Signalling Pathways Important in Human Adipose Tissue Growth and Function

By

Nayan Govindbhai Patel

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Division of Medical Sciences
Faculty of Medicine and Dentistry
University of Birmingham
B15 2TH
UK
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Summary

Adipose tissue is a dynamic tissue which can grow and regress throughout the lifetime of an individual. The aim of this study is to understand the factors important in the regulation of adipocyte growth and function. Using human subcutaneous preadipocytes, the effects of insulin, dexamethasone, T3, and IBMX, alone and in combination, on different aspects of differentiation were examined. All 4 inducers were required in combination to induce a high level of differentiation. The PPARγ activator, rosiglitazone in conjunction with the 4 inducers induced the highest level of adipocyte differentiation. Inhibiting p38 MAPK and $p70^{S6K}$ reduced lipid content and lipogenic activity in differentiated preadipocytes while inhibiting p42/44 MAPK and PI3K had no effect. Inhibiting p38 MAPK, $p70^{S6K}$, p42/44 MAPK and PI3K reduced leptin secretion but had no effect on protein content. Insulin and T3 caused the rapid activation of p42/44 MAPK in preadipocytes and differentiated preadipocytes, but dexamethasone, cortisol, oestrogen and rosiglitazone had no effect. Adipocytes expressed several growth factors i.e. FGF-2, VEGF, and angiopoietins. For FGF and its receptor, expression varied through differentiation. Inhibiting VEGF binding on adipocytes had no effect but inhibiting FGFR signalling and blocking angiopoietin action prevented growth and differentiation suggesting autocrine functions.

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Abbreviations

ADD1	Adipocyte Determination and Differentiation Factor
AMV	Avian Myeloblastosis Virus
Ang	Angiopoietin
ANOVA	One-Way Analysis of Variance
AP-1	Activated Protein Complex-1
aP2	Adipose-Specific Fatty Acid Binding Protein-2
APS	Associated Protein Substrate
BAT	Brown Adipose Tissue
bHLH	basic Helix-Loop-Helix
BMI	Body Mass Index
bp	Base Pair
BRL49653	Rosiglitazone
BSA	Bovine Serum Albumin
C	Celsius
cAMP	Cyclic Adenosine Monophosphate
CAP	Cbl-Associated Adaptor Protein
CBP	CREB-Binding Protein
C/EBP	CCAAT/Enhancer Binding Protein
CHO	Chinese Hamster Ovary
CHOP	C/EBP-Homologous Protein
cm	Centimetre
CO_2	Carbon Dioxide
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dpm	Disintegrations Per Minute
E2	17β Oestradiol

FGFR	Fibroblast Growth Factor Receptor
FKBP-12	FK-506-Binding Protein-12
FLICE	FADD-Like Interleukin-1β Converting Enzyme
g	Gram
g	Gravity
GADD153	Growth Arrest and DNA Damage Inducible Gene 153
GDP	Guanosine Diphosphate
G3PDH	Glycerol-3-Phosphate Dehydrogenase
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
GSK-3	Glycogen Synthase Kinase-3
GTP	Guanosine Triphosphate
h	Hour
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
HLGAG	Heparin-like Glycosaminoglycan
HMG	High-Mobility Group Protein
H_2O_2	Hydrogen Peroxide
HRP	Horseradish Peroxidase
HSL	Hormone Sensitive Lipase
IBMX	3-Isobutyl-1-Methylxanthine
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-Like Growth Factor
IκBA	NFκB Inhibitory Subunit
IL	Interleukin
In	Insulin
IRS	Insulin Receptor Substrate
JAK	Janus Kinase
JNK/SAPK	c-jun N-Terminal/Stress-Activated Protein Kinase
kDa	KiloDalton
K_2HPO_4	Potassium Phophate

mmol	Millimole
min	Minute
MKK	MAP Kinase Kinase
MKP-1	MAPK Phosphatase-1
MMP	Matrix Metalloproteinase
MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
mTOR	Mammalian Target of Rapamycin
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NEFA	Non-Esterified Fatty Acid
NFκB	Nuclear Factor κB
ng	Nanogram
NH_4Cl	Ammonium Chloride
NIDDM	Non-Insulin-Dependent Diabetes Mellitus
NIK	NFκB Inducing Kinase
nM	NanoMolar
nm	Nanometre
NPY	Neuropeptide Y
NSF	N-Ethylmaleimide-Sensitive Factor
OD	Optical Density
PAI-1	Plasminogen Activator Inhibitor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
PDK	3-Phosphoinositide-Dependent Protein Kinase
PEPCK	Phosphophenolpyruvate Carboxylase
pfu	Plaque Forming Unit
15d PGJ_2	15 Deoxy-$\Delta^{12,14}$ Prostaglandin J_2
PH	Pleckstrin Homology
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-Kinase
PKA	cAMP-Dependent Protein Kinase

RAd-sVEGFR1	Recombinant Adenovirus Soluble Vascular Endothelial Growth Factor Receptor1
RAd-sVEGFR2	Recombinant Adenovirus Soluble Vascular Endothelial Growth Factor Receptor2
RIP	Receptor-Interacting Protein
RNA	Ribonucleic Acid
Ros	Rosiglitazone
RSK/ $p90^{RSK}$	p90 Ribosomal S6 Kinase
RT	Real Time
RTK	Receptor Tyrosine Kinase
RT-PCR	Real Time Polymerase Chain Reaction
RXR	Retinoid X Receptor
SCD1	Stearoyl-CoA Desaturase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
SH2	src-Homology-2
SHC	src and Collagen Homologous Protein
siRNA	Small Interfering RNA
SNAP	Soluble NSF Attachment Protein Receptor
SOS	Son of Sevenless
SREBP1	Sterol Regulatory Element Binding protein-1
STAT	Signal Transducer and Activator of Transcription
T3	Triiodothyronine
TBE	Tris Borate/EDTA Electrophoresis Buffer
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween 20
TCF	Ternary Complex Factor
TEMED	N,N,N',N',-Tetramethylethylenediamine
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TNFR	TNF Receptor
TR	Thyroid Hormone Receptor

VSMC	Vascular Smooth Muscle Cells
v-SNARE	Vesicle SNAP
WAT	White Adipose Tissue
WHO	World Health Organisation
wt	Weight

CHAPTER 1

Introduction

1.1 Obesity

Obesity is defined as a significant increase above an ideal weight, where ideal weight is defined as that which maximises life expectancy (Leong and Wilding 1999; Friedman 2000). It is common, affecting one third of the population of developed countries (Campfield and Smith 1999). A recent health survey has found 17% of men and 20% of women to be classed as obese in England and Wales (Prescott-Clarke and Primatesta 1996) while the US National Health and Nutrition Examination Survey (1999-2000) shows that 28% of American men and 34% of American women are obese (Flegal *et al.* 2002). Causation of obesity is due to a number of genetic and environmental factors and their interaction with each other. Most obesity occurs through susceptibility genes, which are attenuated or exacerbated by non-genetic factors. An individual with a susceptibility gene for obesity will only become obese if in an environment where the food supply is plentiful. Obesity-associated syndromes are rare but have been invaluable in delineating important obesity-associated proteins (Kopelman 2000).

Obesity is a significant risk factor in several diseases including hypertension, non-insulin-dependent diabetes mellitus (NIDDM) (Type 2 diabetes), dyslipidaemia, hypercholesterolaemia as well as cardiovascular disease, sleep apnoea, joint diseases and it is correlated with certain cancers such as prostate, colon, and postmenopausal breast cancer (Kopelman 1994; Kopelman 2000; Barnett 2003; Calle *et al.* 2003). This correlation with disease has been shown in an epidemiological study by Willett *et al* (1999), which examined the correlation between body mass index (BMI) and

relative risk of disease with Type 2 diabetes showing the most striking association between obesity and disease (**Fig 1.1**) (Willett *et al.* 1999). The BMI is one method used to estimate adiposity using a formula which combines weight and height. An individual's weight in kilograms is divided by the square of the height in metres (kg/m^2) to give the BMI. The BMI has then been placed into different classifications, which determines levels of adiposity (**Table 1.1**).

As well as the associated health problems, obese people are often stigmatised socially and in the workplace. This is especially true of Western societies, where the desire to be lean is held with high regard. Clearly understanding adipocyte cell biology and the regulation of adipocyte growth and function is required so that this obesity epidemic can be controlled. Similarly an understanding of the role of obesity in other syndromes is essential (Kopelman 2000).

1.2 Insulin Resistance and Type 2 Diabetes

Type 2 diabetes is a complex metabolic disorder and is the most prevalent form of diabetes. Type 2 diabetes typically affects older (>40 years) individuals and is usually associated with obesity. Clinically diabetes is diagnosed by the presence of hyperglycaemia in the fasting state (Gustafson *et al.* 1999).

The development of Type 2 diabetes involves at least two major defects. The first is insulin resistance in peripheral tissues such as liver, fat and skeletal muscle. The second is the failure of the β-cell in the pancreas to compensate for this increased

resistance with increased insulin secretion. During the early development of Type 2 diabetes, individuals develop hyperinsulineamia because of increased release of insulin by the β-cell. This causes downregulation of insulin receptors and desensitization of post-receptor pathways leading to insulin resistance. The β-cell can compensate insulin resistance for many years but eventually the β-cell becomes less sensitive to glucose and begins to secrete less insulin. This eventually leads to β-cell failure and fasting hyperglycaemia becomes apparent, signifying Type 2 diabetes (Gustafson *et al.* 1999). The cause of β-cell failure is unclear, but there are suggestions that the defect is partly inherited (O'Rahilly *et al.* 1986) and malnutrition in early life may be a contributing factor (the thrifty phenotype hypothesis) (Hales and Barker 1992; Leong and Wilding 1999).

The association of obesity with Type 2 diabetes has been recognised for many years. The major basis for this link is the ability of obesity to cause insulin resistance, which precedes Type 2 diabetes in the majority of patients (**Fig 1.1**). Insulin resistance can be related to genetic abnormalities, but in most individuals, it appears to be related to obesity. A probable common link between Type 2 diabetes and obesity is the adipocyte, which stores excess energy in the form of triglyceride, and releases free fatty acids (FFAs) in response to energy requirements such as fasting. In healthy individuals, excess fat is stored in adipocytes and only low amounts of triglyceride are maintained in other tissues. In obese individuals there is an exaggerated release of FFAs due to lipolysis. Although the mechanism for this process remains unclear it may be due to the increased lipolytic activity of specific fat depots, which have increased due to obesity (Reynisdottir *et al.* 1994; Lonnqvist *et al.* 1995). This leads

to abnormal accumulation of triglycerides in other tissues such as muscle, liver, and pancreatic islets resulting in lipotoxicity (Unger 2002). Raised FFA levels reduce insulin-stimulated glucose uptake, and increase glucose output, particularly in the liver and skeletal muscle (Bonadonna *et al.* 1990). In the liver increased levels of FFAs interfere with enzymes involved in glycolysis and glucose oxidation via the Randle Cycle, which converts glucose to fatty acids resulting in increased hepatic glucose output. For example, the impairment of glycolysis by FFA leads to increased levels of glucose-6-phosphate, which inhibits hexokinase, an enzyme involved in glucose uptake (Randle *et al.* 1963; Leong and Wilding 1999). Initially pancreatic β-cells can respond by increasing insulin production, but with ever increasing levels of FFAs in the obese individual, this leads to hyperinsulinaemia and hyperglycaemia typical of Type 2 diabetes. The exaggerated release of FFAs during obesity by adipocytes is an important factor in the causation of Type 2 diabetes implicating the adipocyte and factors which cause FFA release in Type 2 diabetes.

1.3 Adipose Tissue

Commonly referred to as fat, adipose tissue is known to occur in virtually every mammalian organism. It is considered as a type of connective tissue and can be described as a loose association of lipid-filled cells referred to as adipocytes, with associated stromal-vascular cells, held in a matrix of collagen fibres (Greenwood and Johnson 1977).

Adipocytes occur in two major forms, i.e. unilocular where there is a single large inclusion of lipid, and multilocular, where there are many smaller lipid inclusions. The unilocular adipocyte is the characteristic cell type of white adipose tissue (WAT), which is used as an energy storage compartment in mammals, while the multilocular adipocyte is characteristic of brown adipose tissue (BAT), which is used as a heat-producing organ in the mammal (Greenwood and Johnson 1977). WAT and BAT serve different functions. They occur in different animals and are distributed differently in mammals

WAT is characterised by a white or yellow colour and develops from embryonic mesenchyme with the formation of spindle-shaped lipoblasts, which contain small fat vacuoles. These mature into adipocytes, which store fat for use by other body tissues as a source of energy. WAT is the main form of fat-storing cell in the adult and is used as both an energy store and a support tissue. It has a rich capillary blood supply and is innervated by the autonomous nervous system. It possesses receptors for many signalling factors such as growth hormone, insulin, glucocorticoids, thyroid hormones and noradrenaline that modulate the uptake and release of fat (Greenwood and Johnson 1977; Stevens and Lowe 1992).

BAT is characterised by a brown colour and functions as a heat-producing organ by metabolising fat to produce heat. Ultrastructurally multilocular adipose cells contain huge numbers of mitochondria in addition to lipid vacuoles, which correlates with their function of heat generation through mitochondrial metabolism of fatty acids. It does this by the mitochondrial protein uncoupling protein 1 (UCP-1) (Collins *et al.*

2001; Klein *et al.* 2002). BAT does not usually persist in adults but becomes lost during childhood, although small amounts may remain in certain sites (Greenwood and Johnson 1977; Stevens and Lowe 1992). Due to this reason, the thesis will investigate the role of human WAT and not BAT.

1.4 Fat Metabolism

Fat metabolism can be divided into two processes called lipolysis and lipogenesis. Lipogenesis is the process of storing triglycerides in adipose tissue whereas lipolysis is the process by which adipose tissue hydrolyses fatty acids from intracellular triglyceride stores. These processes are closely linked together and play an important role in the balance of fat metabolism (**Fig 1.2**) (Kruszynska 1996; Ramsay 1996).

1.4.1 Lipogenesis

The primary function of the adipocyte is to store triglyceride for later use when energy is required. Adipose tissue triglyceride depots are the major energy stores in the body and account for 10-30% of body weight (Greenwood and Johnson 1977). Triglyceride storage occurs with great efficiency in the adipocyte, utilising glucose to serve as a glycerol-3-phosphate backbone for the esterification of FFA (also referred to as non-esterified fatty acid (NEFA)), with the availability of glycerol-3-phosphate being important in determining lipogenesis. Human adipocytes have relatively low levels of the enzyme glycerokinase, which converts glycerol to glycerol-3-phosphate. Due to the low levels of glycerokinase, glycerol itself cannot be used for triglyceride

synthesis but instead glycerol-3-phosphate in adipocytes is derived from glucose via glycolysis. A recent study has shown that glycerokinase can be induced in human adipocytes by the antidiabetic agents referred to as thiazolidinediones (TZD's) (Guan *et al.* 2002) showing that glycerol can be used for triglyceride synthesis. The vast majority of FFAs are from dietary and hepatic sources and are taken up from the circulation into adipocytes. To facilitate the uptake of FFA from the circulation into the adipocyte, the protein lipoprotein lipase (LPL) is used. LPL is secreted by the adipocyte and binds to the endothelial surface of capillaries and hydrolyses FFA from circulating triglyceride within very low-density lipoproteins (VLDL) and chylomicrons. Hydrolysed FFAs are taken up by the adipocyte by fatty acid-binding proteins and esterification of FFA and glycerol-3-phosphate occurs to form triglycerides (Ramsay 1996). Synthesis of FFA from glucose is a limited function of the adipocyte and hepatocytes are much more active in this regard (Shrago *et al.* 1969). Esterification of FFA with glycerol-3-phophate involves several reactions leading to triglyceride formation and these are shown in **Fig 1.2** (Kruszynska 1996; Ramsay 1996).

Insulin stimulated glucose uptake occurs by facilitated diffusion in response to the induction of the glucose transporter protein GLUT4. Insulin stimulation of adipocytes results in the rapid translocation of GLUT4 to the plasma membrane to allow the uptake of glucose into the adipocyte. GLUT4 will be discussed later. Glucose is then metabolised to form glycerol-3-phosphate for esterification with FFA (Ramsay 1996).

Although the regulation of triglyceride synthesis is incompletely understood, it is known that insulin plays an important role. Insulin stimulates lipogenesis, partly by increasing the activities of several lipogenic enzymes such as pyruvate dehydrogenase, acetyl-CoA carboxylase, and fatty acid synthase. Stimulation of lipogenesis by insulin is partly caused by stimulating glucose uptake and therefore increasing the availability of pyruvate for fatty acid synthesis and of glycerol-3-phosphate for their esterification. As well as this, insulin also inhibits the breakdown of triglyceride by lipolysis (Kruszynska 1996; Ramsay 1996).

1.4.2 Lipolysis

Lipolysis is the process by which adipose tissue triglycerides are hydrolysed to glycerol and fatty acids (**Fig 1.2**). This occurs by the action of two enzymes, hormone sensitive lipase (HSL) and 2-monoacyl-glycerol lipase (2-MAGL), which hydrolyse triglycerides into diglycerides and then monoglycerides and finally glycerol, with release of FFA during each step (Ramsay 1996).

HSL is activated by phosphorylation by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and hormones activating adenylate cyclase and thus they activate lipolysis (Belfrage *et al.* 1981). The catecholamines for example act via β-adrenoreceptors to activate adenylate cyclase and therefore increase cAMP thus activating HSL. α-adrenoreceptor activity however has been shown to inhibit lipolysis (Wahrenberg *et al.* 1989). Growth hormone (Takahashi and Satozawa 2002) activates lipolysis by pathways which do not involve cAMP while a recent study has

implicated extracellular signal-regulated kinase (ERK) in catecholamine-stimulated lipolysis (Greenberg *et al.* 2001). Insulin inhibits lipolysis by inhibiting HSL and by increasing glucose uptake and thus glycerol-3-phosphate availability (Coppack *et al.* 1989; Jensen *et al.* 1989; Kruszynska 1996) and by decreasing cAMP by activating phosphodiesterases (PDE) (Shibata *et al.* 1991; Onuma *et al.* 2002). Neuropeptide Y (NPY), prostaglandins E_1 and E_2, and adenosine are other compounds which have been shown to inhibit lipolysis possibly through G_i proteins (Castan *et al.* 1994).

1.5 Adipocyte Differentiation

WAT formation occurs before birth (Poissonnet *et al.* 1983; Poissonnet *et al.* 1984), but soon after birth there is rapid expansion of WAT (Poissonnet *et al.* 1983; Poissonnet *et al.* 1984; Gregoire *et al.* 1998). The development of adipocytes occurs through a combination of increases in cell number (hyperplasia) and increases in cell size (hypertrophy) (Sorisky 1999). This has been shown *in vivo* when rats fed on a high carbohydrate diet, showed increases in fat mass by an increase in preadipocyte replication as well as differentiation to mature adipocytes (Faust *et al.* 1978; Faust *et al.* 1984). The process of adipocyte acquisition involves the clonal expansion and subsequent differentiation of preadipocytes. Thus, adipocytes represent terminally differentiated cells. The preadipocyte is believed to descend from multipotent mesodermal cells that originate from embryonic stem cells. Preadipocytes are located in the stromal-vascular fraction, which consists of preadipocytes, endothelial cells, and blood cells, while the rest is composed of mature adipocytes. As preadipocytes

can replicate and differentiate into mature adipocytes, these cells are crucial to adipose tissue growth (Ailhaud *et al.* 1992; Sorisky 1999).

1.5.1 The Process of Adipocyte Differentiation

The process of adipocyte differentiation can be divided into a number of stages where numerous genes are switched on and others are repressed. These stages consist of:

- Growth Arrest
- Clonal Expansion
- Early Changes In Gene Expression
- Late Changes In Gene Expression

These stages have been well characterised in a number of *in vitro* systems (**Fig 1.3**).

1.5.1.1 Growth Arrest

In both cell lines and primary cultures, growth arrest and not cell confluence or cell-to-cell contact appears to be essential for adipocyte differentiation. This has been shown in confluent 3T3-F442A cells shifted to methylcellulose-stabilised suspension culture which prevents cell contact but cells still undergo differentiation (Pairault and Green 1979). Rat preadipocytes (Gregoire *et al.* 1998) and human preadipocytes (Hauner *et al.* 1989) plated at low density in serum-free medium still undergo terminal differentiation without cell contact.

The requirement of growth arrest to enable differentiation to proceed has been shown in Ob1771 and 3T3-F442A cells, where, by blocking cell growth, the expression of early markers of adipocyte differentiation such as LPL and pOb24 mRNA occurs (Amri *et al.* 1986). Growth arrest has been shown to be required for differentiation in murine preadipocytes (Krawisz and Scott 1982; Scott *et al.* 1982) and 3T3-L1 preadipocytes (Green and Kehinde 1975; Reichert and Eick 1999). In human preadipocytes, replication inhibits differentiation, and if cells are growth arrested, adipocyte differentiation increases (Entenmann and Hauner 1996).

Two transcription factors, CCAAT/enhancer binding protein alpha (C/EBP-α) and peroxisome proliferator-activated receptor gamma (PPAR-γ) have been shown to activate genes involved in some way in growth arrest as well as those required for adipocyte differentiation (Gregoire *et al.* 1998; Rosen *et al.* 2000). C/EBP-α has been shown to be required for post-mitotic growth arrest in 3T3-L1 preadipocytes (Tao and Umek 2000) and C/EBP-α has been shown to change the expression of genes involved in the cell cycle such as inducing p21 (Waf1/Cip1) (Timchenko *et al.* 1996) and this may cause growth arrest. PPAR-γ has been shown to change the expression of several cyclin-dependent kinase inhibitors, including increasing p18 (INK4c) and p21 (Waf1/Cip1) expression (Morrison and Farmer 1999). Changes in these cyclin-dependent kinase inhibitors are often associated with growth arrest. PPAR-γ has also been shown to induce growth arrest by a decrease in the DNA-binding and transcriptional activity of the E2F/DP complex, a central transcriptional player in the regulation of many genes involved in cell growth (Altiok *et al.* 1997).

1.5.1.2 Clonal Expansion

After growth arrest, preadipocyte cell lines require at least one round of DNA replication and cell doubling, termed clonal expansion, before differentiation can proceed (Pairault and Green 1979). In 3T3-F442A and Ob17 cells, DNA synthesis increases prior to the appearance of adipocyte markers and by inhibiting DNA synthesis, differentiation is prevented (Pairault and Green 1979; Kuri-Harcuch and Marsch-Moreno 1983; Amri *et al.* 1986). This requirement for clonal expansion has been questioned in two studies. In 3T3-L1 cells the p42/44 mitogen-activated protein kinase (MAPK) inhibitor, PD98059 inhibited clonal expansion but did not prevent differentiation (Qiu *et al.* 2001), however a later study found that PD98059 only delayed clonal expansion and did not inhibit it. The use of a more specific and potent inhibitor of p42/44 MAPK, U0126 prevented clonal expansion and differentiation (Tang *et al.* 2003). A cyclin-dependent kinase inhibitor, roscovitine which inhibits the cell cycle, inhibited differentiation suggesting that clonal expansion is required for differentiation. However the possibility that this compound is cytotoxic cannot be ignored (Tang *et al.* 2003). In primary preadipocytes, derived from human adipose tissue, cell division after growth arrest is not required for cells to enter the differentiation process (Entenmann and Hauner 1996). One hypothesis is that a large majority of the isolated human preadipocytes have already undergone potential critical cell divisions *in vivo* and so correspond to a later stage of adipocyte development and are not inhibited by blocking mitosis to undergo differentiation. It is therefore possible that within a population of isolated preadipocytes, there may be some preadipocytes that require clonal expansion (Entenmann and Hauner 1996).

