadou, C. Rice-Evans, R. J. Williams and J. P. Spencer (2007). "Activation of pro-survival Akt and ERK1/2 signalling pathways underlie the anti-apoptotic effects of flavanones in cortical neurons." J Neurochem **103**(4): 1355-67.

Walczak, R. and P. Tontonoz (2002). "PPARadigms and PPARadoxes: expanding roles for PPARgamma in the control of lipid metabolism." J Lipid Res **43**(2): 177-86.

Waltner-Law, M. E., X. L. Wang, B. K. Law, R. K. Hall, M. Nawano and D. K. Granner (2002). "Epigallocatechin gallate, a constituent of green tea, represses hepatic glucose production." J Biol Chem **277**(38): 34933-40.

Watson, R. T. and J. E. Pessin (2001). "Intracellular organization of insulin signaling and GLUT4 translocation." Recent Prog Horm Res **56**: 175-93.

Wiese, R. J., C. C. Mastick, D. F. Lazar and A. R. Saltiel (1995). "Activation of mitogen-activated protein kinase and phosphatidylinositol 3'-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes." J Biol Chem **270**(7): 3442-6.

Williams, R. J., J. P. Spencer and C. Rice-Evans (2004). "Flavonoids: antioxidants or signalling molecules?" Free Radic Biol Med **36**(7): 838-49.

Willson, T. M., M. H. Lambert and S. A. Kliewer (2001). "Peroxisome proliferator-activated receptor gamma and metabolic disease." Annu Rev Biochem **70**: 341-67.

Wise, L. S. and H. Green (1979). "Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells." J Biol Chem **254**(2): 273-5.

Wolf, G. (2004). "Insulin resistance and obesity: resistin, a hormone secreted by adipose tissue." Nutr Rev **62**(10): 389-94.

Wolfram, S., D. Raederstorff, Y. Wang, S. R. Teixeira, V. Elste and P. Weber (2005). "TEAVIGO (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass." Ann Nutr Metab **49**(1): 54-63.

Wu, B. T., P. F. Hung, H. C. Chen, R. N. Huang, H. H. Chang and Y. H. Kao (2005). "The apoptotic effect of green tea (-)-epigallocatechin gallate on 3T3-L1 preadipocytes depends on the Cdk2 pathway." J Agric Food Chem **53**(14): 5695-701.

Wu, C. D. and G. X. Wei (2002). "Tea as a functional food for oral health." Nutrition **18**(5): 443-4.

Wu, L. Y., C. C. Juan, L. T. Ho, Y. P. Hsu and L. S. Hwang (2004). "Effect of green tea supplementation on insulin sensitivity in Sprague-Dawley rats." J Agric Food Chem **52**(3): 643-8.

Xu, A., Y. Wang, J. Y. Xu, D. Stejskal, S. Tam, J. Zhang, N. M. Wat, W. K. Wong and K. S. Lam (2006). "Adipocyte fatty acid-binding protein is a plasma biomarker closely associated with obesity and metabolic syndrome." Clin Chem **52**(3): 405-13.

Yamauchi, T., J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M. L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel and T. Kadowaki (2001). "The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity." Nat Med **7**(8): 941-6.

Yang, C. S., L. Chen, M. J. Lee, D. Balentine, M. C. Kuo and S. P. Schantz (1998). "Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers." Cancer Epidemiol Biomarkers Prev **7**(4): 351-4.

Yang, R. Z., Q. Huang, A. Xu, J. C. McLenithan, J. A. Eisen, A. R. Shuldiner, S. Alkan and D. W. Gong (2003). "Comparative studies of resistin expression and phylogenomics in human and mouse." Biochem Biophys Res Commun **310**(3): 927-35.

Ye, J. M., N. Dzamko, A. J. Hoy, M. A. Iglesias, B. Kemp and E. Kraegen (2006). "Rosiglitazone treatment enhances acute AMP-activated protein kinase-mediated muscle and adipose tissue glucose uptake in high-fat-fed rats." Diabetes **55**(10): 2797-804.

Zhang, H. H., M. Halbleib, F. Ahmad, V. C. Manganiello and A. S. Greenberg (2002). "Tumor necrosis factor-alpha stimulates lipolysis in differentiated human adipocytes through activation of extracellular signal-related kinase and elevation of intracellular cAMP." Diabetes **51**(10): 2929-35.