1.5.1.3 Early Changes in Gene Expression

During adipocyte differentiation, cells convert from a fibroblastic to a spherical shape, accumulating lipid in the process. Dramatic changes occur in cell morphology, cytoskeletal components, and the level and type of extracellular matrix (ECM) components (**Fig 1.3**). These changes in cell shape involve decreases in expression of genes such as tubulin and actin (Spiegelman and Farmer 1982) and changes in expression of collagen genes (Aratani and Kitagawa 1988). These changes may influence expression and the action of genes switched on later during the differentiation process (Jones *et al.* 1993). Adding exogenous ECM components from microvascular endothelial cells has been shown to enhance preadipocyte differentiation (Varzaneh *et al.* 1994). Changes in cell morphology are not a result of lipid accumulation but a distinct process of differentiation because blocking lipid accumulation in 3T3-L1 preadipocytes cells by culturing in biotin-deficient medium did not prevent the morphology changes (Kuri-Harcuch *et al.* 1978). Several genes involved in the ECM have been shown to change during differentiation. A switch in collagen gene expression is an early event in adipocyte differentiation. Fibroblast-expressed type I and type III collagen decline during differentiation, while expression of type IV, type V and type VI increase (Aratani and Kitagawa 1988; Weiner *et al.* 1989; Nakajima *et al.* 2002). Matrix metalloproteinases (MMP) 2 and 9, two key enzymes involved in the modulation of ECM are also upregulated during adipocyte differentiation (Bouloumie *et al.* 2001) while fibronectin is downregulated (Antras *et al.* 1989).

Preadipocyte factor-1 (Pref-1) is a transmembrane protein found in murine preadipocyte cell lines and primary cultures but yet to be identified in humans. It is found exclusively in preadipocytes and may maintain the preadipocyte phenotype. Pref-1 expression is downregulated during differentiation and it is virtually undetectable in mature adipocytes (Smas *et al.* 1999) (**Fig 1.3**). *In vivo* studies in mice have shown that Pref-1 downregulation is required for adipogenesis (Lee *et al.* 2003), but a mechanism of action of Pref-1 has not been identified.

Wnts are secreted signalling proteins that regulate developmental processes and may be a molecular switch governing adipogenesis (Ross *et al.* 2000). Disruption of Wnt signalling induces adipogenesis in a number of cells including 3T3-L1 cells (Bennett *et al.* 2002). Preadipocytes constitutively expressing Wnt-1 fail to differentiate, while activation of Wnt signalling downstream of its receptor also prevents differentiation indicating that Wnt signalling maintains preadipocytes in their undifferentiated state. Wnt is known to inhibit adipogenic transcription factors C/EBP-α and PPAR-γ (Ross *et al.* 2000; Gregoire 2001) which indicates that Wnt must be inhibited in order for adipogenesis to proceed.

LPL expression increases during differentiation and has been cited as an early marker for adipocyte differentiation (Ailhaud *et al.* 1995; MacDougald and Lane 1995). LPL is an enzyme secreted by mature adipocytes and it plays a central role in controlling lipid accumulation (Goldberg 1996; Gregoire *et al.* 1998).

1.5.1.3.1 Transcription Factors Involved in Adipocyte Differentiation

Transcription factors are very important in the process of adipogenesis. At least three families of transcription factors, C/EBP, PPAR-γ, and ADD1/SREBP1 are induced during adipocyte differentiation and are described below.

1.5.1.3.1.1 CCAAT/Enhancer Binding Proteins (C/EBP)

The C/EBP family of transcription factors was the first to be shown to play a major role in adipocyte differentiation. They contain a basic transcriptional activation domain and an adjoining leucine zipper motif, which provides the ability for homo- and heterodimerisation (Lekstrom-Himes and Xanthopoulos 1998).

C/EBPs are expressed in a number of tissues such as granulocytes (Zhang *et al.* 1997a), hepatocytes (Flodby *et al.* 1996), and are not exclusive to adipocytes. There are a number of isoforms present, C/EBP-β and C/EBP-γ being expressed early in the differentiation process while C/EBP-α is expressed relatively late (Cao *et al.* 1991; Yeh *et al.* 1995c) (**Fig 1.3**).

Many experiments have shown the role of C/EBP-β and C/EBP-γ in adipogenesis. Ectopic expression of C/EBP-β induces differentiation of 3T3-L1 cells without the addition of hormonal inducers, while ectopic expression of C/EBP-γ enhances adipogenesis, but there is still a requirement for hormonal inducers (Yeh *et al.* 1995c).

C/EBP-β may also play a role in the determination of cells to the adipocyte lineage. Ectopic expression of C/EBP-β in NIH-3T3 fibroblasts in the presence of hormonal inducers induces adipogenesis (Wu *et al.* 1995), while embryonic fibroblasts show a reduced adipogenic potential if lacking either C/EBP-β or C/EBP-γ and if both are absent, adipocyte development is severely inhibited (Tanaka *et al.* 1997). *In vivo* work in mice lacking C/EBP-β and C/EBP-γ is less convincing because mice lacking C/EBP-β or C/EBP-γ show a reduction in BAT but not WAT. Mice lacking both C/EBP-β and C/EBP-γ show a large reduction in BAT and a slight reduction in WAT, with only 15% surviving the perinatal period (Tanaka *et al.* 1997).

C/EBP-α is important in adipogenesis as shown by *in vitro* data where overexpression of C/EBP-α in 3T3-L1 cells induces differentiation (Freytag *et al.* 1994; Lin and Lane 1994) while blocking C/EBP-α by antisense RNA severely inhibits adipocyte differentiation (Lin and Lane 1992). *In vivo* work with mice carrying a homozygous deletion of C/EBP-α have shown a dramatic reduction of WAT and BAT (Wang *et al.* 1995). C/EBP-α is expressed just before the transcription of most adipocyte-specific genes is initiated and is known to activate the promoters of several adipocyte genes including adipose-specific fatty acid binding protein-2 (aP2) (Herrera *et al.* 1989), stearoyl-CoA desaturase (SCD1) (Ntambi *et al.* 1988), the glucose transporter protein GLUT-4 (Kaestner *et al.* 1990), leptin (Miller *et al.* 1996) and insulin receptor (Gregoire *et al.* 1998).

1.5.1.3.1.2 Peroxisome Proliferator-Activated Receptor-Gamma (PPAR-γ)

PPAR-γ is a member of the nuclear hormone receptor superfamily. PPAR-γ exists as two protein isoforms, PPAR-γ1 and PPAR-γ2, generated by alternative promoter usage and alternative splicing. PPAR-γ1 is expressed in many tissues at low levels (Braissant *et al.* 1996) while PPAR-γ2 is 30 amino acids longer at the amino terminus and is the dominant isoform found in adipose tissue (Tontonoz *et al.* 1994). Its expression is critical for differentiation to proceed (Ren *et al.* 2002) (**Fig 1.3**).

PPAR-γ is ligand-activated by a number of natural and synthetic ligands. Natural ligands include 15 deoxy-$\Delta^{12, 14}$ prostaglandin J_2 (15d PGJ_2), fatty acids, and linoleic acid (Forman *et al.* 1995; Kliewer *et al.* 1995; Kliewer *et al.* 1997) but these natural ligands bind at a lower affinity than other nuclear hormone receptors for their ligands so they may not be the natural, high affinity binding ligand of PPAR-γ. Synthetic compounds called thiazolidinediones e.g. rosiglitazone (BRL 49653), which are used clinically as antidiabetic agents are known to activate PPAR-γ (Lehmann *et al.* 1995). TZD's effects include increased insulin sensitivity, enhanced glucose uptake and improved fatty acid transport and thus increase both the extent and role of adipogenesis (Gregoire *et al.* 1998; Rosen *et al.* 2000). This occurs *in vivo* as well as *in vitro*, so PPAR-γ is very important in the differentiation process of adipocytes (Rosen *et al.* 2000).

PPAR-γ forms a heterodimer with the retinoid X receptor (RXR) becoming transcriptionally active. This can bind to peroxisome proliferator response elements

(PPRE) located on the regulatory regions of a number of genes whose products are associated with lipid metabolism and homeostasis. This includes the promoters of many adipocyte-specific genes such as phosphophenolpyruvate carboxylase (PEPCK) (Tontonoz *et al.* 1994) and LPL (Schoonjans *et al.* 1996). PPAR-γ expression has been shown to be sufficient to induce growth arrest as well as initiate adipogenesis (Adams *et al.* 1997; Altiok *et al.* 1997; Gregoire *et al.* 1998).

Gain and loss-of-function studies have shown the importance of PPAR-γ in adipogenesis. Ectopic expression of PPAR-γ in non adipogenic cells such as fibroblasts (Tontonoz *et al.* 1994), and myoblasts (Hu *et al.* 1995) promoted adipogenesis. Loss-of-function experiments of PPAR-γ in mice have shown that due to a defect in placental development, PPAR-γ knockout led to embryonic lethality (Barak *et al.* 1999; Kubota *et al.* 1999) so the effect on WAT could not be determined. However isolation and induction of adipocyte differentiation in embryonic fibroblasts (Kubota *et al.* 1999) or embryonic stem cells (Rosen *et al.* 1999) from these PPAR-γ null cells resulted in no adipose conversion *in vitro*. Use of a synthetic antagonist of PPAR-γ (Oberfield *et al.* 1999) and the ectopic expression of a dominant-negative PPAR-γ mutant (Gurnell *et al.* 2000) both inhibit adipocyte differentiation confirming the role of PPAR-γ in adipogenesis.

1.5.1.3.1.3 Adipocyte Determination and Differentiation Factor 1/Sterol Regulatory Element Binding Protein-1 (ADD1/SREBP1)

ADD1/SREBP1 is a member of the basic helix-loop-helix (bHLH) family of transcription factors. ADD1 was isolated from rat adipocytes (Tontonoz *et al.* 1993) while the human homolog SREBP1 (Yokoyama *et al.* 1993) was isolated independently from ADD1. A possible role of ADD1/SREBP1 in adipocyte differentiation was suggested by the observation that expression of ADD1/SREBP1 mRNA is induced in preadipocyte cell lines induced to differentiate (Kim and Spiegelman 1996). Overexpression of ADD1/SREBP1 in 3T3-L1 cells in the presence of hormonal inducers of adipocyte differentiation results in enhanced lipid accumulation and adipocyte marker expression probably by inducing PPAR-γ (Fajas *et al.* 1999), while the expression of a dominant-negative form of ADD1/SREBP1 blocks differentiation of 3T3-L1 cells (Kim and Spiegelman 1996). ADD1/SREBP1 also induces expression of genes involved in fatty acid metabolism such as fatty acid synthase and LPL in NIH-3T3 cells ectopically expressing ADD1/SREBP1 (Kim and Spiegelman 1996). Conditioned medium from NIH-3T3 cells expressing ADD1/SREBP1 is able to activate PPAR-γ-mediated transcription suggesting that ADD1/SREBP1 may activate PPAR-γ by the production of an endogenous ligand for PPAR-γ (Kim *et al.* 1998). These studies implicate ADD1/SREBP1 in adipocyte differentiation.

1.5.1.3.2 Cascade of Transcription Factors Involved in Adipogenesis

Induction of adipocyte differentiation involves the cascade of transcription factors. C/EBPs, PPAR-γ and ADD1/SREBP1 have all been implicated in this cascade and a possible model of events has been proposed (**Fig 1.4**). There is initial expression of C/EBP-β and C/EBP-γ, which induces the expression of PPAR-γ (Wu *et al.* 1996). This is probably mediated by C/EBP binding sites in the PPAR-γ promoter (Rosen *et al.* 2000). PPAR-γ then induces the expression of C/EBP-α. The temporal expression profile of C/EBP-α and PPAR-γ demonstrate this possible sequence of expression (Rosen *et al.* 2000). Gain-of-function experiments in which ectopic expression of PPAR-γ or the application of PPAR-γ ligands induces C/EBP-α mRNA expression (Rosen *et al.* 2000). Embryonic fibroblasts (Kubota *et al.* 1999) or embryonic stem cells (Rosen *et al.* 1999) containing PPAR-γ null cells induced to differentiate result in no adipose conversion *in vitro* with low levels of C/EBP-α present. When PPAR-γ is reintroduced into these cells, the ability to differentiate is restored. This suggests that PPAR-γ induces C/EBP-α expression. C/EBP-α null mice show no adipose conversion with little expression of PPAR-γ (Wu *et al.* 1999) suggesting that C/EBP-α and PPAR-γ may co-regulate each other's expression. Another study has shown that PPAR-γ may be the key regulator in adipogenesis. C/EBP-α null embryonic fibroblasts fail to undergo adipogenesis, but if PPAR-γ is overexpressed, adipogenesis is seen (Wu *et al.* 1999). When C/EBP-α is overexpressed in PPAR-γ null embryonic fibroblasts, cells fail to differentiate (Rosen *et al.* 2002), which shows that PPAR-γ is key in adipogenesis. C/EBP-β and C/EBP-γ double-knockout mice express normal

levels of C/EBP-α and PPAR-γ. This implies that there is an independent mechanism for inducing PPAR-γ. ADD1/SREBP1 is able to activate PPAR-γ, so it may be an alternative mechanism for activating PPAR-γ (Kim *et al.* 1998). Activation of C/EBPs and PPAR-γ contribute to the expression of genes that characterise the terminally differentiated adipocyte. The knockout studies have revealed much but confirmation that this is the same temporal sequence of activation of the transcription factors needs to be carried out in human adipocytes.

1.5.1.4 Late Events and Terminal Differentiation of Adipocytes

During the later stages of adipocyte differentiation, adipocytes increase de-novo lipogenesis and acquire sensitivity to insulin. The activity, protein, and mRNA levels for enzymes involved in triacylglycerol metabolism such as glycerol-3-phosphate dehydrogenase (G3PDH), a classic late marker for adipocyte differentiation increase 10-100 fold (Spiegelman *et al.* 1983; Gregoire *et al.* 1998). Glucose transporters such as GLUT-4 (Kaestner *et al.* 1990), insulin receptor (Reed *et al.* 1977), insulin sensitivity (Rubin *et al.* 1977) and β_1-adrenergic receptors decrease, while β-$_2$ and β-$_3$ adrenergic receptors increase (Feve *et al.* 1990; Feve *et al.* 1991; Gregoire *et al.* 1998). As well as proteins involved in lipid metabolism, other adipose tissue-specific products increase in number including aP2, an adipocyte-specific fatty acid binding protein (Spiegelman *et al.* 1983; Bernlohr *et al.* 1984), fatty acid translocase (FAT/CD36), a putative fatty acid transporter (Sfeir *et al.* 1997), adipsin (Cook *et al.* 1987), angiotensin II (Jones *et al.* 1997), plasminogen activator inhibitor (PAI-1)

(Alessi *et al.* 1997), resistin (Steppan *et al.* 2001), adiponectin (Scherer *et al.* 1995; Hu *et al.* 1996b), PEPCK (Tontonoz *et al.* 1995), as well as leptin (MacDougald *et al.* 1995). PPAR-γ and C/EBP have been implicated in activating a number of these genes (Gregoire *et al.* 1998).

1.5.2 *In Vitro* Models of Adipocyte Differentiation

A number of *in vitro* systems have been developed to study adipocyte differentiation. This has allowed many of the molecular and cellular events, which occur during differentiation from fibroblast-like preadipocytes to mature round fat cells to be determined. Preadipose cell lines from rodents and cultures of adipose-derived stromal/vascular precursor cells from various species are used.

Adipocyte precursor cell lines can be segregated into two classes: pluripotent fibroblasts and unipotent preadipocytes. The pluripotent fibroblasts include 10T1/2, Balb/c 3T3, 1246, RCJ3.1 and CHEF/18 fibroblasts and have the ability to be converted into several cell types therefore their cell type is not yet determined (Ntambi and Young-Cheul 2000). For example the 10T1/2 fibroblasts, which are derived from C3H mouse embryos can be converted into to preadipose, premuscle, and precartilage tissue upon treatment with 5-azacytidine, an inhibitor of DNA methylation (Taylor and Jones 1979; Dani *et al.* 1997). This would be a useful system for determining the initial steps required for preadipocyte determination. Immortalised unipotent preadipocyte cell lines capable of undergoing differentiation are useful for studying adipocyte differentiation. Unipotent preadipocytes include

3T3-L1 and 3T3-F422A (Green and Meuth 1974), 1246 (Serrero *et al.* 1992), Ob1771 (Negrel *et al.* 1978), TA1 (Chapman *et al.* 1984) and 30A5 (Konieczny and Emerson 1984) and have undergone determination and can either remain as fibroblasts or can undergo conversion to adipocytes. These preadipocyte cell lines are from different species and at different stages of differentiation. 3T3-L1 and 3T3-F442A cell lines were clonally isolated from disaggregated 17-19 day Swiss 3T3 mouse embryos and are the most frequently used due to their homogeneity and standard conditions required to culture and differentiate. 30A5 preadipocytes were obtained from 5-azacytidine treatment of 10T1/2 fibroblasts and Ob1771 are isolated from genetically obese mice (Ntambi and Young-Cheul 2000). Cell lines can be useful as they are homogeneous so all cells are at the same stage of differentiation allowing a certain level of consistency for experiments. An immortalised preadipocyte cell line of human WAT lineage has not yet been established but there is a BAT human preadipocyte cell line (PAZ6) (Zilberfarb *et al.* 1997). Attempts to immortalised WAT in humans have been unsuccessful because cells show limited differentiation (Forest *et al.* 1983) or they evolve with passage and lose the ability to differentiate (Wabitsch *et al.* 2001). Therefore much research is carried out on cell lines of mouse and rat origin. Cell lines can be passaged indefinitely and therefore provide an unlimited number of preadipocytes of a homogeneous nature. However the stage of development and the lineage of preadipocyte cell lines have not been well established and different cell lines may correspond to different unidentified stages of development. Furthermore cell lines evolve with passage number and time in culture. They are not static which may explain why conflicting data has been found from different laboratories.

Primary preadipocytes have been successfully cultured from a number of species including humans (Deslex *et al.* 1987b), rat (Deslex *et al.* 1987a), mouse (Litthauer and Serrero 1992), rabbit (Reyne *et al.* 1989), sheep (Soret *et al.* 1999), and pig (Hausman and Martin 1989). They reflect the *in vivo* process of adipogenesis better as they are diploid just like the cells *in vivo*, rather than the many aneuploid cell lines whose characteristics may have been altered when they were immortalised. Primary preadipocytes can be difficult to culture as the numbers of preadipocytes available from adipose tissue is limited and they cannot be passaged indefinitely as their characteristics may change. There can be substantial heterogeneity in the extent of differentiation between samples from different donors. This can be disadvantageous if consistency is required. Several studies have shown that adipocytes from different adipose depots show different characteristics. The use of primary cultures allows these specific depots to be analysed and compared. For example the expression of leptin is higher in subcutaneous depots than omental depots and higher in females than males (Montague *et al.* 1997; Zhang *et al.* 1999), and levels of adipogenesis vary between subcutaneous, mesenteric, and omental differentiated preadipocytes when looking at markers of differentiation (Tchkonia *et al.* 2002). Adipocytes from different stages of development can be isolated allowing comparisons of differentiation potential and metabolic activity to be determined. For primary preadipocyte cultures, differentiation capacity is donor-dependent and decreases with age (Bjorntorp *et al.* 1982; Deslex *et al.* 1987b; Kirkland *et al.* 1990; Gregoire *et al.* 1995). There are species differences between rodents and humans so the use of

human primary preadipocytes is important to validate rodent data in human preadipocytes.

In vitro preadipocyte cell line models can be extrapolated to the *in vivo* system. This has been shown when nude mice are given subcutaneous injections of preadipocytes leading to the development of mature fat pads, indistinguishable from WAT (Green and Kehinde 1979). However these systems are limited due to the lack of paracrine and intercellular interactions between cell types in adipose tissue (Lau *et al.* 1996). There is mounting evidence that these interactions are important in adipocyte growth and development. Microvascular endothelial cells have been reported to secrete factors that promote proliferation in preadipocytes in both human and rat cells (Lau *et al.* 1990; Hausman *et al.* 1996; Hutley *et al.* 2001). These factors are also known to promote differentiation with a possible role of extracellular matrix factors in this process (Bjorntorp 1983; Varzaneh *et al.* 1994). The lack of paracrine interactions suggests that *in vitro* models of differentiation do not reflect the *in vivo* system completely. For example the levels of secreted leptin in mice are higher *in vivo* than *in vitro* highlighting the differences between the two systems and the possible role of paracrine interactions (MacDougald *et al.* 1995). This suggests care must be taken when extrapolating data from cell lines to the *in vivo* context.

External inducers are required for the induction of differentiation of preadipocytes. However the complement of inducers required facilitating differentiation differs with cell line used. This, as well as other differences such as the timing of expression of certain transcription factors during differentiation may be due to the stage of

development or differentiation that the cell line was arrested at for cloning. Many different hormones and growth factors play a role in adipocyte differentiation acting via specific receptors to enable the process of differentiation to occur. Initial differentiation protocols required the presence of serum, but serum components are neither well-characterised nor easily-controllable. The development of chemically defined serum-free medium suitable for the differentiation of preadipocyte cell lines and primary preadipocytes has helped to assess the precise hormonal requirement for differentiation. **Table 1.2** shows a list of preadipocyte cell lines and primary preadipocytes and the differentiation protocols used. Although the complement of inducing agents required for differentiation varies with each cell culture model, there are some similarities such as the need for insulin, agents to increase intracellular cAMP such as 3-isobutyl-1-methylxanthine (IBMX), and glucocorticoids such as dexamethasone or cortisol. This suggest that these agents are prerequisites for differentiation and variations in the agents required for differentiation between cell lines and primary cultures are due to differences in the stage of development that the preadipocytes are at. Species differences will also influence the agents required for differentiation (**Table 1.2**).

In this thesis human primary subcutaneous preadipocytes will be utilised because primary cultures are more likely to reflect the *in vivo* context better than rodent cell lines as discussed before. To differentiate human primary preadipocytes in serum-free medium, the current model involves the addition of insulin, glucocorticoids, IBMX, and triiodothyronine (T3) (Petruschke and Hauner 1993). The TZD rosiglitazone has been shown to enhance adipocyte differentiation *in vitro* (Adams *et*

al. 1997) and has been added to the differentiation protocol in several studies (Adams *et al.* 1997; Sen *et al.* 2001; Tchkonia *et al.* 2002). The signalling pathways activated by these differentiating agents will be discussed below.

1.5.2.1 Insulin

Insulin is an essential peptide hormone that regulates metabolism, growth and differentiation. It elicits a broad array of biological responses in almost all cells of the body. As a primary hormone responsible for signalling the storage and utilisation of many basic nutrients, insulin activates transport systems and enzymes involved in intracellular use and storage of glucose, amino acids and fatty acids, and inhibits the catabolic processes such as the breakdown of glycogen, fat, and protein evoked by counter-regulatory hormones (Kahn and Folli 1993; Nystrom and Quon 1999). The ability of insulin to promote glucose storage in muscle and fat is crucial to the maintenance of glucose homeostasis and impairment in the ability of insulin to stimulate glucose uptake contributes to the development of insulin resistance and Type 2 diabetes in an individual (Pederson and Rondinone 2000).

Adipocytes are involved in lipid homeostasis and energy balance, but also play a central role in metabolism being a target for insulin action (Pederson and Rondinone 2000). Although the relevant *in vivo* stimuli for adipocyte differentiation has not yet been identified, studies on preadipocyte cells in culture have shown that insulin or insulin-like growth factor (IGF-1) is required for adipocyte differentiation (Smith *et*

al. 1988). Therefore insulin seems to be a very important factor for *in vitro* adipocyte differentiation.

The signalling pathway of insulin is very complex and is still undergoing elucidation. **Fig 1.5** is a representation of the insulin signalling pathway, but care must be taken with this proposed model as elements of the pathway have been determined in many different cell lines and different species and so may not represent insulin signalling in human adipocytes. The insulin signalling pathway involves protein phosphorylations and dephosphorylations leading to multiple signalling cascades responsible for translating the binding of insulin to its receptor into endpoint biological responses such as glucose transport, glycogen synthesis, protein synthesis, fatty acid synthesis, gene transcription, anti-apoptosis, and anti-lipolytic functions.

1.5.2.1.1 Insulin Receptor

The insulin receptor belongs to the family of receptors called receptor tyrosine kinases (RTK) (Yarden and Ullrich 1988). Insulin receptors are expressed on the plasma membrane of virtually all mammalian cells, including liver, adipose, muscle, and blood cells. The insulin receptor consists of a heterotetramer made up of two α-subunits (135 kiloDalton (kDa) each) which are entirely extracellular and contain the insulin binding sites and two β-subunits (95 kDa) which possess tyrosine kinase activity, and are embedded in the membrane by the transmembrane domains located within the β-subunits. These four subunits are disulphide linked and are therefore

present as a dimer prior to hormone binding (Kahn and Folli 1993; Taha and Klip 1999).

Insulin binding the α-subunit causes the activation of tyrosine kinase leading to autophosphorylation of tyrosine residues in several regions of the intracellular β-subunit. Further tyrosine phosphorylation occurs of endogenous receptor substrates such as the insulin receptor substrate (IRS) proteins, which transmit the insulin signal downstream to several different pathways (**Fig 1.5**).

1.5.2.1.2 Insulin Receptor Substrates (IRS)

Several docking proteins have been identified which are phosphorylated by the insulin receptor to transmit the insulin signal. Insulin receptor substrate-1 (IRS-1) was the first identified but recently related proteins have been identified, including IRS-2, IRS-3, IRS-4, *et al* although they may not all be involved in insulin signalling. All the IRS-proteins have the same overall structure i.e. an NH_2-terminal pleckstrin homology (PH) and/or phosphotyrosine-binding (PTB) domain which contain multiple tyrosine residues that facilitate insulin receptor binding and subsequent phosphorylation. IRS-proteins contain src-homology-2 (SH2) protein binding sites which bind to signalling proteins downstream containing SH2 domains such as Grb-2 or the p85 subunit of phosphatidylinositol-3-kinase (PI3K) to further transmit the insulin signal (Gustafson *et al.* 1999) (**Fig 1.5**).

IRS-1 and IRS-2 are widely distributed throughout the body where their relative expression levels in different tissues vary but they can be found in muscle, liver and adipose tissue. IRS-3 expression has been demonstrated in adipose tissue, fibroblasts, and liver cells, while IRS-4 appears to be restricted to embryonic kidney cells (Gustafson *et al.* 1999; Taha and Klip 1999; Rhodes and White 2002).

IRS-1 and IRS-2 activate various proteins including PI3K, phosphotyrosine phosphatase (PTP), Grb2, Nck, and Crk (White 1998). IRS-3 binds PI3K with a higher affinity than IRS-1 in rat adipose tissue (Ross *et al.* 1998), while for IRS-4, PI3K binding activity has yet to be demonstrated (Taha and Klip 1999).

In the human adipocyte, IRS-1 is upregulated during differentiation to a greater extent than IRS-2 indicating that it may play a more significant role in adipocyte differentiation (Pederson and Rondinone 2000). The same is true in 3T3-L1 cells where there is low expression of IRS-2 so IRS-1 is predominant (Sun *et al.* 1997).

Another member of the IRS family and substrate of the insulin receptor is the src and collagen homologous protein (SHC) which exists in three isoforms 46 kDa, 52 kDa, and 66 kDa and contains SH2 and PTB domains. SHC can activate Ras signalling independently of IRS-1. Both SHC and IRS-1 activate Ras via an adaptor protein called Grb-2 which contains an SH2 domain for this purpose (Pelicci *et al.* 1992; Giorgetti *et al.* 1994) (**Fig 1.5**).

1.5.2.1.3 Phosphatidylinositol-3-kinase (PI3K)

PI3K is a member of the lipid kinase family. The p85-p110 dimer, discussed below, plays the most important role in insulin signalling in the adipocyte. It is composed of a heterodimeric p110 kDa catalytic subunit associated with a p85 kDa regulatory subunit. It selectively phosphorylates phosphatidylinositol (PI), PI(4) phosphate, and PI(4,5) bisphosphate on the D-3 position on the inositol ring producing PI(3)P, $PI(3,4)P_2$, and $PI(3,4,5)P_3$ respectively. The p85 subunit of PI3K contains an SH2 domain which is activated by IRS-1 thus generating lipid products, which can interact with other proteins downstream of PI3K (Alessi *et al.* 1998; Gustafson *et al.* 1999) **(Fig 1.5)**.

One lipid product of PI3K action, PI(3)P, is constitutively present in cells and its presence is largely unaltered by hormonal stimulation. On the other hand, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ are undetectable in resting cells, but their intracellular concentrations rise rapidly with insulin stimulation (Endemann *et al.* 1990; Ruderman *et al.* 1990). In 3T3-L1 preadipocytes, $PI(3,4,5)P_3$ but not $PI(3,4)P_2$ levels increase with insulin stimulation, which would imply that $PI(3,4,5)P_3$ is the important mediator of insulin signalling in the adipocyte (Sorisky *et al.* 1996).

The products of PI3K action act as secondary messengers so that PI3K is central to cellular processes such as mitogenesis, transformation, differentiation, endocytosis, glucose transport, cytoskeletal organisation, and prevention of apoptosis (Toker and Cantley 1997; Sorisky 1999). For example, a number of studies have shown that the

lipid product $PI(3,4,5)P_3$ produced by PI3K plays a critical role in insulin-stimulated glucose transport and translocation of the glucose transporter protein, GLUT-4 (**Fig 1.5**). By inhibiting PI3K by use of PI3K inhibitors such as wortmannin and LY294002, or by adenoviral-mediated expression of a dominant-negative mutant of p85, which cannot interact with the p110 catalytic subunit, insulin-stimulated glucose transport and GLUT-4 translocation were blocked in 3T3-L1 adipocytes (Evans *et al.* 1995; Haruta *et al.* 1995; Yeh *et al.* 1995a; Morris *et al.* 1996; Katagiri *et al.* 1997; Gustafson *et al.* 1999). Other signalling proteins activated by PI3K and its lipid products include protein kinase B (PKB), protein kinase C (PKC), and p70 S6 kinase ($p70^{S6K}$), as well as GLUT4, which are discussed below (**Fig 1.5**).

1.5.2.1.4 Protein Kinase B (PKB)/Akt

PKB (also known as Akt) is widely expressed in mammalian tissues and is an important downstream target of PI3K. There are three mammalian isoforms of PKB, which have been identified and these are termed PKBα, PKBβ, and PKBγ, which possess 85% sequence identity and are widely expressed in human tissues. They contain an N-terminal PH domain, a kinase catalytic domain and a C-terminal tail and they belong to the AGC subfamily group of protein kinases (Alessi and Cohen 1998).

PKB provides one of the critical links between upstream signals generated upon activation of insulin receptors and their cellular consequences with all three PKB isoforms being activated in response to insulin or growth factors within a minute. Activation of PI3K leads to the formation of $PI(3,4,5)P_3$ and $PI(3,4)P_2$ and these

secondary messengers play at least three roles in the activation of PKB. The first role is that $PI(3,4,5)P_3$ interaction with PKB is proposed to recruit PKB to the plasma membrane, targeting it to the activating enzymes 3-phosphoinositide-dependent protein kinase (PDK) –1 and PDK2. The second role is to induce a conformational change that makes PKB a good substrate for PDK1 and PDK2. Thirdly it is also likely that $PI(3,4,5)P_3$ interacts with the PH domain of PDK1 and/or PDK2 leading to their activation (Alessi and Cohen 1998; Alessi and Downes 1998; Sorisky 1999) (**Fig 1.5**).

PKB may be involved in glucose uptake by regulating the translocation of the GLUT4 protein to the plasma membrane, which promotes glucose transport. In 3T3-L1 adipocytes, the expression of a constitutively active PKB elicits a high level of glucose transport and GLUT4 translocation in the absence of insulin stimulation (Kohn et al 1996). Constitutively active PKB also triggered the spontaneous differentiation of 3T3-L1 adipocytes (Magun *et al.* 1996). Insulin stimulates the association of PKBβ with GLUT4 vesicles in rat adipocytes resulting in the phosphorylation of several proteins associated with GLUT4 translocation and glucose uptake (Calera *et al.* 1998). Inhibiting PKB activity in 3T3-L1 adipocytes partially blocked insulin-stimulated GLUT4 translocation suggesting that PKB is important in translocation, but other factors may also be involved (Hill *et al.* 1999).

PKB in normal insulin-responsive tissues phosphorylates several proteins that mediate the metabolic actions of insulin. One direct substrate of PKB is glycogen synthase kinase-3 (GSK-3). PKB phosphorylates GSK-3 on a single regulatory serine residue,

causing GSK-3 inhibition (Cross *et al.* 1995), which is thought to contribute to the insulin-induced stimulation of glycogen synthesis by the activation of glycogen synthase. Normally glycogen synthase is inhibited by GSK-3 but by blocking GSK-3, glycogen synthase is activated (**Fig 1.5**).

Insulin promotes protein synthesis in muscle and fat cells by stimulating the initiation and elongation steps in protein translation. PKB modulates the activity of translational components in protein synthesis as constitutively active PKB increases protein synthesis in L6 muscle cells and 3T3-L1 adipocytes (Hajduch *et al.* 1998; Ueki *et al.* 1998). PKB can activate the protein synthesis initiation factor eIF2B to aid in protein synthesis as well as other aspects of the protein synthesis machinery (Alessi and Cohen 1998). PKB has also been implicated in activating $p70^{S6K}$, a protein downstream of PKB which is involved in protein synthesis (Scott *et al.* 1998) (**Fig 1.5**).

Bad, a member of the Bcl-2 gene family that regulates apoptosis is known to be a substrate for PKB. It is believed that PKB phosphorylation of Bad inactivates its pro-apoptotic effects (Sorisky 1999) (**Fig 1.5**).

1.5.2.1.5 Protein Kinase C (PKC) -ξ and -λ

Activation of PKCλ and PKCξ induces insulin-stimulated glucose uptake in human (Bandyopadhyay *et al.* 2002) and rat adipocytes (Standaert *et al.* 1999). Insulin stimulation of PKCλ and PKCξ can be blocked by PI3K inhibitors (Bandyopadhyay

et al. 1997; Standaert *et al.* 1997) and recent reports suggest that the lipid product produced by PI3K, $PI(3,4,5)P_3$ and downstream PDK1 activate PKCλ and PKCξ. By blocking PKB, PKCλ activation of glucose transport is not affected. Therefore PKCλ and PKCξ represent an alternative pathway for activating glucose uptake to PKB (Kotani *et al.* 1998) (**Fig 1.5**).

1.5.2.1.6 GLUT4

Glucose entry into mammalian cells is mediated by a family of 14 facilitative glucose transport proteins referred to as GLUTs. They are integral membrane proteins that possess 12 membrane spanning domains. Many GLUTs are expressed in adipose tissue including GLUT1, GLUT4, GLUT5, GLUT8, GLUT10, and GLUT12 (Wood et al 2003; Wood & Trayhurn 2003; Joost et al 2001) and are involved in the uptake of glucose or in the case of GLUT5, fructose into cells. GLUTs are expressed in different tissues to provide the uptake of nutrients such as glucose. GLUT1 is widely expressed and is found predominantly at the cell surface, and is believed to function in providing the basal requirement of glucose to cells. GLUT4 is the most widely studied glucose transporter in adipose tissue and is involved in insulin-stimulated glucose uptake and for that reason it will be the only transporter that will be discussed in detail. GLUT4 is expressed in insulin-sensitive tissue such as adipose and muscle where it functions in glucose uptake (Cushman and Wardzala 1980; Gustafson *et al.* 1999). Unlike GLUT1, GLUT4 resides in an intracellular compartment referred to as the GLUT4 vesicle and very little GLUT4 is present in the plasma membrane. Insulin

stimulation causes the translocation of the GLUT4 vesicles from the intracellular compartment to the plasma membrane facilitating the uptake of glucose.

GLUT4 knockout mice show insulin resistance with mild impairment of glucose tolerance, growth retardation and decreased adipose tissue (Katz *et al.* 1995). In mice which have selective reduction of GLUT4, there is normal growth and adipose mass but markedly impaired insulin-stimulated glucose uptake in adipocytes. Although GLUT4 expression is preserved in muscle, these mice develop insulin resistance in muscle and liver leading to glucose intolerance and hyperinsulinaemia (Abel *et al.* 2001). Overexpression of GLUT4 in transgenic mice results in increased glucose tolerance with increases in both basal and insulin-stimulated glucose uptake in isolated adipocytes (Shepherd *et al.* 1993). These studies demonstrate the importance of GLUT4 in glucose transport.

Two pathways, a PI3K-dependent and a PI3K-independent pathway exist to mediate insulin-stimulated uptake of glucose by GLUT4 (**Fig 1.5**). The PI3K pathway can activate GLUT4 translocation by PKB, PKCλ and PKCξ. However the exact contribution of these signalling proteins in GLUT4 translocation is unclear. A PI3K-independent pathway exists enabling insulin-stimulated translocation of GLUT4. The protein product of the Cbl protooncogene Cbl (Ribon and Saltiel 1997) is tyrosine phosphorylated by the insulin receptor. Cbl is associated with two adaptor proteins called Cbl-associated adaptor protein (CAP) and associated protein substrate (APS) (Ribon *et al.* 1998; Moodie *et al.* 1999; Liu *et al.* 2002). Upon phosphorylation, the Cbl/CAP complex translocates to lipid raft domains in the plasma membrane. Lipid

rafts are domains within the plasma membrane that are enriched in cholesterol and lipids with saturated acyl chains and specific proteins. Flotillin is a lipid raft protein which associates with CAP and serves to anchor the Cbl/CAP complex in the membrane compartment. Translocation of phosphorylated Cbl recruits the adaptor protein CrkII complexed with the guanyl-exchange protein C3G to the lipid raft (Bickel 2002). Once inside the lipid raft, C3G localises to the lipid raft and activates the G-protein, TC10, by the exchange of GTP for GDP (Chiang *et al.* 2001; Watson *et al.* 2001). Activated TC10 mediates the translocation of GLUT4 by some, yet unknown, mechanism (**Fig 1.5**).

GLUT4 translocation involves the exocytosis of GLUT4 vesicles to the membrane surface. When cells are stimulated by insulin, the levels of exocytosis increase while GLUT4 recycling by endocytosis decreases resulting in a net increase of GLUT4 transporter proteins at the membrane surface to facilitate the uptake of glucose (Holman and Cushman 1994; Pessin *et al.* 1999; Shepherd and Kahn 1999). The process of exocytosis and endocytosis involves small GTP-binding proteins of the Rab family, which act as regulators. Rab4, Rab5 and Rab11 have all been implicated in the endosomal recycling, sorting, and exocytic movement of GLUT4. Rab4 seems to be involved in the translocation of GLUT4 to the membrane surface (Shibata *et al.* 1996), while Rab5 may be involved in the endocytosis of GLUT4 from the cell membrane (Cormont *et al.* 1996). The role of Rab11 is unclear with possible roles in both the endocytosis and exocytosis of GLUT4 (Kessler *et al.* 2000; Zeigerer *et al.* 2002).

Once GLUT4 reaches the cell membrane, fusion of the GLUT4 vesicle and the membrane has to occur. This involves the pairing of protein complexes in the vesicle compartments (v-SNAREs, for vesicle SNAP (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) with cognate receptor complexes on the target membrane (t-SNAREs, for vesicle SNAP (soluble NSF attachment protein receptors) resulting in fusion of the vesicle with the target membrane. In GLUT4 membrane fusion, the identified v-SNARE is predominately vesicle associated membrane protein 2 (VAMP2) (Cain *et al.* 1992; Martin *et al.* 1996), while the predominant t-SNARE is SNAP-23 (St-Denis *et al.* 1999; Kawanishi *et al.* 2000). VAMP2 and SNAP-23 complex together resulting in membrane fusion and GLUT4 is embedded in the membrane surface enabling the uptake of glucose.

1.5.2.1.7 p70 S6 Kinase ($p70^{S6K}$)

The 70 kDa ribosomal S6 kinase ($p70^{S6K}$) is a serine/threonine kinase. Full activation of $p70^{S6K}$ occurs through a series of phosphorylation events on eight or more serine or threonine residues (Pullen and Thomas 1997). These phosphorylation sites occur on the C-terminal autoinhibitory domain but there are also phosphorylation sites on the catalytic domain of $p70^{S6K}$, which are critical for catalytic activity. Autoinhibitory sites are thought to be phosphorylated by members of the MAPK family including p38 and extracellular signal-regulated kinases ERK. Phosphorylation at these sites induces a conformational change that allows phosphorylation of catalytic sites (Berven and Crouch 2000).

There is mounting evidence that PI3K and the subsequent activation of PKB are involved in $p70^{S6K}$ activation. Inhibitors of PI3K block $p70^{S6K}$ activation, while expression of a constitutively active PI3K results in increased basal $p70^{S6K}$ activity (Cheatham *et al.* 1994; Petritsch *et al.* 1995; Weng *et al.* 1995). PKB may also be involved in activating $p70^{S6K}$ through the mammalian target of rapamycin, mTOR, because rapamycin, an inhibitor of mTOR inhibits $p70^{S6K}$ activation (Burgering and Coffer 1995; Sehgal 1995; Scott *et al.* 1998). PDK1 has also been implicated in $p70^{S6K}$ activation as it can directly phosphorylate $p70^{S6K}$ and results in its activation (Alessi and Cohen 1998; Pullen *et al.* 1998). A PI3K-independent pathway which activates $p70^{S6K}$ was demonstrated in Chinese hamster ovary (CHO) cells overexpressing dominant negative PI3K p85 subunit. Activation of $p70^{S6K}$ was still possible even when PI3K activity was inhibited (Hara *et al.* 1995). In L6 muscle cells, $p70^{S6K}$ is activated by insulin even when PI3K and PKB are inhibited by wortmannin (Somwar *et al.* 1998). This suggests several pathways leading to the activation of $p70^{S6K}$ (**Fig 1.5**).

The physiological target of growth factor and insulin-activated $p70^{S6K}$ is the 40S subunit of the S6 ribosomal protein. $p70^{S6K}$ phosphorylates the S6 ribosomal protein, which has been implicated in stimulating the translation of mRNA transcripts that contain a polypyrimidine tract at their 5' transcriptional start site. These transcripts mainly encode ribosomal proteins and protein synthesis elongation factors, thus $p70^{S6K}$ plays an important role in protein synthesis (Alessi and Cohen 1998; Berven and Crouch 2000). Also it has been recently shown that $p70^{S6K}$ plays a key role in the progression of the cell cycle through G_1 to S phase transition (Chou and Blenis 1995).

1.5.2.1.8 Ras

Ras is a small G-protein that acts as a molecular switch to mediate the effects of many growth factors such as insulin. The Ras pathway has been implicated in cellular responses such as proliferation and differentiation (Ceresa and Pessin 1998). The inactive form of Ras is bound by guanosine diphosphate (GDP) and can be switched on when SOS (Son of Sevenless) is brought into proximity with it. SOS stimulates the exchange of GDP for guanosine triphosphate (GTP) on Ras, resulting in Ras activation and downstream signalling (Chardin *et al.* 1993; Li *et al.* 1993). SOS is associated with Grb2, which is a protein containing SH2 domains, and this association is important in allowing mSOS to interact with Ras. Grb2 can be activated by both IRS-1 or by SHC, which are both activated by insulin-bound insulin receptor, thus transmitting the insulin signal (Skolnik *et al.* 1993; Taha and Klip 1999) (**Fig 1.5**).

Ras is involved in proliferation and differentiation and active mutants of Ras lead to uncontrolled cell proliferation and transformation. Activating mutations are found in a variety of human cancers (Cantley *et al.* 1991; Gibbs *et al.* 1994; Downward 2003). Similarly mutant Ras induces adipocyte differentiation, while dominant-negative forms of Ras inhibit differentiation (Benito *et al.* 1991; Porras and Santos 1996). The mechanism by which Ras induces differentiation has yet to be determined but Ras is known to activate another protein called Raf, which results in a cascade of kinase activations including MAP/ERK Kinase (MEK) (Taha and Klip 1999) (**Fig 1.5**).

1.5.2.1.9 Mitogen-Activated Protein Kinase (MAPK)

At present there are at least three MAPK pathways, which are defined according to the MAPK that is activated. These are the ERK pathway, c-jun N-terminal/stress-activated protein kinase (JNK/SAPK) pathway, and the p38 MAPK pathway. This allows a diverse number of stimuli such as growth factors, cytokines, stress stimuli, as well as insulin to be coupled to the MAPK signalling cascade.

1.5.2.1.9.1 p42/44 Mitogen-Activated Kinase (p42/44 MAPK)

p42/44 MAPK, also referred to as ERK1 (p44 MAPK) and ERK2 (p42 MAPK) are members of the MAPK family, which consists of a 41 kDa and 43 kDa protein. They share an 85% homology and are ubiquitously expressed in many tissues (Pearson *et al.* 2001).

MAPK are regulated by phosphorylation cascades. Two upstream protein kinases are activated in series leading to the activation of MAPK. In all currently known MAPK cascades, the kinase immediately upstream of the MAPK is a member of the MAP/ERK kinase (MEK or MKK (MAP kinase kinase)) family. These are dual specificity enzymes that can phosphorylate hydroxyl side chains of serine/threonine and tyrosine residues in their MAPK substrates leading to dual phosphorylation. MEKs are also activated by phosphorylation of two residues, either serine or threonine by MEK kinases (MEKK). MEKKs are many and diverse, which explains why many stimuli can activate the MAPK pathway (Pearson *et al.* 2001).

p42/44 MAPKs are stimulated by many ligand-activated receptors and other perturbations, with some cell specificity. Insulin is known to activate p42/44 MAPK in a wide variety of cells e.g. 3T3-L1 cells (Porras *et al.* 1994), rat lymphocytes (Pawelczyk *et al.* 2003), L6 myotubes (Tsakiridis *et al.* 2001) *et al.* Factors such as serum, growth factors, cytokines, stresses and others are able to activate p42/44 MAPK. For example, p42/44 MAPK is activated by oestrogen in rat adipocytes (Dos Santos *et al.* 2002), while in vascular smooth muscle cells, oxygen radicals can activate p42/44 MAPK (Baas and Berk 1995). Activators of PPAR-γ such as thiazolidinediones were found to activate p42/44 MAPK in vascular smooth muscle cells (Takeda *et al.* 2001), and leptin can activate p42/44 MAPK in rat preadipocytes and human peripheral blood mononuclear cells (Machinal-Quelin *et al.* 2002a; Najib and Sanchez-Margalet 2002).

The most well characterised signalling pathway from the cell membrane to p42/44 MAPK is that used by receptor tyrosine kinases such as insulin. As mentioned previously, insulin binding to its receptor results in the activation of IRS-1 and SHC, which interact with Grb2, which then activates Ras. Ras activates a MEKK called Raf by recruiting Raf to the plasma membrane and phosphorylating Raf (Stokoe *et al.* 1994; Pearson *et al.* 2001). Raf can now activate MEK1 and MEK2, which are dual specificity kinases. MEK1/2 phosphorylates p42/44 MAPK on tyrosine and threonine residues thus activating p42/44 MAPK (Pearson *et al.* 2001) (**Fig 1.5**).

p42/44 MAPK has a number of roles in mammalian cells including proliferation, survival, motility and differentiation. p42/44 MAPK phosphorylates a wide range of substrates leading to their activation. p42/44 MAPK are known to phosphorylate substrates in the nucleus such as transcription factors involved in the cell cycle. For example p42/44 MAPK can regulate a number of transcription factors such as c-Fos (Chen *et al.* 1993), and the ternary complex factor $p62^{TCF}$ (Gille *et al.* 1992), leading to changes in gene expression. In addition, p42/44 MAPK and its downstream kinases are implicated in eliciting rapidly targeted alterations in the chromatin environment of specific genes by modulating the phosphorylation and/or methylation of nucleosomal and chromatin proteins such as histone H3 (Sassone-Corsi *et al.* 1999) and the nucleosome-bound high-mobility group protein HMG-14 (Thomson *et al.* 1999). This could result in increased transcription factor accessibility to DNA binding sites (Thomson *et al.* 1999). Insulin action, which causes activation of the p42/44 MAPK cascade for example, results in the activation of enzymes such as p90 ribosomal S6 kinase (RSK, or $p90^{RSK}$). This cascade results in regulation of kinases that may lead to changes in nuclear protein phosphorylation, leading to changes in expression of other insulin-dependent enzymes regulated at the transcriptional level but the exact role of RSK has not yet been elucidated (Wang *et al.* 2001).

1.5.2.1.9.2 p38 Mitogen-Activated Kinase (p38 MAPK)

The p38 MAPKs are stress-regulated protein kinases related to JNK/SAPK and part of the superfamily of MAPKs. Mammalian p38 mitogen-activated protein kinase (p38 MAPK) was originally identified in macrophages that were stimulated with

lipopolysacharides (LPS) (Han *et al.* 1994). This isoform of p38 MAPK is called p38α MAPK and is a 38 kDa protein which is rapidly tyrosine phosphorylated when stimulated. Three other isoforms of p38 MAPK have been identified which are p38β MAPK (Jiang *et al.* 1996), p38δ MAPK (Jiang *et al.* 1997), and p38γ MAPK (Lechner *et al.* 1996). The p38α and p38β are ubiquitously expressed while p38γ and p38δ differentially expressed in different tissues (Jiang *et al.* 1996; Lechner *et al.* 1996; Li *et al.* 1996).

Diverse extracellular stimuli including irradiation, ultraviolet (UV) light, heat shock, high osmotic stress, proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), and certain mitogens have been shown to activate p38 MAPK (Ono and Han 2000). For example, p38 MAPK activation has been observed in many inflammatory responses such as LPS-treated macrophages (Han *et al.* 1994), TNF-α-stimulated endothelial cells (Pietersma *et al.* 1997), interleukin-17 stimulated chondrocytes (Shalom-Barak *et al.* 1998), and in human platelets stimulated with thrombin (Saklatvala *et al.* 1996). Growth factors such as fibroblast growth factor (FGF) (Tan *et al.* 1996) and vascular endothelial growth factor (VEGF) (Rousseau *et al.* 1997), as well as insulin can activate p38 MAPK. Activation of p38 MAPK is also dependent on cell type. For example, insulin can stimulate p38 MAPK in 3T3-L1 adipocytes (Sweeney *et al.* 1999), but downregulates p38 MAPK in chick forebrain neuron cells (Heidenreich and Kummer 1996). Most of the research done has been on p38α MAPK activation while little work has been done on the activation of the other isoforms of p38 MAPK in different cells under different conditions.

Like all MAPKs, p38 MAPK is activated by dual kinases, the MKKs. These phosphorylate and activate p38 MAPK on threonine/tyrosine residues and include MKK3 and MKK6 (Raingeaud *et al.* 1996). There are many more MKKS and other upstream activators of p38 MAPK, which explains why p38 MAPK can be activated by a diverse number of stimuli (Ono and Han 2000).

p38 MAPK is involved in a number of biological responses including inflammation, stress, apoptosis, differentiation, and proliferation. The p38 MAPK pathway controls the activity of multiple transcription factors and the expression of many genes such as SRF accessory protein 1 (Sap1), p53, and many more (Ono and Han 2000). For example, p38 MAPK can control transcription factors of the C/EBP family. C/EBP-homologous protein 10 (CHOP 10) (growth arrest and DNA damage inducible gene 153, or GADD153) is a member of the C/EBP family and is known to be involved in the regulation of cell growth and differentiation (Wang and Ron 1996), while C/EBPβ is also a substrate of p38 MAPK and may play a role in 3T3-L1 adipocyte differentiation (Engelman *et al.* 1998). Some of these are direct substrates of p38 MAPK, while others are phosphorylated by downstream protein kinases. The p38 MAPK pathway is important in the generation of cellular responses to certain cytokines. TNF-α can activate p38 MAPK (Suzuki *et al.* 1999), but p38 MAPK has been shown to regulate the production of TNF-α and other cytokines and therefore play an important role in inflammation (Lee *et al.* 1994). In addition, p38 MAPK is shown to be involved in apoptosis in human FS-4 fibroblasts, where inhibition of p38 MAPK activity resulted in the prevention of apoptosis (Schwenger *et al.* 1997). p38 MAPK is involved in differentiation, observed in C2C12 myotube differentiation

(Zetser *et al.* 1999), while the differentiation of PC12 cells into neurons also requires the p38 MAPK pathway (Morooka and Nishida 1998). The p38 MAPK pathway may be important in GLUT4 activation leading to the uptake of glucose by insulin stimulation in 3T3-L1 adipocytes and L6 myotubes as inhibiting p38 MAPK, does not prevent GLUT4 translocation but does prevent its activity (Sweeney *et al.* 1999).

1.5.2.1.9.3 c-jun N-terminal/stress-activated protein kinase (JNK/SAPK)

JNK/SAPK is a subgroup of MAPK which consists of a 46 kDa and 54 kDa isoform. JNK/SAPK are activated by UV light, antibiotics, cytokines, and other environmental stresses. JNK/SAPKs are important for cytokine biosynthesis and are involved in cell transformation, stress responses and apoptosis (English *et al.* 1999).

JNK/SAPK has been implicated in promoting insulin resistance and obesity. TNF-α induces insulin resistance and a possible mechanism for this is by the activation of JNK/SAPK. JNK/SAPK inhibits IRS-1 by phosphorylating the serine 307 residue, causing the inactivation of the insulin signalling pathway leading to insulin resistance (Aguirre *et al.* 2000; Hirosumi *et al.* 2002).

1.5.2.2 Glucocorticoids

The differentiation of many adipocyte cell lines requires the presence of a glucocorticoid (**Table 1.2**). Glucocorticoids are lipophilic hormones which include

cortisol and the synthetic glucocorticoid, dexamethasone. Glucocorticoids have been shown to induce the C/EBP transcription factors, which play a major role in adipocyte differentiation (Wu *et al.* 1996), and they exert stimulatory effects on a number of genes involved in adipogenesis such as LPL and G3PDH (Hauner *et al.* 1989). Dexamethasone can inhibit TNF-α induced apoptosis of adipocytes promoting adipocyte mass (Zhang *et al.* 2001), and inhibition of pref-1 mRNA transcripts by dexamethasone induces adipogenesis (Smas *et al.* 1999).

Glucocorticoids belong to the nuclear hormone superfamily and exert their effects through binding to the intracellular glucocorticoid receptor (GR). The GR is complexed with other proteins, such as heat shock proteins, in the inactive state (Sanchez *et al.* 1990). As glucocorticoids are lipophilic hormones, they can pass through the plasma membrane and bind to the GR causing the dissociation of the heat shock proteins. Bound GR homodimerises with another active GR and the complex translocates into the nucleus where it binds via its DNA-binding domain regions to specific sites on genomic DNA called glucocorticoid response elements (GREs) (Tsai and O'Malley 1994). Binding by the receptor-ligand complex to the DNA upstream from transcription initiation sites stimulate or repress gene transcription allowing glucocorticoids to have a major role in controlling many genes containing GREs.

Glucocorticoids play an important role in inducing obesity. Glucocorticoids increase leptin secretion in adipose tissue (De Vos *et al.* 1995; Slieker *et al.* 1996) but inhibit the central effects of leptin on food intake and body weight regulation favouring the effects of NPY which is normally inhibited by leptin. NPY promotes food intake and

decreases energy expenditure (Wang *et al.* 1997), so elevated levels of cortisol which occur in conditions like Cushing's syndrome are associated with the development of central obesity as well as having direct effects on adipocytes (Lamberts and Birkenhager 1976; Leal-Cerro *et al.* 2001).

1.5.2.3 Triiodothyronine (T3)

Thyroid hormones such as T3 influence many metabolic pathways. They are known to increase basal energy expenditure acting on protein, carbohydrate, and lipid metabolism in many tissues such as adipose, liver, and skeletal muscle (Freake and Oppenheimer 1995). Thyroid hormones are thought to be involved in lipolysis. Hyperthyroidism results in enhanced lipolysis, while in hypothyroidism, lipolysis is decreased possibly through reductions in β-adrenergic receptor density and postreceptor events as demonstrated in both human (Wahrenberg *et al.* 1994) and murine adipocytes (Germack *et al.* 2000). Hyperthyroidism also results in adipose tissue hyperplasia, concomitant with cell size reduction, and hypothyroidism induces opposite effects (Levacher *et al.* 1984). Hypothyroidism results in decreased food intake and a reduction in basal metabolic rate as well as an increase in body weight while the opposite is true for hyperthyroidism (Krotkiewski 2000).

T3 has been shown to be important in the differentiation of Ob17/Ob1771 preadipocyte cell line and in suppressing mitogenesis in ob17 preadipocytes (Gharbi-Chihi *et al.* 1981; Darimont *et al.* 1993). However the requirement of T3 in adipocyte differentiation may be species specific. For example, in pig preadipocytes, T3 is not

required for differentiation (Suryawan *et al.* 1997), but in human preadipocyte differentiation, T3 is usually included (Hauner *et al.* 1989) (**Table 1.2**). T3 has been shown to stimulate glucose transport in 3T3-L1 adipocytes, possibly by increasing GLUT1 and GLUT4 proteins. However studies in primary rat adipocytes show contradictory results with decreased glucose uptake. This could be due to species differences or due to the fact that 3T3-L1 cells are a clonal cell line, while this study used primary rat adipocytes (Goto *et al.* 1997).

Like glucocorticoids, T3 is a member of the nuclear hormone superfamily and exerts its effects through two isoforms of the thyroid hormone receptors (TR), TRα1 and TRβ1. TRs heterodimerise with the retinoid X receptor (RXR) and are bound to thyroid hormone response elements (TRE) on DNA. TR-RXR heterodimers in the absence of T3 are bound by a number of proteins, which make up the corepressor complex, and together with TR-RXR bind TREs repressing or silencing basal target gene expression. When the TR-RXR heterodimer is bound by T3, the corepressor complex is replaced by a coactivator complex allowing the target gene to be activated and transcribed. Many genes involved in signal transduction, lipid metabolism, apoptosis and inflammatory responses including ADD1/SREBP1are controlled by T3 leading to its effects in adipose and other tissues (Harvey and Williams 2002; Viguerie *et al.* 2002).

1.5.2.4 cyclic 3'5'-Adenosine Monophosphate (cAMP)

IBMX accelerates the differentiation of many preadipocyte cell lines and is used in all differentiation protocols (**Table 1.2**). IBMX inhibits cAMP phosphodiesterases and blocks inhibitory A1 adenosine receptors in a competitive manner resulting in an increase in cAMP (Parsons *et al.* 1988). Other agents such as forskolin, which increase cAMP, can replace IBMX in the differentiation protocol (Yarwood *et al.* 1995), however not all adipose cell culture models require the increase in cAMP by forskolin or IBMX as shown in porcine adipocytes where both forskolin and IBMX had no effect on differentiation (Boone *et al.* 1999). This implies that mechanisms regulating adipocyte differentiation may differ among species.

cAMP acts as a secondary messenger and is able to activate a large number of genes. The cellular responses of cAMP are mediated through PKA. In the basal state, PKA resides in the cytoplasm as an inactive heterotetramer comprising of two paired regulatory (R) and catalytic (C) subunits. Induction of cAMP liberates the C-subunits, which diffuse into the nucleus and phosphorylate cAMP response element binding protein (CREB). CREB forms a heterodimer with CREB-binding protein (CBP), which acts as a coactivator and this binds DNA-binding domain regions to specific sites on genomic DNA called cAMP response elements (CRE). This allows activation of the target gene by the initiation of transcription. Elevations in cAMP ultimately activate genes which contain CRE within them (Mayr and Montminy 2001).

cAMP increases the expression of C/EBPβ and this is required for the subsequent activation of PPARγ and adipocyte differentiation (Tae *et al.* 1995). cAMP can inhibit TNF-α effects in preadipocytes, which could explain why it is adipogenic as it can suppress autocrine TNF-α, which is inhibitory to adipogenesis (Hube *et al.* 1999b). In a study by Reusch *et al* (2000) in 3T3-L1 cells, CREB activation induces differentiation and a number of adipocyte-specific genes were shown to contain CRE, implicating cAMP in adipogenesis (Reusch *et al.* 2000).

1.6 The Adipocyte as an Endocrine Organ; The Role of Autocrine and Paracrine Factors on Regulation of Adipocyte Growth and Function

Adipocytes are highly specialised cells that play a critical role in energy regulation and homeostasis. However recent evidence indicates that the adipocyte is an endocrine cell which can influence other cells, including the brain, liver and muscle, as well as itself. Adipocytes secrete a variety of factors such as leptin, adiponectin, TNF-α, angiotensinogen, resistin, adipsin, agouti, *et al* which can act in a paracrine and/or autocrine manner (**Fig 1.6**). Leptin is a known late marker of adipocyte differentiation, while TNF-α is important in adipocyte lipid metabolism. Adipose tissue secretes a number of proteins such as FGFs, VEGFs and angiopoietins, which mediate the angiogenesis, required for adipocyte growth and differentiation. Whether these secreted factors could have autocrine roles in human adipocytes has yet to be determined.

1.6.1 Autocrine Factors

1.6.1.1 Leptin

Leptin is a cytokine-like protein produced by the adipocyte, which has an autocrine effect. Leptin is the protein product of the *ob* gene, which was discovered by positional cloning and shown to be localised to chromosome 6 in mice and 7q31.3 in humans (Zhang *et al.* 1994; Isse *et al.* 1995). The human *ob* gene encodes an mRNA of 4.5 kilobases (kb) that is translated as a 167 amino acid product with an amino-terminal secretory signal sequence of 21 amino acids. This is cleaved and leptin circulates as a protein of 16 kDa (Zhang *et al.* 1994; Masuzaki *et al.* 1995). The tertiary structure of leptin suggests it is a globular protein which consists of four antiparallel α-helices and two short β-sheets held together by a single disulfide bond between cysteine-96 and cysteine-146 (Madej *et al.* 1995; Zhang *et al.* 1997b). Leptin is highly expressed in adipose tissue (Masuzaki *et al.* 1995), but has been detected in the placenta (Masuzaki *et al.* 1997), gastric fundic mucosa (Bado *et al.* 1998), skeletal muscle (Wang *et al.* 1998), and mammary epithelium (Casabiell *et al.* 1997).

The leptin receptor gene (OB-R) belongs to the class I cytokine receptor family and is distributed in many tissues throughout the body such as adipose, hypothalamus, liver, pancreas, kidneys, spleen, and heart. OB-R are produced in several alternatively spliced forms, designated OB-Ra, OB-Rb, OB-Rc, OB-Rd, and OB-Re with OB-Rb and OB-Ra being the major forms (Tartaglia 1997). The receptors share an identical

extracellular ligand-binding domain of 840 amino acids at the amino terminus, as well as a transmembrane domain of 34 amino acids but they vary at the carboxy terminus. A variable intracellular domain, characteristic for each of the five receptor isoforms has been identified which contains sequences for binding of Janus kinase (JAK). Activation of OB-R by leptin induces activation of both JAK and signal transducer and activator of transcription (STAT) proteins which are able to activate specific gene transcription in the nucleus (Ghilardi *et al.* 1996; Vaisse *et al.* 1996).

Plasma leptin concentrations and mRNA levels are strongly correlated with estimates of obesity, total fat mass, percentage body fat and BMI. Mutations in the ob gene and the gene for OB-R result in obese mice referred to as *ob/ob* mice and *db/db* mice respectively. Addition of exogenous leptin to the *ob/ob* mouse model reverses the obese phenotype (Pelleymounter *et al.* 1995; Chen *et al.* 1996) implying that leptin is involved in obesity and regulating body weight. In obese individuals, levels of plasma leptin and *ob* mRNA are increased, while they decrease with weight loss (Maffei *et al.* 1995). Consistent with this and the animal stuides, leptin-deficient (Strobel *et al.* 1998) and leptin-receptor-deficient (Lahlou *et al.* 2002) human subjects are obese. There is a reversal of the obese phenotype in leptin-deficient human subjects with recombinant leptin administration (Farooqi *et al.* 1999). Glucose infusion and lipid infusion increase leptin expression in adipose tissue and muscle (Wang *et al.* 1998). Adipocyte size is an important determinant of leptin synthesis, as larger adipocytes contain more leptin than smaller adipocytes (Chen *et al.* 1997; Ahima and Flier 2000; Couillard *et al.* 2000) and levels of leptin increase during preadipocyte differentiation with leptin being a late marker for differentiation (Chen

et al. 1997; Zhang *et al.* 2000). Leptin levels are higher in subcutaneous adipocytes than omental preadipocytes and there is higher expression of leptin in women over men (Montague *et al.* 1997; Zhang *et al.* 1999). All this implies that leptin plays a role in fuel homeostasis and is acting as a sensor of nutrient flux in tissues.

Leptin exerts its major effects on body weight by affecting the expression of many hypothalamic neuropeptides, thus the hypothalamus is the primary site for the action of leptin on body weight (Flier and Maratos-Flier 1998). The effects of leptin are mediated at least in part by NPY, a potent stimulator of food intake (Wang *et al.* 1997). Leptin inhibits NPY leading to a signal to inhibit food intake and increases in energy expenditure (Schwartz *et al.* 1996). However NPY is not the sole regulator of body composition as the administration of leptin to NPY-knockout mice also inhibited food intake (Erickson *et al.* 1996). In addition to NPY, leptin modulates the levels of several other neuropeptides that control food intake, including corticotrophin-releasing hormone, galanin melanin-concentrating hormone and pro-opiomelanocortins (Flier and Maratos-Flier 1998).

Leptin has been shown to have an effect on the lipolytic activity of adipocytes (Fruhbeck *et al.* 1997). Leptin has been shown to repress acetyl-CoA carboxylase gene expression, insulin-mediated glucose uptake, fatty acid synthesis and lipid synthesis thus inhibiting lipogenesis (Shimabukuro *et al.* 1997; Wang *et al.* 1998; Zhang *et al.* 1999). Therefore leptin is directly regulating adipose tissue metabolism by stimulating lipolysis and inhibiting lipogenesis.

Insulin has been shown to stimulate leptin expression. In rats, injection of insulin, and the addition of leptin to isolated rat adipocytes resulted in an increase of *ob* mRNA (Saladin *et al.* 1995). In humans, insulin stimulation of leptin occurs but it is more of a chronic response rather than an acute effect on leptin both *in vitro* and *in vivo* (Kolaczynski *et al.* 1996). Expression of leptin is also regulated by glucocorticoids. Addition of hydrocortisone or dexamethasone increased *ob* mRNA in rat adipose tissue (De Vos *et al.* 1995), and incubation of isolated rat adipocytes with dexamethasone resulted in increased leptin release by increased *ob* mRNA expression (Slieker *et al.* 1996). Thiazolidinediones such as troglitazone decreased *ob* mRNA in both human and murine adipocytes (Nolan *et al.* 1996; Zhang *et al.* 1996b) as do β-adrenergic receptor agonists and agents which increase intracellular cAMP (Slieker *et al.* 1996) while oestrogen has been shown to increase *ob* mRNA and leptin secretion (Machinal-Quelin *et al.* 2002b).

Leptin is implicated in insulin signalling and possibly the induction of insulin resistance (Fruhbeck *et al.* 1997). Leptin can act through some components of the insulin signalling cascade such as IRS-1 and IRS-2, PI3K and ERK, and can modify insulin-induced changes in gene expression *in vitro* and *in vivo* (Kim *et al.* 2000; Szanto and Kahn 2000). In the rat hepatoma cell line, Fao, leptin pre-treatment enhances insulin-induced tyrosine phosphorylation and PI3K binding to IRS-1, while inhibiting tyrosine phosphorylation and PI3K binding to IRS-2. Leptin induces serine phosphorylation of Akt and glycogen synthase 3 (Szanto and Kahn 2000).

Leptin has diverse effects in addition to appetite and body weight regulation. It has been shown to be involved in reproduction, haematopoiesis, angiogenesis, and immune responsiveness. In leptin deficient *ob/ob* mice, administration of leptin restores puberty and fertility and it accelerates puberty in wild-type mice facilitating the reproductive behaviour of rodents (Chehab *et al.* 1996). Functional OB-R is capable of signalling for cell survival, proliferation, and differentiation in macrophages (Cioffi *et al.* 1996; Gainsford *et al.* 1996), while leptin has a direct proliferative effect on T cells (Lord *et al.* 1998). Leptin can be angiogenic as it causes cultured endothelial cells to aggregate and form tubes similar to tissue vasculature (Bouloumie *et al.* 1998; Sierra-Honigmann *et al.* 1998). Leptin also accelerates wound healing which is a process depending on blood vessel growth (Sierra-Honigmann *et al.* 1998).

1.6.1.2 Tumour Necrosis Factor α (TNF-α)

TNF-α is a cytokine which plays an important role in lipid metabolism in the adipocyte. Studies have shown that TNF-α reduces FFA uptake, inhibits lipogenesis, and stimulates lipolysis. TNF-α can inhibit the activity and protein expression of LPL, a protein involved in the uptake of FFA and lipogenesis both *in vitro* (Cornelius *et al.* 1988; Hauner *et al.* 1995a) and *in vivo* (Semb *et al.* 1987). TNF-α also decreases expression of FFA transporters such as fatty acid transport protein (FATP) and FAT in adipose tissue (Memon *et al.* 1998). TNF-α therefore decreases the uptake of FFAs from circulation which possibly contributes to hyperlipidaemia

observed in obesity. Expression of enzymes such as acetyl-CoA carboxylase (Pape and Kim 1988) and fatty acid synthase (Doerrler *et al.* 1994), which are involved in lipogenesis, may be decreased by TNF-α. TNF-α can stimulate lipolysis mediated by its receptor TNF Receptor 1 (TNFR1), but the mechanism by which this occurs is not very well understood (Kawakami *et al.* 1987; Green *et al.* 1994; Hauner *et al.* 1995a; Lopez-Soriano *et al.* 1997; Sethi *et al.* 2000). Activation of the ERK pathway and elevation of cAMP are two possible pathways leading to increased lipolysis (Zhang *et al.* 2002).

TNF-α was first identified as a macrophage product implicated in the metabolic disturbances of chronic inflammation and malignancy. It has a wide range of biological functions including control of gene expression, induction of apoptosis, and regulation of cell growth and differentiation. The cytokine is synthesised as a 25 kDa transmembrane pro-hormone which undergoes proteolytic cleavage to yield the mature 17 kDa soluble TNF-α form (Jue *et al.* 1990). TNF-α was first shown to be expressed in adipose tissue in rodent models of obesity (Hotamisligil *et al.* 1993). Clinical studies have shown TNF-α to be expressed in humans with higher expression of TNF-α in subcutaneous fat depots than omental fat depots (Hotamisligil *et al.* 1995; Kern *et al.* 1995; Hube *et al.* 1999a).

The cellular actions of TNF-α are attributed to the activities of two distinct receptors: TNFR1, a 55 or 60 kDa peptide in rodents and humans respectively (Loetscher *et al.* 1990) and TNF receptor 2 (TNFR2), a 75 or 80 kDa peptide in rodents and humans respectively (Smith *et al.* 1990). Binding of TNF-α to TNFR1 and TNFR2, leads to

ligand-induced receptor trimerisation and subsequent signalling cascades (Tartaglia and Goeddel 1992). The extracellular domains of these two receptors exhibit some sequence homology, while the intracellular domains appear to be quite dissimilar, suggesting that the receptors utilise distinct signalling pathways (Tartaglia *et al.* 1991). Both receptors activate multiple kinases and phosphoprotein phosphatases and can utilise all major transduction pathways (Vilcek and Lee 1991). Activation of TNFR1 recruits the adaptor protein, TNFR1-associated death domain protein (TRADD) which in turn recruits two downstream signal transducers: Fas-associated death domain protein (FADD), and TNFR-associated factor 2 (TRAF2) (Hsu *et al.* 1996). FADD interacts directly with the apoptotic protease FADD-like interleukin-1β converting enzyme (FLICE) to trigger apoptosis. TRAF2 activates two separate pathways and appears to be essential for mediation of survival. In the first pathway, nuclear factor κB (NFκB) is activated by NFκB inducing kinase (NIK) which phosphorylates NFκB inhibitory subunit (IκBA) causing its degradation and allows NFκB to translocate to the nucleus and promote survival (Malinin *et al.* 1997). The serine/threonine kinase receptor-interacting protein (RIP) is also recruited by TRAF2 and can activate NFκB (Kelliher *et al.* 1998). The second pathway, independent of NIK, results in the activation of JNK/SAPK which in turn activates transcription factors such as c-Jun and mediates the induction of activated protein complex-1 (AP-1) (Natoli *et al.* 1997). Both NFκB and AP-1 mediate the induction of cytokines and inflammatory responses. Recent studies have shown that TNFR1 associates with MAPK death domain protein (MADD) which activates p42/44 MAPK and is another pathway enabling TNF-α to elicit its effects (Schievella *et al.* 1997). TNFR1 is involved primarily in insulin receptor signalling and glucose transport by inhibiting

insulin receptor and IRS-1 tyrosine phosphorylation preventing their activation (Peraldi *et al.* 1996), while TNFR2 is involved in the pathogenesis of TNF-induced insulin resistance possibly through interacting with the insulin signalling pathway (Hotamisligil *et al.* 1995; Liu *et al.* 1998).

Elevated levels of TNF-α have been postulated to induce insulin resistance in a variety of disease states including cancer (McCall *et al.* 1992), sepsis, trauma (Shangraw *et al.* 1989), and obesity (Hotamisligil *et al.* 1995). This has been observed in healthy individuals, and in isolated cells exposed to TNF-α, insulin resistance is induced (Hotamisligil *et al.* 1994; Hauner *et al.* 1995a). In obesity-related insulin resistance and rodent models of obesity, TNF-α is overexpressed (Hotamisligil *et al.* 1993; Yamakawa *et al.* 1995), and levels of TNF-α expression strongly correlate with hyperinsulinaemia and decreased insulin sensitivity. Furthermore, dietary and chemical treatment of obesity results in improved insulin sensitivity and decreased TNF-α levels (Hotamisligil *et al.* 1993; Dandona *et al.* 1998). Administration of thiazolidinediones to obese, insulin-resistant mice results in improved insulin sensitivity and decreased TNF-α expression in fat (Hofmann *et al.* 1994). One possible mechanism for TNF-α involvement in insulin resistance is by inhibition of the insulin receptor thus blocking its kinase activity by altering IRS-1 (Hotamisligil *et al.* 1996). This results in the inhibition of downstream insulin signalling pathways, which may lead to insulin resistance. Insulin-stimulated glucose uptake has been shown to be inhibited by the downregulation of GLUT4 gene expression by TNF-α (Hauner *et al.* 1995a). In addition to adipocytes, TNF-α-induced insulin resistance is not limited to adipocytes and has been demonstrated in a

number of cell types including muscle cells (Storz *et al.* 1998) and hepatocytes (Feinstein *et al.* 1993).

TNF-α has been shown to be a potent inhibitor of adipocyte differentiation (Petruschke and Hauner 1993) at least in part by its inhibitory effects on PPARγ and C/EBPα which are master regulators of adipocyte differentiation (Stephens *et al.* 1997; Xing *et al.* 1997). This suppression may result in the downregulation of many adipocyte specific genes downstream, such as LPL (Hauner *et al.* 1995a), GLUT4 (Hauner *et al.* 1995a), and leptin (Zhang *et al.* 2000). Treatment of differentiated preadipocytes with TNF-α results in delipidation and the reversion of cells to a fibroblast-like morphology (Petruschke and Hauner 1993; Zhang *et al.* 2000). TNF-α has also been implicated in apoptosis of adipocytes (Prins *et al.* 1994; Prins *et al.* 1997; Zhang *et al.* 2001). This implies that TNF-α plays an important role in the regulation of adipose tissue mass.

1.6.1.3 Fibroblast Growth Factors (FGFs)

FGFs are a large family of small polypeptide growth factors expressed in invertebrates and vertebrates. FGFs are secreted by cells of the mesenchyme including preadipocytes but there are relatively few studies examining the role of FGFs in the adipocyte. In vertebrates, at least 23 members of the FGF family have been identified sharing a common sequence homology, which ranges from a 13-71% shared amino acid identity, and range in molecular mass from 17-34 kDa (Ornitz and

Itoh 2001). The defining features of the FGF family include a strong affinity for heparin and heparin-like glycosaminoglycans (HLGAGs) of the ECM, as well as a central core of 140 amino acids that is highly homologous between different family members. This central core folds into 12 antiparallel β-strands that together form a cylindrical barrel closed by the more variable amino- and carboxy-terminal stretches (Ago *et al.* 1991; Zhang *et al.* 1991; Powers *et al.* 2000).

In mammals, the members of the FGF family are differentially expressed in most tissues, but the pattern and timing of expression vary. Most FGFs (FGFs 3-8, 10, 15, 17-19, 21-23) have amino-terminal signal peptides and are readily secreted from cells. FGF 9, 16, and 20 lack this amino terminal signal sequence but are still secreted (Miyamoto *et al.* 1993; Miyake *et al.* 1998; Ohmachi *et al.* 2000), while FGF 11-14 lack these signal sequences and are thought to remain intracellular (Smallwood *et al.* 1996; Yamamoto *et al.* 1998; Munoz-Sanjuan *et al.* 2000; Wang *et al.* 2000). FGF 1 and FGF 2 are found on the cell surface and within the ECM but lack the classical signal sequence directing secretion. They may be released from damaged cells or by an exocytotic mechanism independent of the endoplasmic-reticulum-Golgi pathway (Mignatti *et al.* 1992; Ornitz and Itoh 2001).

FGFs bind to specific RTKs called fibroblast growth factor receptors (FGFR) (**Fig 1.7**). Four high affinity tyrosine kinase receptors have been characterised (FGFR 1-4) which bind FGF. They consist of three components, an extracellular section that comprises three domains (immunoglobulin (Ig) domains I, II, and III), a transmembrane section, and an intracellular domain that contains the tyrosine kinase.

Multiple isoforms of the receptor also arise as a result of alternative mRNA splicing of the FGFR gene leading to ligand-binding specificity due to differences in the extracellular domains in FGFR1-4 (Miki *et al.* 1992; Chellaiah *et al.* 1994; Ornitz and Itoh 2001). FGFRs are distributed in many tissues and have a temporal and spatial expression of receptors. This allows many different FGFs to signal through FGFRs and elicit different effects.

FGF signalling involves the binding of FGF to its receptor leading to receptor dimerisation and tyrosine autophosphorylation. Many models have been proposed to explain FGF signalling (Pantoliano *et al.* 1994; Spivak-Kroizman *et al.* 1994). The most recent models (Plotnikov *et al.* 1999; Venkataraman *et al.* 1999; Stauber *et al.* 2000) take into account the essential HLGAG-FGFR interaction required for receptor dimerisation which these previous models did not (Kan *et al.* 1993). In these models, FGF binds to FGFR, which dimerises with another FGF-bound FGFR. This complex is stabilised by HLGAG binding across the FGF-bound FGFR dimer complex. Autophosphorylation of the intracellular domain occurs by the tyrosine kinases and these phosphorylated tyrosines activate target enzymes such as phospholipase C-γ (PLCγ), FRS2, PKC, Src, Grb2, SHC and Crk leading to the activation of many signalling pathways which facilitate the actions of FGF (**Fig 1.7**) (Boilly *et al.* 2000; Powers *et al.* 2000). For example FGF can activate the p42/44 MAPK signalling pathway in a variety of cells including 3T3-L1 preadipocytes (Prusty *et al.* 2002), rat intestine (Fu *et al.* 2003), and human endometrial carcinoma cells (Taniguchi *et al.* 2003).

FGF-2 (basic FGF (bFGF)) is one of the most widely studied FGFs. It has numerous effects in different cell and organ systems. It is a potent angiogenic molecule *in vivo* and *in vitro* stimulating smooth muscle growth, wound healing, and tissue repair. In addition it may play a role in haematopoiesis and in the differentiation and function of the nervous system, the eye, and the skeleton (Bikfalvi *et al.* 1997).

FGF-2 was first identified as an 18 kDa heparin-binding protein (Bohlen *et al.* 1984). However alternative isoforms with higher molecular weights of 22, 22.5, 24, and 34 kDa have also been identified (Bikfalvi *et al.* 1995). The 18 kDa form of FGF-2 is found mainly in the cytosol while the high molecular weight forms of FGF-2 are found in the nucleus and are non-migratory mitogens being inducers of anchorage dependent growth and growth in low serum (Bikfalvi *et al.* 1995). These different isoforms may enable FGF-2 to elicit its varied effects in different tissues.

FGF-2 mRNA has been found in omental human adipose tissue and this was decreased in differentiation however the protein product identified in these studies was abnormally large (66 kDa) for bona fide FGF-2 (18 kDa) (Teichert-Kuliszewska *et al.* 1992; Teichert-Kuliszewska *et al.* 1994). Recent work using quantitative PCR to examine mRNA levels in adipose depots in obese men have described the existence of several FGF family members i.e. FGF 1, 2, 7, 9, 10, 18, and FGFR1 but the cell types expressing the mRNA, the protein levels and importance of these to adipocyte growth and function were not addressed (Gabrielsson *et al.* 2002). FGF 10 has been shown to play an important role in mouse WAT and 3T3-L1 preadipocyte differentiation. FGF-10 mRNA and protein were found in 3T3 cells and mouse

preadipocytes and blocking its actions was shown to inhibit differentiation (Sakaue *et al.* 2002). Exogenous FGF-2 is mitogenic to rat adipocytes (Serrero 1987), chicken preadipocytes (Butterwith *et al.* 1993), and human preadipocytes (Teichert-Kuliszewska *et al.* 1992), but there were contradictory reports of the effects of FGF-2 on adipocyte differentiation. In rat cells, FGF-2 was found to either have no effect (Vassaux *et al.* 1994) or stimulated differentiation (Serrero 1987; Hauner *et al.* 1995b). In sheep preadipocytes FGF 2 stimulated differentiation, but in several preadipocyte cell lines such as TA1 adipocytes and human preadipocytes, FGF 2 inhibited differentiation (Navre and Ringold 1989; Teichert-Kuliszewska *et al.* 1992; Hauner *et al.* 1995b; Krieger-Brauer and Kather 1995). Effects may have been variable because different cell lines and concentrations of FGF-2 used. The expression and role of FGFs in adipose is therefore unresolved and may differ between different depots and different species.

1.6.2 Paracrine Factors

1.6.2.1 Vascular Endothelial Growth Factor (VEGF)

VEGF was initially identified due to its ability to induce vascular permeability (Senger *et al.* 1983), and to promote vascular endothelial cell proliferation (Plouet *et al.* 1989). Many cells secrete VEGF including rat adipocytes (Tonello *et al.* 1999; Asano *et al.* 2001; Mick *et al.* 2002) and human adipocytes (Zhang *et al.* 1997c) with VEGF induced during 3T3-L1 differentiation (Claffey *et al.* 1992). VEGF plays a fundamental role in the growth and differentiation of vascular and lymphatic

endothelial cells being mitogenic for vascular endothelial cells derived from arteries, veins, and lymphatics (Ferrara and Henzel 1989; Plouet *et al.* 1989). It has a pronounced angiogenic response in a wide variety of *in vivo* models (Connolly *et al.* 1989; Leung *et al.* 1989) and is a survival factor for endothelial cells (Alon *et al.* 1995; Gerber *et al.* 1998). There is considerable evidence that VEGF is a major tumour angiogenesis factor. VEGF mRNA is upregulated in a number of human tumours such as lung cancer (Volm *et al.* 1997), breast cancer (Yoshiji *et al.* 1996), and many other cancers (Ferrara 1999). VEGF is secreted in a number of tumour cell lines and inhibition of VEGF activity in tumour cell lines leads to a suppression of growth (Asano *et al.* 1995; Ferrara 1999; Ferrara and Alitalo 1999). VEGF is therefore an important regulator of the process of angiogenesis, which is the sprouting of new blood vessels from pre-existing capillaries by the multiplication of endothelial cells, their migration, remodelling of the ECM, tube formation and the recruitment of surrounding structures to maintain newly formed vessels.

Human VEGF gene is approximately 14 kb and is localised to chromosome 6p21.3 (Houck *et al.* 1991; Vincenti *et al.* 1996). There are several different members of the VEGF family, VEGF-A, -B, -C, and –D (Carmeliet *et al.* 1996). Alternative exon splicing of the VEGF-A gene results in the generation of four different VEGF-A species, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$. $VEGF_{165}$ predominates, but $VEGF_{121}$ and $VEGF_{189}$ are detected in the majority of cells and tissues and $VEGF_{206}$ is rare (Houck *et al.* 1991). Native VEGF is a basic, heparin binding, homodimeric glycoprotein of 45 kDa, whose properties correspond to $VEGF_{165}$ (Ferrara 1999).

The main receptors that are involved in initiating signal transduction cascades in response to VEGFs are a family of closely related receptor tyrosine kinases consisting of three members termed vascular endothelial growth factor receptor 1(VEGFR1) (Flt-1), VEGFR2 (Flk-1/KDR) and VEGFR3 (Flt-4). VEGFR1 binds to VEGF-A and -B, VEGFR2 binds to VEGF-A, -C, and –D, while VEGFR3 binds to VEGF-C and –D. VEGFR2 seems to mediate the growth and permeability actions of VEGF, whereas VEGFR1 may have a negative role, either by acting as a decoy receptor or by suppressing signalling through VEGFR2 (**Fig 1.8**). VEGFR3 may be important during blood vessel development and is lymphatic-specific so is probably important for lymphatic development (Yancopoulos *et al.* 2000).

Knockout studies of VEGF and VEGFR2 show the important role of VEGF. Knockouts of VEGF even of a single allele in mice lead to embryonic lethality due to severe vascular abnormalities (Carmeliet *et al.* 1996; Ferrara *et al.* 1996). Knockouts of VEGFR2 in mice also prevent the development of vasculature with very few endothelial cells present, which demonstrates the role of VEGF in the formation of vasculature (Shalaby *et al.* 1995).

Interaction of VEGF through VEGFR2 leads to endothelial cell growth by the activation of MAPK. However VEGFR2 does not utilise the Ras-activation pathway to activate MAPK, but uses the PLCγ-PKC pathway to mediate its effect. PLCγ directly binds autophosphorylated VEGFR2, and is tyrosine phosphorylated and activated. Activated PLCγ stimulates PKC activation, which subsequently activates

raf-1 and then the MAPK cascade (Takahashi and Shibuya 1997; Takahashi *et al.* 1999).

By blocking angiogenesis, adipose tissue mass can be regulated (Rupnick *et al.* 2002). This implies that VEGF has a potential paracrine role in the adipocyte, probably being involved in adipose tissue growth by inducing angiogenesis required to lay down new adipose tissue.

1.6.2.2 Angiopoietins

The angiopoietin ligands and Tie receptors belong to the receptor tyrosine kinase family, and play a critical role in blood vessel formation during angiogenesis. They are involved in a diverse number of cellular processes such as cell migration, proliferation and survival, and reorganisation of the actin cytoskeleton. There are four main members of the angiopoietin family referred to as Angiopoietin-1, -2,-3, and -4, with Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) being well characterised (Davis *et al.* 1996; Maisonpierre *et al.* 1997). Both Ang-1 and Ang-2 are 70 kDa secreted proteins with a hydrophobic signal peptide sequence directing secretion at the amino terminus followed by a coiled-coil domain, and a carboxyl terminus fibrinogen-like domain containing significant levels of sequence homology (Ward and Dumont 2002). Ang 1 and Ang- 2 were identified as the ligands to the previously discovered Tie-2 receptor (Davis *et al.* 1996; Maisonpierre *et al.* 1997).

Tie receptors are RTKs predominantly expressed by vascular endothelial cells. There are two members known as Tie-1 (also known as Tie) and Tie-2 (also known as Tek). These highly related genes have unique extracellular domains, consisting of two immunoglobulin-like loops which flank three epidermal growth factor repeats, followed by three fibronectin-type III repeats. The cytoplasmic region of the molecules contains conserved tyrosine kinase domains (Dumont *et al.* 1992; Partanen *et al.* 1992; Iwama *et al.* 1993; Maisonpierre *et al.* 1993; Sato *et al.* 1993; Schnurch and Risau 1993).

No ligands have been identified for Tie-1, but it can physically associate with Tie-2 and may possibly play a role in Tie-2 signalling (Marron *et al.* 2000). Interestingly mice lacking Tie-1 die during embryogenesis having subcutaneous oedema, with localised haemorrhaging thus the leakage of interstitial fluid and erythrocytes suggests a role for Tie-1 in maintenance of blood vessel integrity (Puri *et al.* 1995; Sato *et al.* 1995). Both Ang-1 and Ang-2 are ligands for Tie-2 but have different effects. Ang-1 specifically binds Tie-2 and induces its tyrosine phosphorylation thus activating Tie-2, while Ang-2 also binds Tie-2, but does not induce tyrosine phosphorylation, but instead blocks Ang-1-mediated Tie-2 receptor activation (**Fig 1.9**) (Davis *et al.* 1996; Maisonpierre *et al.* 1997).

Virtually all endothelial cells during embryonic development express Tie-2. Expression of Tie-2 persists in quiescent adult endothelial cells although at reduced levels in some organs such as brain and heart (Dumont *et al.* 1992; Sato *et al.* 1993). During early embryonic development Ang-1 is expressed in the heart myocardium

surrounding the endocardium but distributes more widely in the mesenchyme of surrounding developing vessels and in close association with endothelial cells (Davis *et al.* 1996). However Ang-2 is not readily detected in the developing heart but is abundant in the dorsal aorta and major aortic branches, specifically in the smooth muscle layer immediately beneath the vessel endothelium (Maisonpierre *et al.* 1997). In the adult, Ang-1 mRNA is expressed in organs undergoing vascular remodelling such as the placenta and the ovary, while Ang-2 mRNA is more restricted with expression in those sites undergoing remodelling (Maisonpierre *et al.* 1997).

Tie-2 knockout mice die at the embryonic stage and exhibit extensive malformations of the vasculature in a variety of tissues suggesting the importance of Tie-2 in blood vessel development (Dumont *et al.* 1994). Ang-1 knockout mice have a very similar, though less severe phenotype compared to mice lacking Tie-2, which implies that other factors may activate Tie-2 than just Ang-1 (Suri *et al.* 1996). Overexpression of Ang-2 has a very similar but more severe phenotype than the Ang-1 and Tie-2 knockouts, implying that Ang-2 is the antagonist for the Tie-2/Ang-1 pathway (Maisonpierre *et al.* 1997).

Unlike VEGF or FGF-2, Ang/Tie-2 signalling does not have a mitogenic effect (Davis *et al.* 1996). Activation of Tie-2 by Ang-1 results in the activation of PI3K and PKB and this has been found to be important in cell survival of endothelial cells thus being an antiapoptotic factor (Kim *et al.* 2000). Ang-1 activation of Tie-2 induces endothelial cell sprouting to form tubule-like structures (Koblizek *et al.* 1998). Ang-1 may also be involved in cell migration as it induces migration of endothelial cells and

this effect can be abolished by inhibiting PI3K (Fujikawa *et al.* 1999). Tie-2 interacts with a variety of molecules, which may mediate its role. Tie-2 activates Dok-R, which activates molecules involved in Ras signalling which may play a role in cell migration, proliferation, and organisation of the cytoskeleton. Tie-2 can also activate other proteins such as adaptor protein Grb2, Grb7, Grb14, PI3K, and the transcription factors STAT3 and STAT5, which may mediate effects on growth, differentiation, migration and survival (Huang *et al.* 1995; Kontos *et al.* 1998; Jones *et al.* 1999; Korpelainen *et al.* 1999). Tie-2 therefore plays a crucial role in network formation and stabilisation of the vasculature by both cell-to-cell and cell-to-ECM interactions.

The antagonistic relationship of Ang 2 on Ang 1/Tie-2 signalling enables Ang 2 to be involved in vascular regression and remodelling. By antagonising Ang 1 action at the Tie-2 receptor, Ang 2 causes vessel destabilisation loosening the adherence between endothelial cells and the underlying support tissue (Maisonpierre *et al.* 1997; Goede *et al.* 1998; Stratmann *et al.* 1998; Holash *et al.* 1999a; Holash *et al.* 1999b; Zagzag *et al.* 1999). In the absence of angiogenic factors, Ang 2 destabilises vessels causing their regression by destabilising the survival effects of Ang-1 which are mediated through PI3K. Vessel regression by Ang 2 has been demonstrated in the eye around the lens during eye development and in malignant glioma cells (Otani *et al.* 1999; Hackett *et al.* 2000; Yancopoulos *et al.* 2000; Koga *et al.* 2001). In the presence of other angiogenic factors such as VEGF, Ang 2 causes vessel regression and VEGF is able to stimulate angiogenesis and vessel sprouting (Holash *et al.* 1999a; Holash *et al.* 1999b; Zagzag *et al.* 1999).

Ang 1 and Ang 2 have been shown to mediate cell adhesion through integrins (Carlson *et al.* 2001). Integrins such as β_1 and $\alpha_v\beta_5$ integrins are cell surface receptor proteins which mediate adhesion to the ECM. Angiopoietins can bind integrins as well as ECM proteins such as vitronectin and facilitate cell-cell and cell-ECM adhesion independent of Tie-2 (Carlson *et al.* 2001). This presents an alternative route to which angiopoietins mediate their action.

Except for angiogenesis that occurs during hormonal stimulation of the ovary and uterus, the endothelium of the vasculature normally remains quiescent during adult life. However conditions such as growing tumours, wound healing, and vascular malformations do require the process of angiogenesis. Numerous tumours show the expression of the angiopoietin ligands and/or Tie receptors such as in brain tumours and breast cancer (Hatva *et al.* 1995; Holash *et al.* 1999b; Hayes *et al.* 2000). Adipose tissue can grow and regress throughout adulthood being highly vascularised so the role of angiogenesis would be important in adipose tissue remodelling (Rupnick *et al.* 2002). Both Ang-1 and Ang-2 have been shown to be expressed in rat adipose tissue and 3T3-L1 cells (Stacker *et al.* 2000; Cohen *et al.* 2001). This implies that angiopoietins and Tie-2 signalling may be important in the regulation of adipose tissue mass through angiogenesis.

1.7 Aims and Rationale

Adipose tissue is a dynamic tissue which can grow and regress throughout the lifetime of an individual. The replication of preadipocytes and their differentiation results in

this increased mass. The aim of this study is to understand the factors important in the regulation of adipocyte growth and function. In order to achieve this, a robust primary preadipocyte cell culture model which can be differentiated effectively into mature adipocytes is required. Several preadipocyte models from different adipose sites and different species have been described to study the processes of adipocyte growth and differentiation. The complement of factors required for differentiation may however vary between different preadipocyte cell models. Signalling pathways which facilitate the growth and differentiation of adipocytes are activated. As well as exogenous compounds, the adipocyte secretes paracrine and autocrine factors which may have a significant role in adipocyte growth and differentiation.

The aims of this study are:

1 To characterise the role of the components used to induce differentiation and to optimise differentiation in primary human subcutaneous preadipocytes. The effects of specific inducers of differentiation on different aspects of differentiation such as lipid accumulation, lipogenesis, leptin secretion, protein content and DNA content will be determined.

2 To determine the role of signalling pathways involved in the process of differentiation by inhibiting parts of signalling pathways with specific inhibitors and studying their effect on parameters of preadipocyte differentiation. The effects of different adipogenic compounds on p42/44

MAPK in human preadipocytes and differentiated preadipocytes will be determined.

3 To determine the expression and function of secreted paracrine and autocrine factors on adipocyte growth and differentiation. Factors examined include the ligands FGF, VEGF and angiopoietins and their cognate receptors, FGFR, VEGFR1 and VEGFR2 and Tie2.

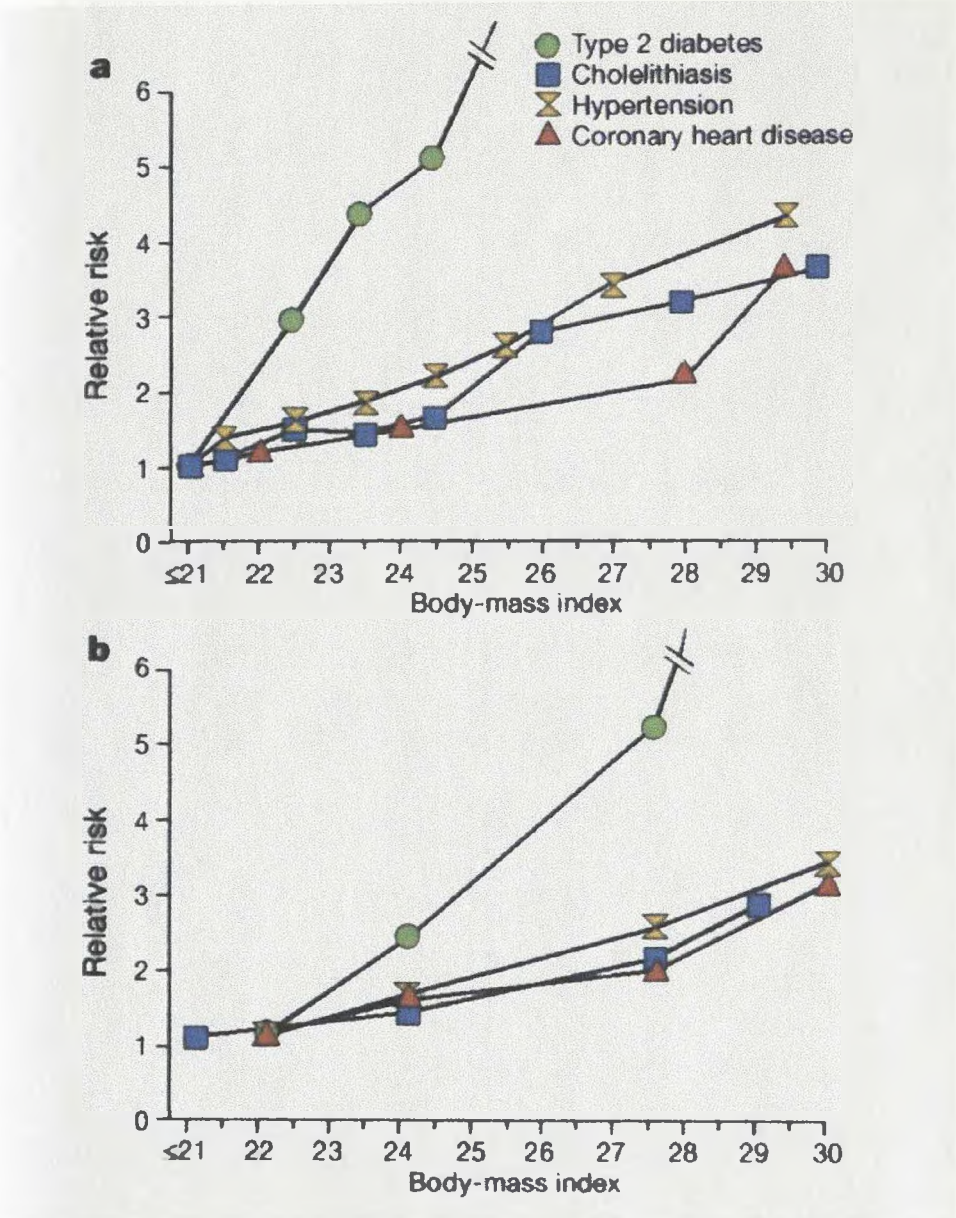
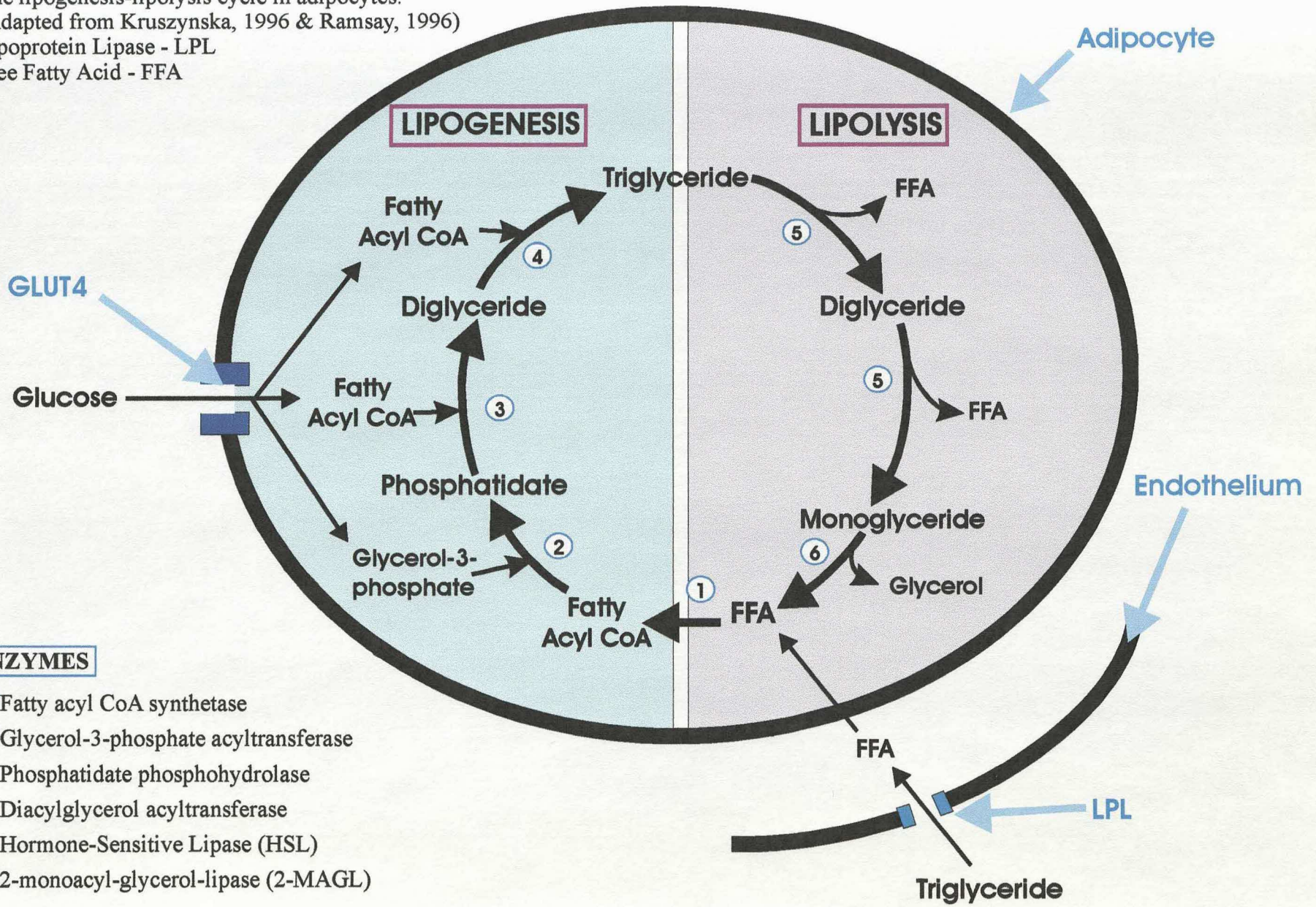


Fig 1.1 Relationship between BMI (≤ 30) and the relative risk of type 2 diabetes, hypertension, coronary heart disease, and cholelithiasis. Graph A shows the relative risk for women, while Graph B shows the relative risk for men. Reproduced from Willet *et al.*, (1999)

Fig 1.2 The lipogenesis-lipolysis cycle in adipocytes.
(Adapted from Kruszynska, 1996 & Ramsay, 1996)
Lipoprotein Lipase - LPL
Free Fatty Acid - FFA



ENZYMES

1. Fatty acyl CoA synthetase
2. Glycerol-3-phosphate acyltransferase
3. Phosphatidate phosphohydrolase
4. Diacylglycerol acyltransferase
5. Hormone-Sensitive Lipase (HSL)
6. 2-monoacyl-glycerol-lipase (2-MAGL)

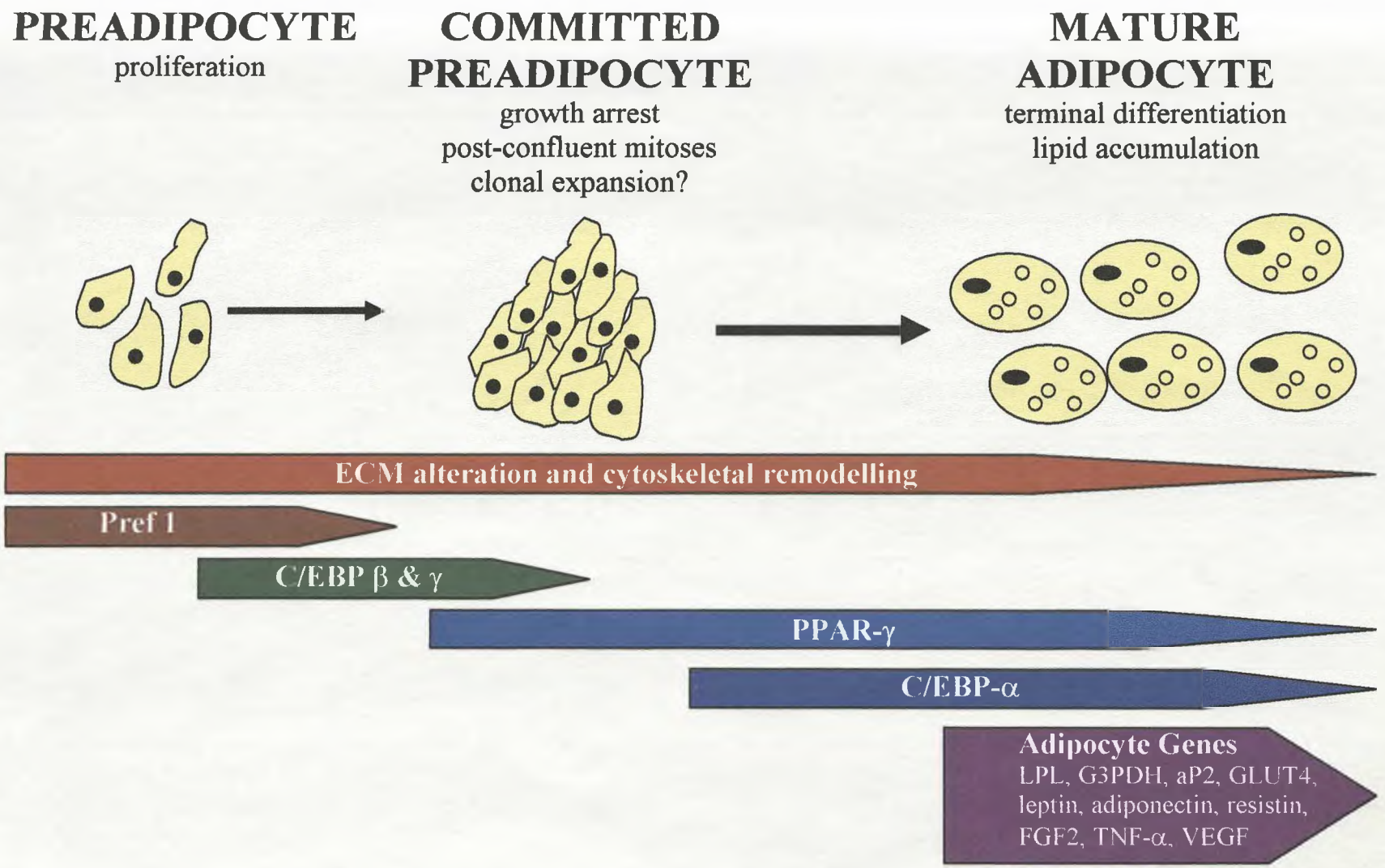


Fig 1.3 Overview of stages in adipocyte differentiation. Adapted from Gregoire *et al* 1998

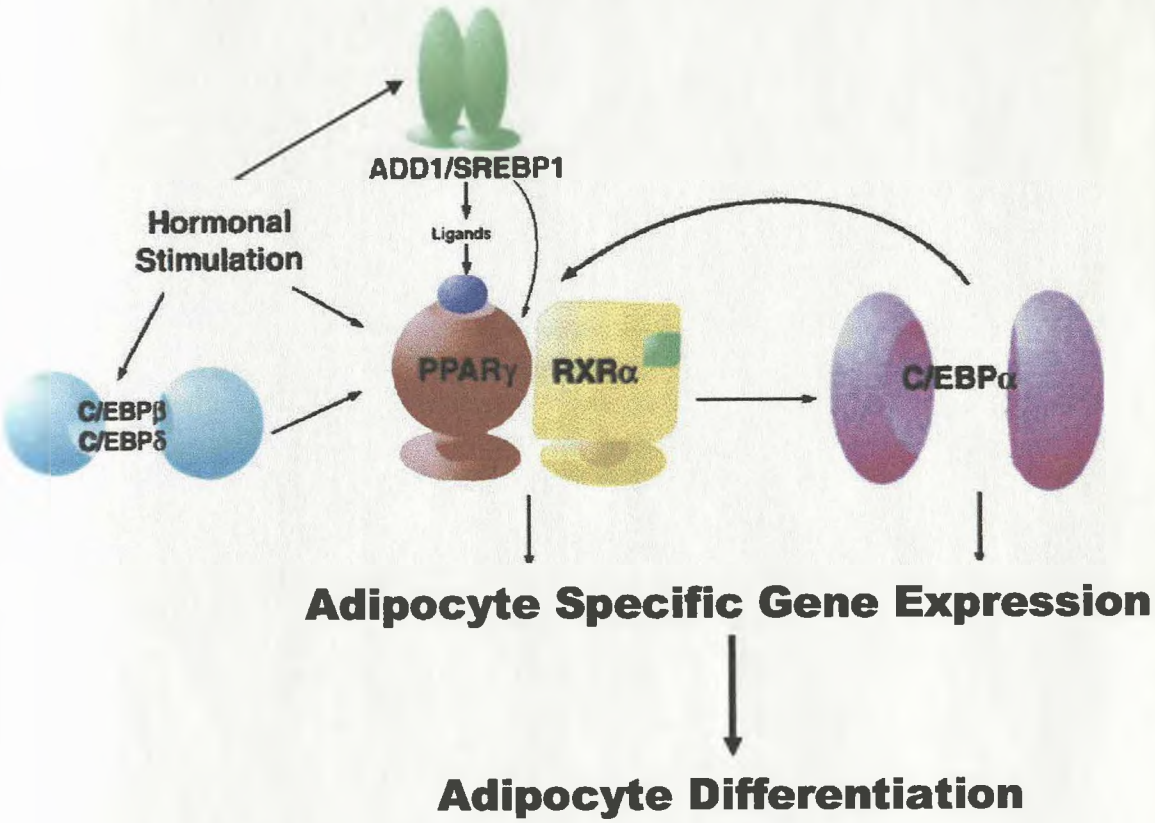
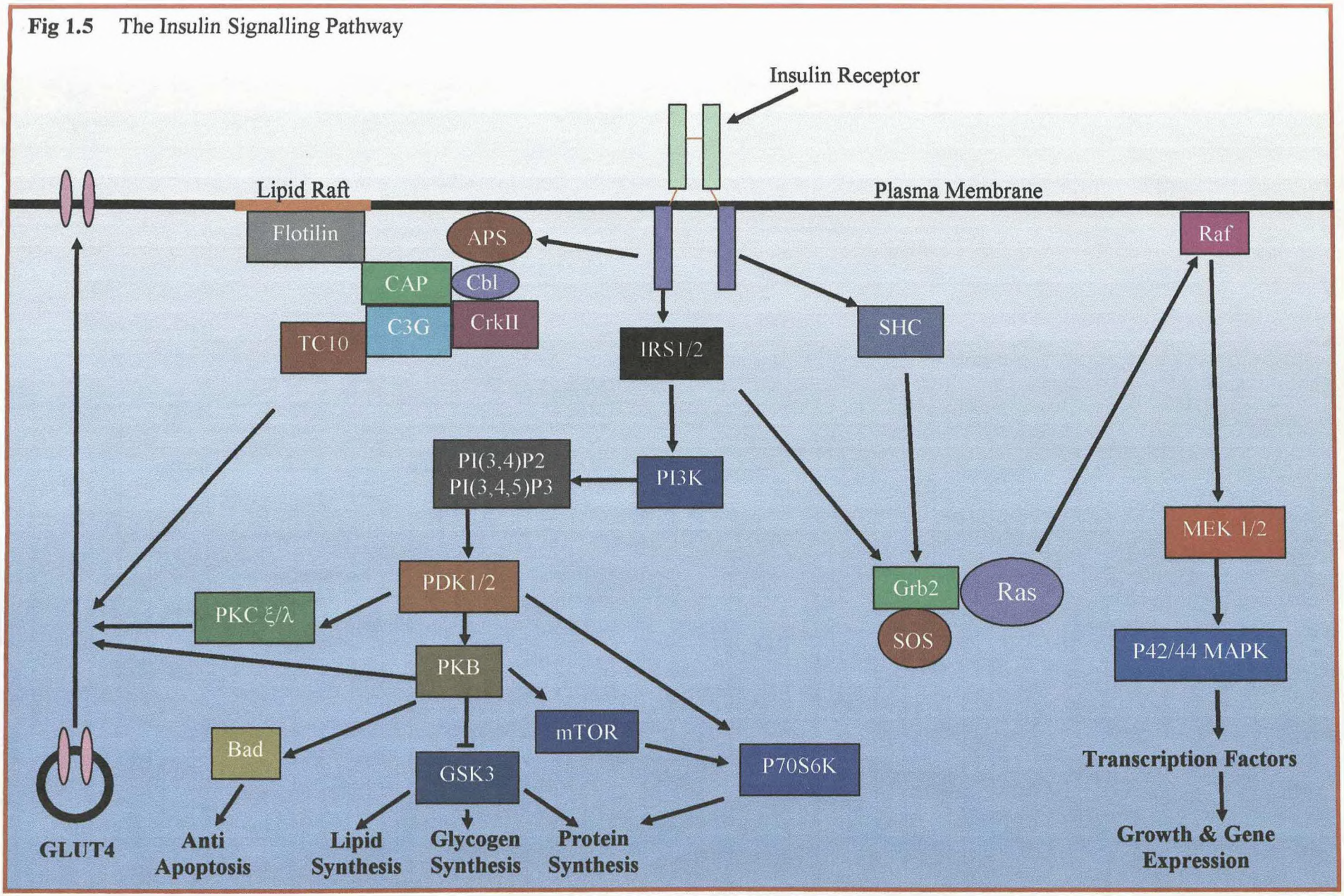


Fig 1.4 Cascade of transcriptional factors involved in adipogenesis. After the hormonal induction of adipocyte differentiation, C/EBP-β and C/EBP-δ are the earliest induced transcription factors. These two induce the expression of PPAR-γ. ADD1/SREBP1 is another transcription factor induced by hormonal stimulation which induces the expression of PPAR-γ. ADD1/SREBP1 does this either directly or by promoting the expression of an unknown endogenous PPAR-γ ligand. PPAR-γ induces C/EBP-α expression, and both PPAR-γ and C/EBP-α induce the expression of adipocyte specific genes leading to differentiation. Figure adapted from Rosen & Spiegelman, 2000.

Fig 1.5 The Insulin Signalling Pathway



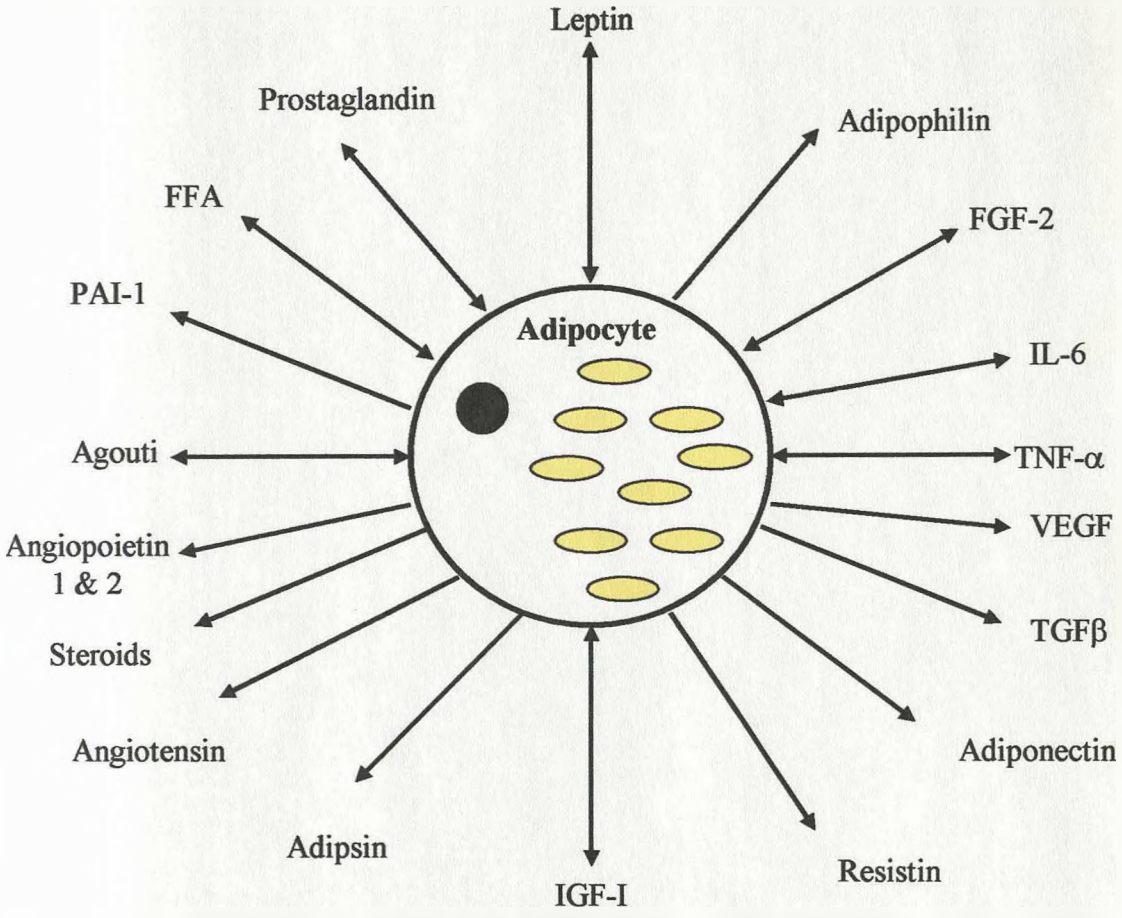


Fig 1.6 The adipocyte as an endocrine organ. Paracrine and autocrine factors secreted by adipocytes.
FGF-2, fibroblast growth factor-2; IL-2, interleukin-6; TNF-α, tumour necrosis factor α; TGFβ, transforming growth factor β; IGF-I, insulin-like growth factor-I; PAI-1, Plasminogen activator inhibitor-1; FFA, free fatty acid; VEGF, vascular endothelial growth factor

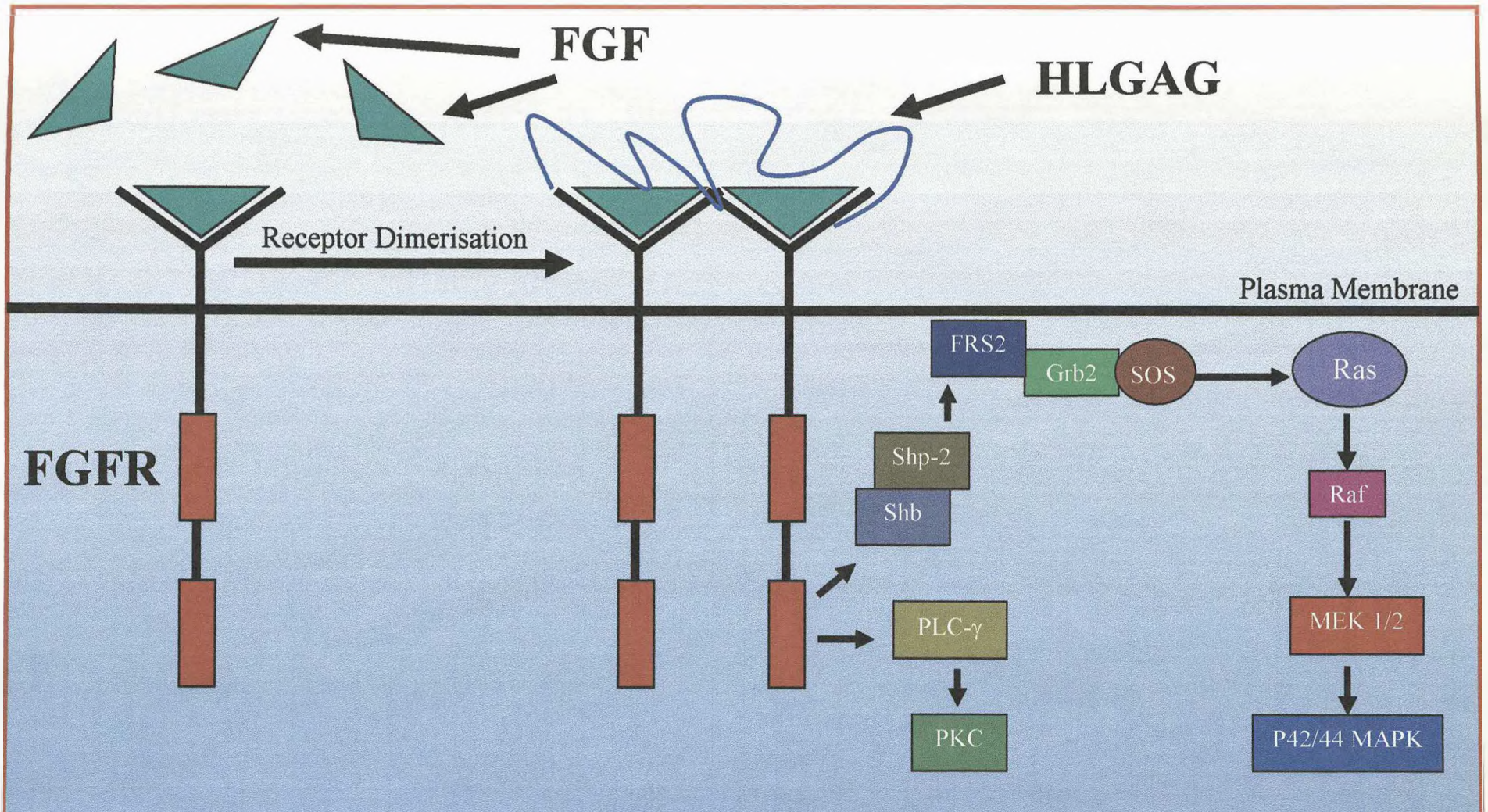


Fig 1.7 Model of FGFR signalling. FGF binds to FGFR, which dimerises with another FGF-bound FGFR. This complex is stabilised by HLGAG binding across the FGF-bound FGFR dimer complex. This causes autophosphorylation of the intracellular domain of FGFR leading to activation of downstream signalling pathways such as p42/44 MAPK and PKC.

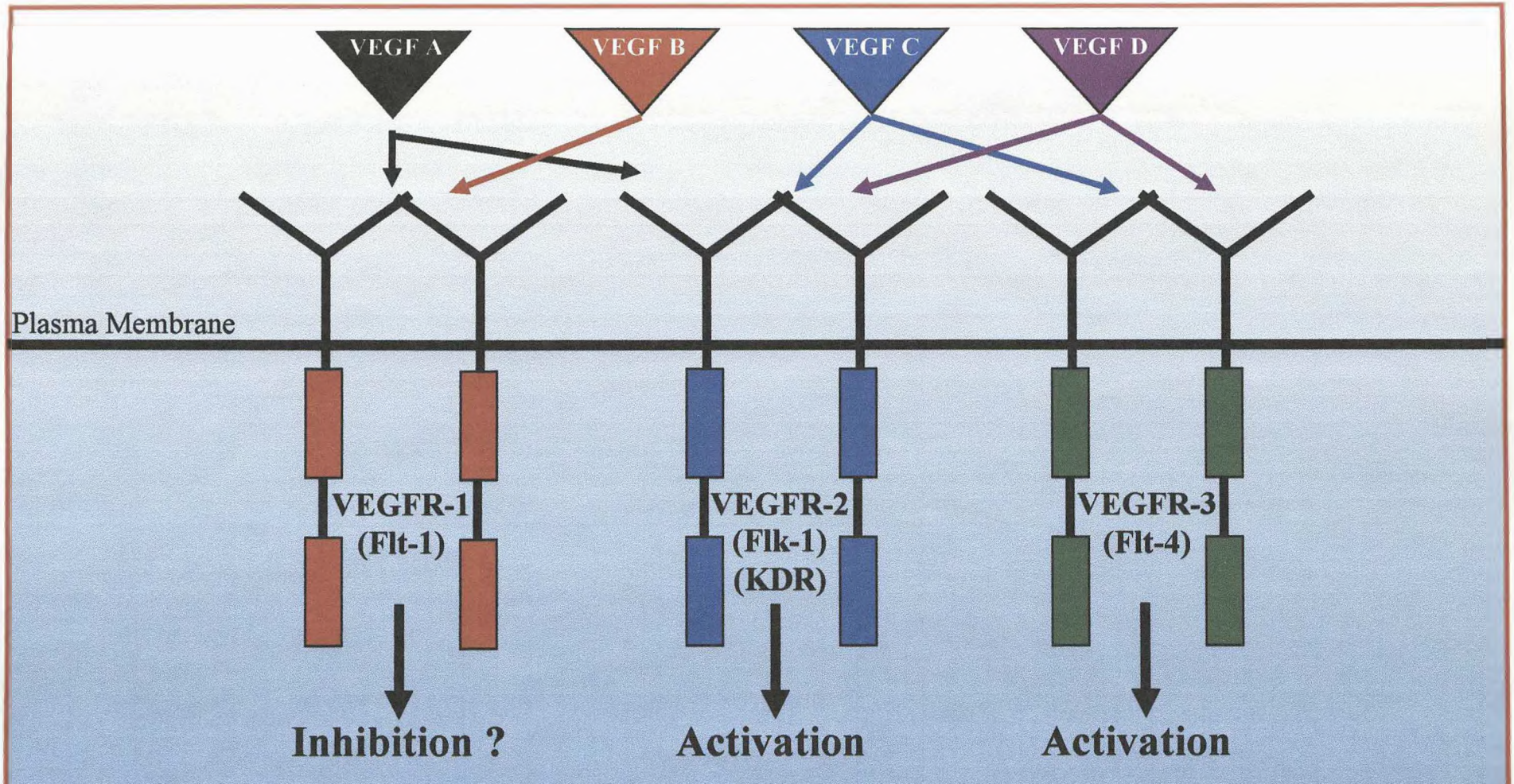


Fig 1.9 Model of VEGF signalling. After binding by VEGF, VEGFRs undergo dimerisation and autophosphorylation of the intracellular domain of VEGFR leading to activation of downstream signalling pathways. VEGFR2 is involved in the growth and permeability actions of VEGF, while VEGFR1 may have a negative role in VEGF activation. VEGFR3 may be important in blood vessel development.

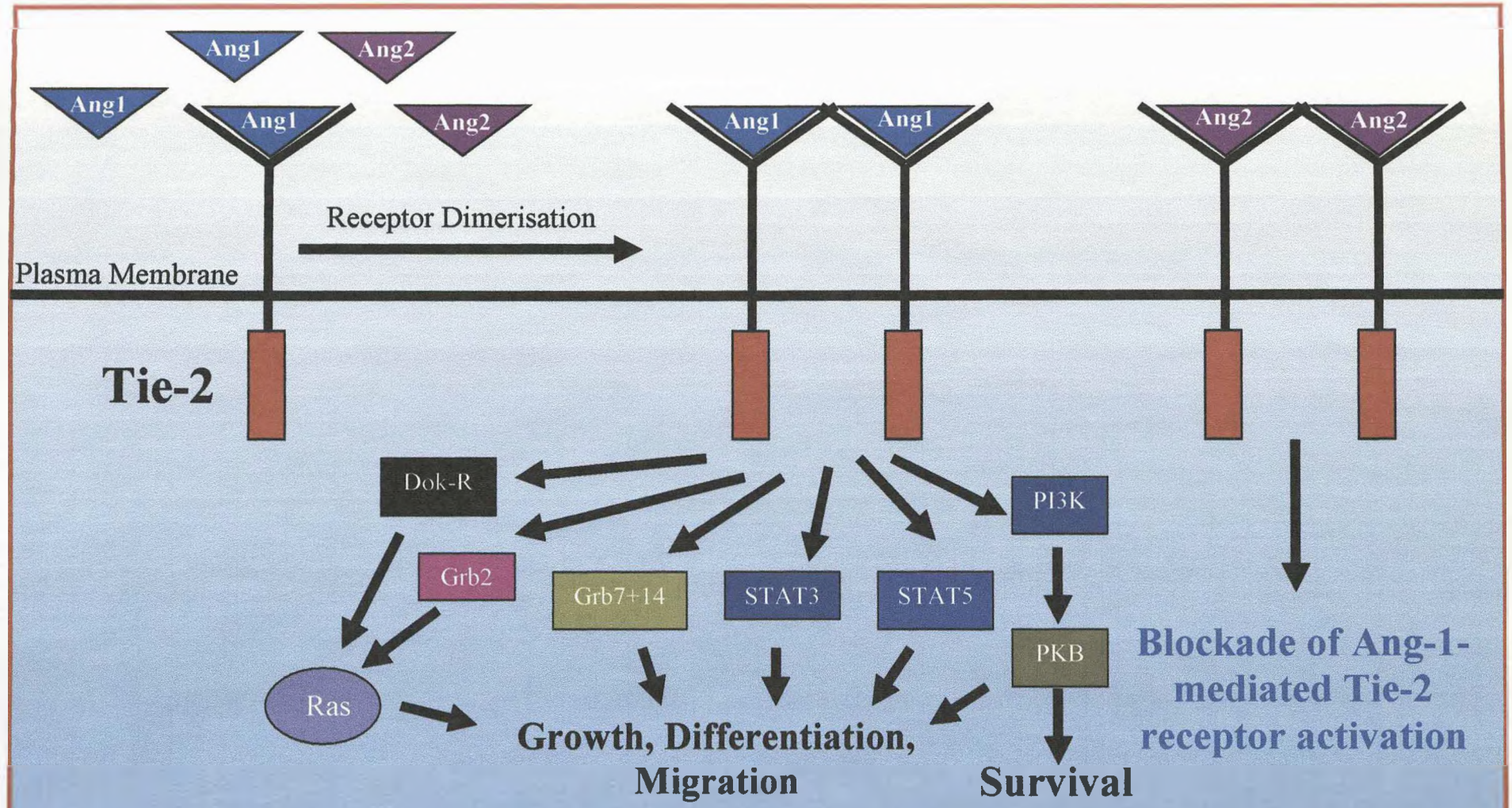


Fig 1.9 Model of Tie-2 signalling. Ang 1 binds to Tie-2, which dimerises with another Tie-2 receptor. This causes autophosphorylation of the intracellular domain of Tie-2 leading to activation of downstream signalling pathways. If Ang 2 binds Tie-2, Ang-1-mediated Tie-2 receptor activation is prevented.

BMI (kg m^{-2})	WHO Classification	Popular Description
<18.5	Underweight	Thin
18.5-24.9	—	Normal
25.0-29.9	Grade 1 overweight	Overweight
30.0-39.9	Grade 2 overweight	Obesity
≥40.0	Grade 3 overweight	Morbidly Obesity

Table 1.1 World Health Organisation (WHO) classification of BMI (adapted from Koppelman, 2000)

PREADIPOCYTE MODEL & SPECIES	DIFFERENTIATION PROTOCOL	REFERENCE
Pluripotent Fibroblasts		
Embryonic Stem Cells (Mouse)	FBS+In+T3+Retinoic acid	Dani *et al*, 1997
NIH3T3 (Mouse)	Ectopic expression of PPARγ, C/EBPα or C/EBPβ+In+Dex+IBMX	Freytag *et al*, 1994; Brun *et al*, 1996; Wu *et al*, 1995
Swiss 3T3 (Mouse)	Ectopic expression of C/EBPα	Freytag *et al*, 1994
Balb/3T3 (Mouse)	Ectopic expression of C/EBPα	Freytag *et al*, 1994
C3H10T1/2 (Mouse)	Demethylating agent 5'-azacytidine or PPARγ ligands such as indomethacin	Lehmann *et al*, 1997
C2C12 (Mouse)	Thiazolidinediones	Teboul *et al*, 1995
G8 (Mouse)	Ectopic expression of PPARγ or C/EBPα+In+Dex	Hu *et al*, 1995
Unipotent Preadipocytes		
3T3-L1 (Mouse)	FBS+In+Dex+IBMX	Student *et al*, 1980
3T3-F442A (Mouse)	FBS+In	Spiegelman & Green 1980
Ob1771 (Mouse)	FBS+In+T3	Gharbi-Chihi *et al*, 1981
TA1 (Mouse)	FBS+In+Dex	Chapman *et al* 1984
30A5 (Mouse)	FBS+In+Dex+IBMX	Pape & Kim 1988
1246 (Mouse)	DMEM+In+Dex+IBMX	Serrero & Khoo 1982
Primary Preadipocytes		
Human	DMEM+In+Dex+IBMX+T3	Hauner *et al*, 1989
Mouse	DMEM+In+Dex+HDL	Litthauer & Serrero, 1992
Rat	DMEM+In+DEX+IBMX	Kim *et al*, 2001
Rabbit	DMEM+In+Dex+T3	Reyne *et al*, 1989
Sheep	DMEM+In+Dex+T3	Soret *et al*, 1999
Pig	DMEM+In+Dex	Suryawan *et al*, 1997

Table 1.2 *In vitro* models of adipocyte differentiation. FBS, foetal bovine serum; In, insulin; Dex, dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; T3, triiodothyronine; HDL, high density lipoprotein. Adapted from Hwang *et al* 1997; Gregoire *et al*, 1998.

CHAPTER 2

Materials and Methods

2.1 Materials

Table 2.1 Materials utilised and their suppliers

Material	Supplier
ABC reagent	Sigma, Poole, Dorset, UK
Acetone	Fisher Scientific Ltd, Loughborough, UK
Acridine Orange	Sigma, Poole, Dorset, UK
Agarose	Sigma, Poole, Dorset, UK
Ammonium Chloride (NH_4Cl)	Sigma, Poole, Dorset, UK
Ammonium Persulphate	Sigma, Poole, Dorset, UK
AmpErase UNG	Applied Biosystems, Warrington, UK
AmpliTaqGold Polymerase	Applied Biosystems, Warrington, UK
Anti-Goat IgG-horseradish peroxidase (HRP) Conjugate	Santa Cruz Biotechnology, CA, USA
Anti-Mouse IgG-Biotin Conjugate	Santa Cruz Biotechnology, CA, USA
Anti-Mouse IgG-HRP Conjugate	Santa Cruz Biotechnology, CA, USA
Anti-Rabbit IgG-HRP Conjugate	Santa Cruz Biotechnology, CA, USA
Autoradiography Film	GRI Ltd, Essex, UK
Avian Myeloblastosis Virus (AMV) Reverse Transcriptase	Promega, Southampton, UK
Bovine Foetal Calf Serum (FCS)	First Link Ltd, Brierley Hill, UK
Bovine Serum Albumin (BSA)	First Link Ltd, Brierley Hill, UK
Bromophenol Blue	Sigma, Poole, Dorset, UK

Borate	Sigma, Poole, Dorset, UK
Chloroform	Sigma, Poole, Dorset, UK
Cortisol	Sigma, Poole, Dorset, UK
D_C Protein Assay	BioRad, Preston, UK
Deoxynucleotide Triphosphates (dNTPs)	Promega, Southampton, UK
Dexamethasone	Sigma, Poole, Dorset, UK
3,3'Diaminobenzidine	Sigma, Poole, Dorset, UK
Dimethyl Sulfoxide (DMSO)	Sigma, Poole, Dorset, UK
DMEM/Ham's F-12 medium	Invitrogen Ltd, Paisley, UK
DNA Ladder (100 bp)	New England Biolabs, Hertfordshire, UK
Enhanced Chemiluminescence (ECL)	Insight Biotechnology, Middlesex, UK
Ethanol	Fisher Scientific Ltd, Loughborough, UK
Ethylenediaminetetraacetic Acid (EDTA)	Sigma, Poole, Dorset, UK
FGF-2 Antibody	Sigma, Poole, Dorset, UK
FGF-2 ELISA Kit	R&D Systems, Oxon, UK
FGFR1 Antibody (C-Term)	Santa Cruz Biotechnology, CA, USA
FGFR1 Antibody (N-Term)	Santa Cruz Biotechnology, CA, USA
Glucose	Sigma, Poole, Dorset, UK
D-[U-^{14}C] Glucose	Amersham Biosciences, Bucks, UK
Glycine	Sigma, Poole, Dorset, UK
Hank's Balanced Salt Solution (HBSS)	Invitrogen Ltd, Paisley, UK
Hydrochloric Acid (HCl)	Fisher Scientific Ltd, Loughborough, UK
Hydrogen Peroxide (H_2O_2)	Sigma, Poole, Dorset, UK
2-Hydroxypropyl-cyclodextrin	Sigma, Poole, Dorset, UK

Insulin	Sigma, Poole, Dorset, UK
3-isobutyl-1-methylxanthine (IBMX)	Sigma, Poole, Dorset, UK
Isopropanol	Fisher Scientific Ltd, Loughborough, UK
Leptin ELISA Kit	DRG Diagnostics, Marburg, Germany
Manganese Chloride ($MgCl_2$)	Promega, Southampton, UK
$Mn(OAc)_2$	Applied Biosystems, Warrington, UK
Methanol	Fisher Scientific Ltd, Loughborough, UK
β-Mercaptoethanol	Sigma, Poole, Dorset, UK
Normal Goat Serum	Sigma, Poole, Dorset, UK
17β Oestradiol (E2)	Sigma, Poole, Dorset, UK
Oil Red O	Sigma, Poole, Dorset, UK
Optiphase Scintillation Fluid	Fisher Scientific Ltd, Loughborough, UK
Phospho-p38 MAPK Antibody	New England Biolabs, Hertfordshire, UK
p42/44 MAPK Antibody	New England Biolabs, Hertfordshire, UK
Phospho-p42/44 MAPK Antibody	New England Biolabs, Hertfordshire, UK
PD166866	Parke-Davis, Detroit, USA
PD98059	Calbiochem, Nottingham, UK
Penicillin G	Sigma, Poole, Dorset, UK
Phosphate Buffered Saline (PBS)	Sigma, Poole, Dorset, UK
Polyacrylamide	Helena Biosciences, Sunderland, UK
Polyvinylidene Difluoride (PVDF) Hybond Membrane	Amersham Biosciences, Bucks, UK
Potassium Phosphate (K_2HPO_4)	Sigma, Poole, Dorset, UK
PPARγ Antibody (C-Term)	Santa Cruz Biotechnology, CA, USA

RAd-β-gal	Provided by Cancer Studies, Birmingham
RAdDN-FGFR1	Provided by Dr Emma Davies
RAdExTie2	Provided by Dr James Ramsden
RAdExTek	Provided by Dr Mulligan
RAd-sVEGFR1	Provided by Lin *et al* (1998)
RAd-sVEGFR2	Provided by Lin *et al* (1998)
Random Hexamer Primers	Promega, Southampton, UK
Rapamycin	Calbiochem, Nottingham, UK
Reverse Transcription Buffer	Promega, Southampton, UK
18S RNA Primers & Probes	Applied Biosystems, Warrington, UK
RNAse-free water	Promega, Southampton, UK
RNAsin Ribonuclease Inhibitor	Promega, Southampton, UK
Rosiglitazone	GSK, Harlow, Essex, UK
RT-PCR Primers	Invitrogen Ltd, Paisley, UK
SB203580	GSK, Harlow, Essex, UK
Sodium Chloride (NaCl)	Sigma, Poole, Dorset, UK
Sodium Dodecyl Sulphate (SDS)	Sigma, Poole, Dorset, UK
SDS Molecular Weight Standards	Sigma, Poole, Dorset, UK
Sodium Hydroxide (NaOH)	Sigma, Poole, Dorset, UK
Streptomycin Sulphate	Sigma, Poole, Dorset, UK
Taq DNA polymerase	Promega, Southampton, UK
Taqman Primers & Probe	Applied Biosystems, Warrington, UK
TaqMan Universal PCR Master Mix	Applied Biosystems, Warrington, UK

TEMED (N,N,N',N',-tetramethylethylenediamine)	Sigma, Poole, Dorset, UK
Tie 2 Antibody (C-Term)	Santa Cruz Biotechnology, CA, USA
Tie 2 Antibody (N-Term)	Santa Cruz Biotechnology, CA, USA
Triiodothyronine	Sigma, Poole, Dorset, UK
Tris	Sigma, Poole, Dorset, UK
Triton X-100	Sigma, Poole, Dorset, UK
TRIzol reagent	Invitrogen Ltd, Paisley, UK
TNF α	PeprotechEC Ltd, London, UK
Tween 20	Sigma, Poole, Dorset, UK
Type I Collagenase	Worthington, Freehold NJ, USA
VEGFR2 Antibody	Calbiochem, Nottingham, UK
Virkon	Antec International Ltd, Suffolk, UK
Wortmannin	Alexis Corporation, San Diego, CA

2.2 Subjects

Subcutaneous abdominal adipose tissue was obtained from female subjects (age 49.3 ± 9.9 years [mean ± SEM]; BMI 27.30 ± 6.42 kg/m^2) undergoing elective surgery in accordance with the guidelines of the South Birmingham ethics committee. None of the subjects had diabetes or severe systemic illness, and none were taking medications known to influence adipose tissue mass, distribution or metabolism.

2.3 Isolation and Culture of Human Preadipocytes

Preadipocytes were isolated by a variation of the method of Rodbell (1964). Adipose tissue was digested with type I collagenase, (1mg/ml) in Hank's balanced salt solution (HBSS), for 1 h at 37°C, and shaken at 100 cycles/min. The disrupted tissue was filtered through a double-layered cotton mesh and isolated cells were washed with HBSS and centrifuged at 250 x g for 5 min to give a pellet containing preadipocytes. The cell pellet was resuspended in erythrocyte lysis buffer (154mM NH_4Cl, 5.7mM K_2HPO_4, 0.1mM EDTA, pH 7.0) for 10 min and centrifuged at 250 x *g* for 5 min to remove erythrocyte contamination. The resulting pellet was washed in HBSS and centrifuged at 250 x *g* for 5 min and resuspended in DMEM/Ham's F-12 medium supplemented with 15% bovine foetal calf serum (FCS). ~10^5cells were plated in 12 well (4.5cm^2) tissue culture dishes and grown until confluent (10^6 cells per well). All media used was supplemented with 100U/ml penicillin G, and 0.1 mg/ml streptomycin sulphate.

2.4 Differentiation of Human Preadipocytes

Unless otherwise stated, confluent preadipocytes (referred to as Day 0 preadipocytes) were washed twice with HBSS and cultured in DMEM/Ham's F-12 medium containing 100nM insulin, 100nM dexamethasone (Dex), 0.2nM triiodothyronine (T3), and for the first four days of culture, 0.25mM 3-isobutyl-1-methylxanthine (IBMX) (Petruschke and Hauner 1993). This is referred to as normal differentiation medium. Cells were incubated in 5% CO_2:95% air at 37°C and differentiation medium was changed every 2-3 days until cells had accumulated visible lipid droplets.

2.5 Adenovirus

2.5.1 Recombinant Adenovirus-β-galactosidase (RAd-β-gal)

Recombinant adenovirus expressing β-galactosidase (RAd-β-gal) was used as a control E1/E3 deleted adenovirus. RAd-β-gal obtained from the Department of Cancer Studies, University of Birmingham, UK.

2.5.2 Recombinant Adenovirus-Dominant Negative-Fibroblast Growth Factor Receptor-1 (RAdDN-FGFR1)

Recombinant adenovirus expressing the extracellular and transmembrane domain of FGFR1, but deficient in the kinase domain (RAdDN-FGFR1) was produced within our laboratory by Dr Emma Davies (Davies *et al.* 2003). The truncated recombinant protein produced by RAdDN-FGFR1 acts as a dominant negative knockout by dimerising with native full length FGFR receptors. However as the truncated recombinant FGFR1 protein lacks the kinase domain, it prevents activation of downstream signalling pathways when bound by its ligand (**Fig 2.1**).

2.5.3 Recombinant Adenovirus Extracellular Tie2 (RAdExTie2)

Recombinant adenovirus expressing the extracellular domain of human Tie2 truncated at the C-terminus was utilised (RAdExTie2). This recombinant protein is the full length of the extracellular domain except for the first 6 amino acids and the 11 amino acids before the transmembrane domain. RAdExTie2 binds and sequesters native Ang 1 and Ang 2 preventing them from activating signalling pathways (**Fig 2.2**). This adenovirus was constructed within our laboratory by Dr James Ramsden.

2.5.4 Recombinant Adenovirus Extracellular Tek (RAdExTek)

Recombinant adenovirus expressing the extracellular domain of murine Tie2 that is 89.6% homologous to the human extracellular domain of Tie2 was used (RAdExTek). This recombinant protein is the full length of the extracellular domain, minus 8 amino acids and binds and sequesters native Ang 1 and Ang 2 preventing them from activating signalling pathways (**Fig 2.2**). This adenovirus was constructed by Lin *et al* (1998).

2.5.5 Recombinant Adenovirus Soluble Vascular Endothelial Growth Factor Receptor1 (RAd-sVEGFR1)

Recombinant adenovirus expressing the signal sequence of VEGFR1, and the first 3 immunoglobulin-like repeats, with an added C-terminus histidine tag was utilised (RAd-sVEGFR1). This binds and sequesters native VEGF preventing it from activating signalling pathways (**Fig 2.2**) (Kuo *et al.* 2001). RAd-sVEGFR1 provided by Dr Mulligan (Children's Hospital, Boston, USA).

2.5.6 Recombinant Adenovirus Soluble Vascular Endothelial Growth Factor Receptor2 (RAd-sVEGFR2)

Recombinant adenovirus expressing the signal peptide and the ectodomain of VEGFR2 fused to a murine IgG2α Fc fragment (RAd-sVEGFR2). This binds and

sequesters native VEGF preventing it from activating signalling pathways (Fig 2.2) (Kuo et al, 2001). RAd-sVEGFR2 provided by Dr Mulligan (Children's Hospital, Boston, USA).

2.5.7 Adenovirus Growth

Standard virological practice was used when handling adenoviruses in the laboratory with Class II containment facilities, including the use of 1% Virkon or 0.5% SDS to disinfect.

Replication deficient adenoviruses were grown in the 293 cell line (human embryonic kidney cells) transformed with the Ad5 E1 region, which is missing from the adenovirus, thus allowing adenoviral growth. 293 cells were cultured in DMEM medium supplemented with 10% FCS, and were split 1:10 once per week.

293 cells were grown in 150cm^2 flasks and at 90% confluence, cells were infected for 90 minutes with ~10^5 plaque forming units (pfu) of adenovirus in a small volume (3ml) of infection medium (DMEM medium supplemented with 2% FCS). Flasks were tilted on a regular basis to prevent desiccation. After 90 minutes, 15ml of infection medium was added. Cells were cultured until a cytopathic effect was observed, usually 3-4 days, shown by cells rounding up and detaching from the culture dish. Floating cells were collected by centrifugation for 10 minutes at 2000 x *g*, and the supernatant discarded. To release adenovirus from 293 cells, the cell pellet was suspended in a small volume of infection medium (1ml per 150cm^2 flask) and

freeze-thawed by immersion in dry ice/acetone followed by a water bath at 37°C and repeated 3 times. Cell debris was removed by centrifugation for 10 minutes at 2000 x *g* and supernatant, containing the virus was collected, aliqotted and stored at -40°C in screw cap vials until use. A plaque assay was carried out (plaque assay carried out by James Ramsden) to determine the pfu, which is the number of particles of adenovirus per unit volume that can infect 293 cells and form new viral particles to infect surrounding cells and is a measure of potency of adenoviral preparations.

2.5.8 Transduction of Replication-defective Adenovirus in Human Preadipocytes

Unless otherwise stated, confluent preadipocytes were washed twice with HBSS and a 0.5ml serum-free medium. Cells were infected with replication defective (E1/E3 deleted) adenovirus at a known multiplicity of infection (MOI) for 90 minutes with gentle agitation. MOI is the ratio of infectious particles to each mammalian cell present. The virus-containing medium was removed and replaced with fresh medium.

2.6 Lipogenesis Assay

Lipogenesis was measured in cells using a variation of the method described by Moody *et al* (1974). Briefly cells were washed in HBSS and incubated overnight at 37°C, 5% CO_2:95% air, in DMEM containing 5 mM glucose, appropriate medium components and treatments used during each experiment, and 2μCi/ml D-[U-^{14}C]

glucose (specific activity 291mCi/mmol). Cells were washed twice with HBSS and lipid was extracted using 250µl ethanol and placed into scintillation vials containing 4ml Optiphase scintillation fluid, and D-[U-^{14}C] glucose radioactivity measured as disintegrations per minute (dpm) was determined on a LKB scintillation counter.

2.7 Glucose Uptake Assay

Glucose uptake was measured. Briefly cells were washed in HBSS and incubated overnight at 37°C, 5% CO_2:95% air, in DMEM containing 5mM glucose, appropriate medium components and treatments used during each experiment, and 2µCi/ml D-[U-^{14}C] glucose (specific activity 291mCi/mmol). Cells were washed twice with HBSS and the cell layer was dissolved in sample buffer containing 2% sodium dodecyl sulphate (SDS) solution (2% SDS, 62.5mM Tris-HCl pH 6.8) and placed into scintillation vials containing 4ml Optiphase scintillation fluid, and D-[U-^{14}C] glucose radioactivity measured as disintegrations per minute (dpm) was determined on a LKB scintillation counter.

2.8 Lipid Staining of Cells

To help visualize and quantitate lipid content of cells, lipid staining was carried out using a variation of the method described by Culling (1963). Briefly cells were washed with HBSS, and cells were stained with 2.5% Oil Red O dissolved in isopropanol for 15 minutes at room temperature. Cells were briefly washed with 60%

isopropanol at room temperature and then washed twice with distilled water. Cells were viewed under a light microscope. Lipid and Oil Red O were extracted using 250µl ethanol and the absorbance was measured on a spectrophotometer at 520nm wavelength.

2.9 Protein Assay

Protein content of cells was determined using a colorimetric assay kit (D_C Protein Assay) described by Lowry *et al* (1951). The assay involves the reaction of protein with an alkaline copper tartrate solution and Folin reagent resulting in a colour development, which can be measured at 690nm. The cell layer was dissolved in sample buffer containing 2% SDS solution (2% SDS, 62.5mM Tris-HCl pH 6.8). Stock standards of bovine serum albumin (BSA) were prepared in sample buffer containing 2% SDS solution covering a range of concentrations (0.25-16mg/ml). In a 96 well plate 5µl of standard or sample, 25µl of copper tartrate solution, and 200µl of Folin reagent were added into each well, mixed for 5 seconds and left to incubate for 15 minutes at room temperature. The absorbance was measured on a spectrophotometer at 690nm wavelength. Quantification of protein was extrapolated from a standard curve.

2.10 Leptin ELISA

Conditioned medium was collected from cells depending upon each experiment. The total amount of secreted leptin was measured using an ELISA kit. The procedure was modified by the addition of 0.1% Triton added to samples and standards. Conditioned medium was not diluted and 50µl of sample was assayed directly and standards were diluted in differentiation medium. These samples remained on the linear scale of the standard curve throughout the collection period.

2.11 FGF-2 ELISA

Conditioned medium was collected from cells depending upon each experiment. The total amount of secreted FGF-2 was measured using an ELISA kit according to the manufacturer's instructions.

2.12 Determination of DNA Content

Nuclei of cells fixed in 95% ethanol were stained with acridine orange (10µg/ml in HBSS) for 1 minute in the dark at room temperature. Cells were washed twice with HBSS and the cell layer was taken up in 1M NaOH. DNA content was determined by measuring the excitation and emission wavelength at 485nm and 535nm in a fluorescence microplate reader.

2.13 Western Blot Analysis

2.13.1 Protein Sample Preparation

2.13.1.1 Cell Layer Protein Isolation

Cell layer proteins isolated from preadipocytes and differentiated preadipocytes were dissolved in sample buffer containing 2% SDS solution (2% SDS, 62.5mM Tris-HCl pH 6.8). The protein concentration was assayed using the D_C Protein Assay as described previously and 100μg of protein reduced with 10% β-mercaptoethanol and bromophenol blue and heated for 5 min at 95°C.

2.13.1.2 Conditioned Medium Protein Isolation

To analyse proteins secreted by cells into the medium, cells were grown in the absence of serum. 1ml of the conditioned medium was added to 2ml ethanol and left at -20°C for 2 h to allow proteins to be precipitated. The precipitated proteins were collected by centrifugation at 1500 x *g* at 4°C, and resuspended in sample buffer (1% SDS, 62.5mM Tris-HCl, 2% β-mercaptoethanol, bromophenol blue, pH 6.8). Samples were heated at 70°C for 30 minutes, then 95°C for 5 minutes.

2.13.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were run through a 1.5mm x 100mm polyacrylamide gel by electrophoresis. Pre-stained molecular weight markers were used as standards and run alongside the samples to allow determination of molecular weights of detected proteins. The running gel was 12.5% polyacrylamide and the stacking gel 7.5% polyacrylamide, both in 0.75M Tris, 0.2% wt/vol SDS, 0.05% ammonium persulphate, 0.1% TEMED (N,N,N',N',-tetramethylethylenediamine), pH 8.8. The electrophoresis buffer contained 40mM Tris, 0.3M glycine, and 0.08% SDS, pH 8.3. The gel was run at 80V until samples reached the running gel, and 130V until the end.

2.13.3 Electroblotting of Proteins

The SDS-PAGE separated proteins were transferred to a polyvinylidene difluoride (PVDF) hybond membrane by electroblotting. The PVDF membrane was pre-soaked in methanol and both the membrane and gels were equilibrated in transfer buffer (25mM Tris, 200mM glycine, pH 8.3) for 10 minutes prior to use. The protein transfer was carried out in a vertical transfer apparatus containing transfer buffer for 2.5 h at 425mA.

2.13.4 Protein Detection

The blotted membrane was blocked by incubating in 10% BSA in Tris-buffered saline-Tween 20 (TBS-T) (10 mM Tris-HCl pH 7.5, 100mM NaCl, 0.1% Tween 20) for 1 h at room temperature on a shaker to prevent non-specific binding. The membrane was washed 3 times for 5 min with TBS-T, and placed in primary antibody at a dilution of 1:1000 in TBS-T with 10% BSA overnight at 4°C. The membrane was washed three times for 5 min in TBS-T and incubated with secondary antibody conjugated with horseradish peroxidase (HRP) at a dilution of 1:10,000 in TBS-T for 1 h at room temperature. The membrane was washed three times for 5 min in TBS-T and antigens were detected by the enhanced chemiluminescence (ECL) system after exposure to autoradiography film with intensifying screens. The time of exposure was determined empirically, and the film developed and fixed.

2.13.5 Membrane Stripping

Membranes were stripped with stripping buffer (62.5mM Tris-HCl, 2% SDS and 10% β-mercaptoethanol, pH 6.8) at 60°C for 30 min. The membrane was washed 4 times for 15 min with TBS-T, and then reprobed.

2.14 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

2.14.1 RNA Isolation

Cells grown in a 3.5cm Petri dish as a monolayer were lysed by the addition of 1ml of TRIzol reagent and passing the cell lysate several times through a pipette. The homogenised samples were transferred to a 1.5ml microfuge tube and left at 15-30°C for 5 minutes to allow dissociation of nucleoprotein complexes. 200µl of chloroform per ml of TRIzol Reagent initially used was added and the tubes were shaken vigorously by hand for 15 seconds and incubated at 15-30°C for 2-3 minutes. The samples were centrifuged at 12,000 x *g* for 15 minutes at 2-8°C and the resultant mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA in the upper aqueous phase was transferred to a new microfuge tube and RNA was precipitated by mixing with 500µl of isopropanol per ml of TRIzol Reagent initially used. The samples were incubated at 15-30°C for 10 minutes and centrifuged at 12,000 x *g* for 15 minutes at 2-8°C. The supernatant was removed and the pellet was washed with 1ml 75% ethanol per ml of TRIzol Reagent initially used. The pellet was mixed by vortexing and centrifuged at 7,500 x *g* for 5 minutes at 2-8°C. The pellet was air dried for 10 minutes and dissolved in 50µl of RNAse-free water and incubated for 10 minutes at 55°C.

2.14.2 Quantification of RNA

RNA quantification was determined by the measurement of the optical density (OD) at 260nm using a spectrophotometer. An OD of 1 is equivalent to an RNA concentration of 40μg/ml.

2.14.3 Reverse Transcription (RT)

Prior to use, RNA was heated at 70°C for 10 minutes to denature secondary structure. Synthesis of cDNA was performed using 1μg of total RNA in 5mM $MgCl_2$, 1x reverse transcription buffer, 1mM of each dNTP, 1U recombinant RNAsin ribonuclease inhibitor, 15U avian myeloblastosis virus (AMV) reverse transcriptase and 0.5μg random hexamer primers made up to a total volume of 20μl with RNAse-free water . This was incubated in a thermal cycler at 42°C for 45 minutes and then for 5 minutes at 95°C.

2.14.4 Primer Design

Gene sequences for Tie2, Angiopoietin 1, Angiopoietin 2, FGF-10 were obtained from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi (PubMed Database, National Library of Medicine). The primers were constructed using the Primer 3 software available on http://www-genome.wi.mit.edu/genome_software/other/primer3.html.

Primers used were:

Primer	Forward	Reverse	Product Size
Tie2	ccatcgagtcactgaattacagtgt	ctcgtagagttctgcacaagtcatc	131 bp
Angiopoietin 1	tggaacatgtgatggaaaattatactc	gggccatctccgacttca	84 bp
Angiopoietin 2	cgttgattttcagaggacttgga	acaaactcatttcccagccaata	84 bp
FGF-10	tcttcttcctcctccttctc	tcctctccttcagcttacag	313 bp

2.14.5 Polymerase Chain Reaction (PCR)

PCR reaction was performed with 100ng of cDNA in 1mM $MgCl_2$, 0.2mM dNTP, 2x PCR buffer, 2.5U Taq DNA polymerase, 50pmol reverse and forward primers. For Tie2, Angiopoietin 1, and Angiopoietin 2 primers the samples were initially heated to 95°C for 1 minute, then through 35 cycles of 95°C (1 minute), 58°C (1 minute), and

72°C (1 minute), with a final extension step of 72°C for 5 minutes. For the FGF-10 primer, the samples were initially heated to 95°C for 1 minute, then through 35 cycles of 95°C (1 minute), 50°C (1 minute), and 72°C (1 minute), with a final extension step of 72°C for 5 minutes.

2.14.6 Agarose Gel Electrophoresis

The products of the PCR reaction were separated by electrophoresis using a 2% wt/vol agarose gel in Tris Borate/EDTA electrophoresis buffer (TBE) (Tris 89mM, Borate 89mM, EDTA 2.5mM) at 100V for 1-2 h, and imaged on a UV gelbox. Molecular standards of a known molecular size were run to allow sizing of the resulting products.

2.15 Quantitative PCR

I thank Dr K. Boleart for carrying out the Quantitative PCR. Expression of specific mRNAs was determined using the ABI PRISM 7700 Sequence Detection System. RNA was isolated and RT was carried out to produce cDNA as previously described. Quantitative PCR was carried out in 25µl volumes on 96-well plates in a reaction buffer containing 1 x TaqMan Universal PCR Master Mix, 3mM $Mn(OAc)_2$, 200µM deoxynucleotide triphosphates, 1.25U AmpliTaqGold polymerase, 1.25U AmpErase UNG, 100-200nM Taqman probe, 900nM primers and 50ng cDNA. All reactions

were multiplexed with a probe and primers for 18S ribosomal RNA, provided as a preoptimised, primer-limited control system allowing data to be expressed in relation to an internal control reference, to allow for differences in RT efficiency. All target gene probes were labelled with FAM-6-carboxyfluorescein, and the 18S RNA gene probe was labelled with VIC. Reactions were carried out in the ABI PRISM 7700 Sequence Detector under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes; then 44 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt (ΔCt = Ct of target gene minus Ct of 18S RNA housekeeping gene). All statistics were carried out on ΔCt values before being transformed through the equation $2^{-\Delta\Delta Ct}$ to give fold changes in gene expression.

Gene sequences for VEGF and VEGFR2 were obtained from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi (PubMed Database, National Library of Medicine). The primers were constructed using the Primer Express software.

Primers used were:

Primer	Forward	Reverse	Product Size
VEGF	TACCTCCACCATGCCAAGTG **Probe:** TCCCAGGCTGCACCCATGGC	TGATTCTGCCCTCCTCCTTCT	62 bp
VEGFR2	CATGTACGGTCTATGCCATTCCT **Probe:** CATCACATCCACTGGTATTGGCAGTTGG	CGTTGGCGCACTCTTCCT	74 bp

2.16 Imunnohistochemistry

Preadipocytes and differentiated preadipocytes were grown on glass coverslips. Cells were immersed in methanol at -20°C for 10 minutes. The cells were aspirated and were incubated in blocking buffer (5% normal goat serum in TBS-T) for 1 h at room temperature. Cells were then washed once in Tris-buffered saline (TBS) (10mM Tris-HCl pH 7.5, 100mM NaCl) and incubated with primary antibody at a dilution of 1:50 made up in 3% BSA in TBS overnight at 4°C. Control cells were set up which were incubated over night in 3% BSA in TBS without primary antibody. Cells were washed twice for 5 minutes in TBS-T and once for 5 minutes in TBS and incubated for 1 h at room temperature in biotinylated secondary antibody at a dilution of 1:500 made up in 3% BSA in TBS. Cells were washed three times for 5 minutes in TBS-T and once for 5 minutes in TBS and incubated for exactly 30 minutes in 0.6% H_2O_2 in

TBS at room temperature. Slides were washed three times again for 5 minutes in TBS-T and once for 5 minutes in TBS and incubated for 1 h with 0.5-1.0ml ABC reagent made up in phosphate-buffered saline (PBS). Slides were washed twice for 5 minutes in TBS and then antigen immunoreactivity was detected using freshly prepared 3,3'diaminobenzidine (1.89 mM containing 0.03% of 30% v/v H_2O_2). Reaction progress was monitored until staining was visible and terminated by adding an equal volume of H_2O. Slides were aspirated and washed once with H_2O and viewed by light microscopy.

2.17 Statistics

All experiments in the study were performed using adipose tissue from at least three patients ($n \geq 3$). At least 3 replicates per experiment were carried out. Statistics were carried out using GraphPad InStat version 3.00 for Windows 95, GraphPad Software, San Diego California USA, (www.graphpad.com). Data were transformed to percentage mean values compared to control values to compensate for the variation in data between different patient samples. Data range of lower and upper values from which percentage values are derived are shown in the **Appendix**. One-way analysis of variance (ANOVA) was used for data analysis in this study and Dunnett post test was used to compare against control unless otherwise stated. Data are shown as mean ± standard error of the mean (SEM). *P* values <0.05 were considered significant.

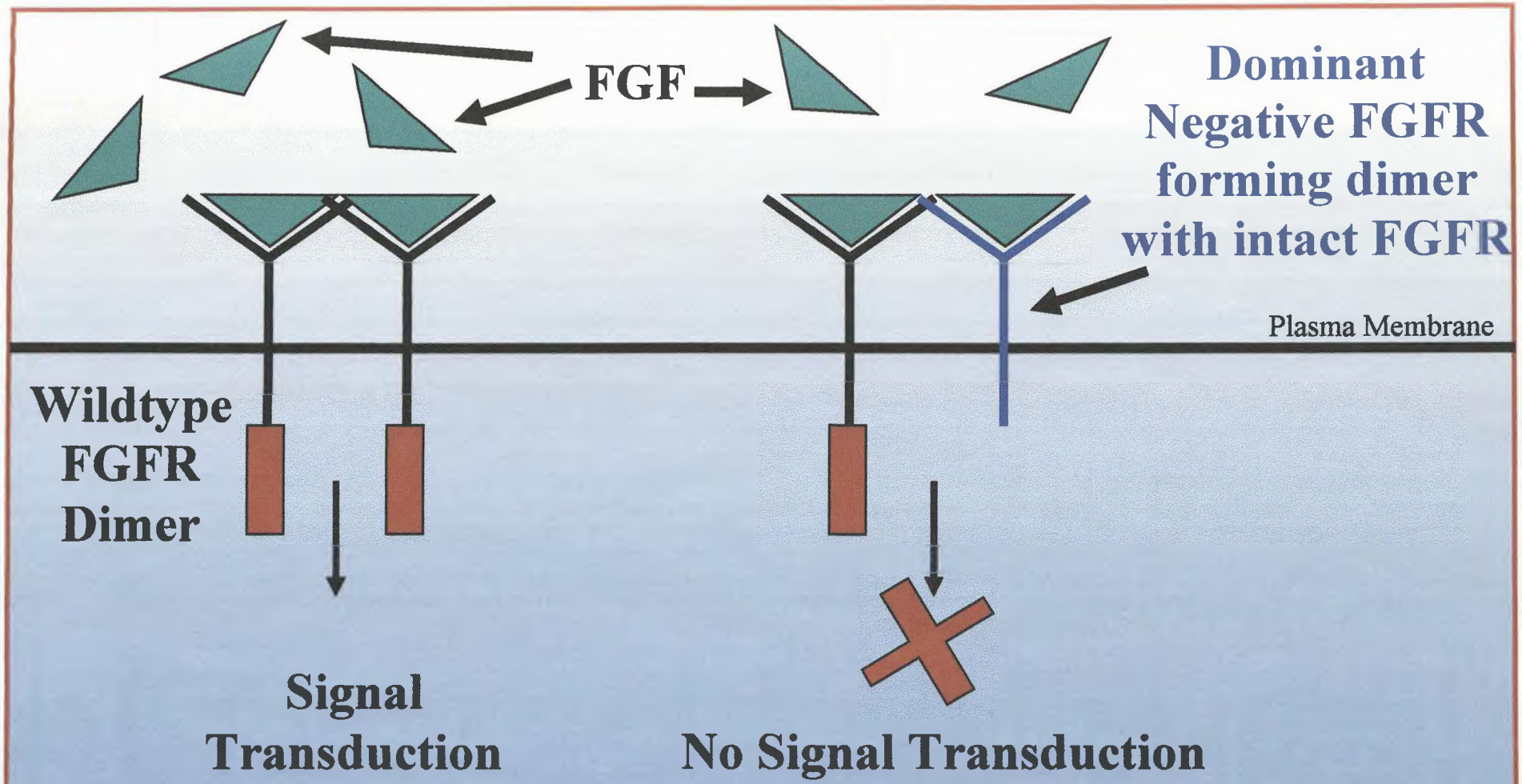


Fig 2.1 RAdDN-FGFR1 blocks FGF signalling by producing a truncated kinase-deficient FGFR1 which dimerises with native full length FGFR receptors. This dimer is no longer able to transduce signals by FGF.

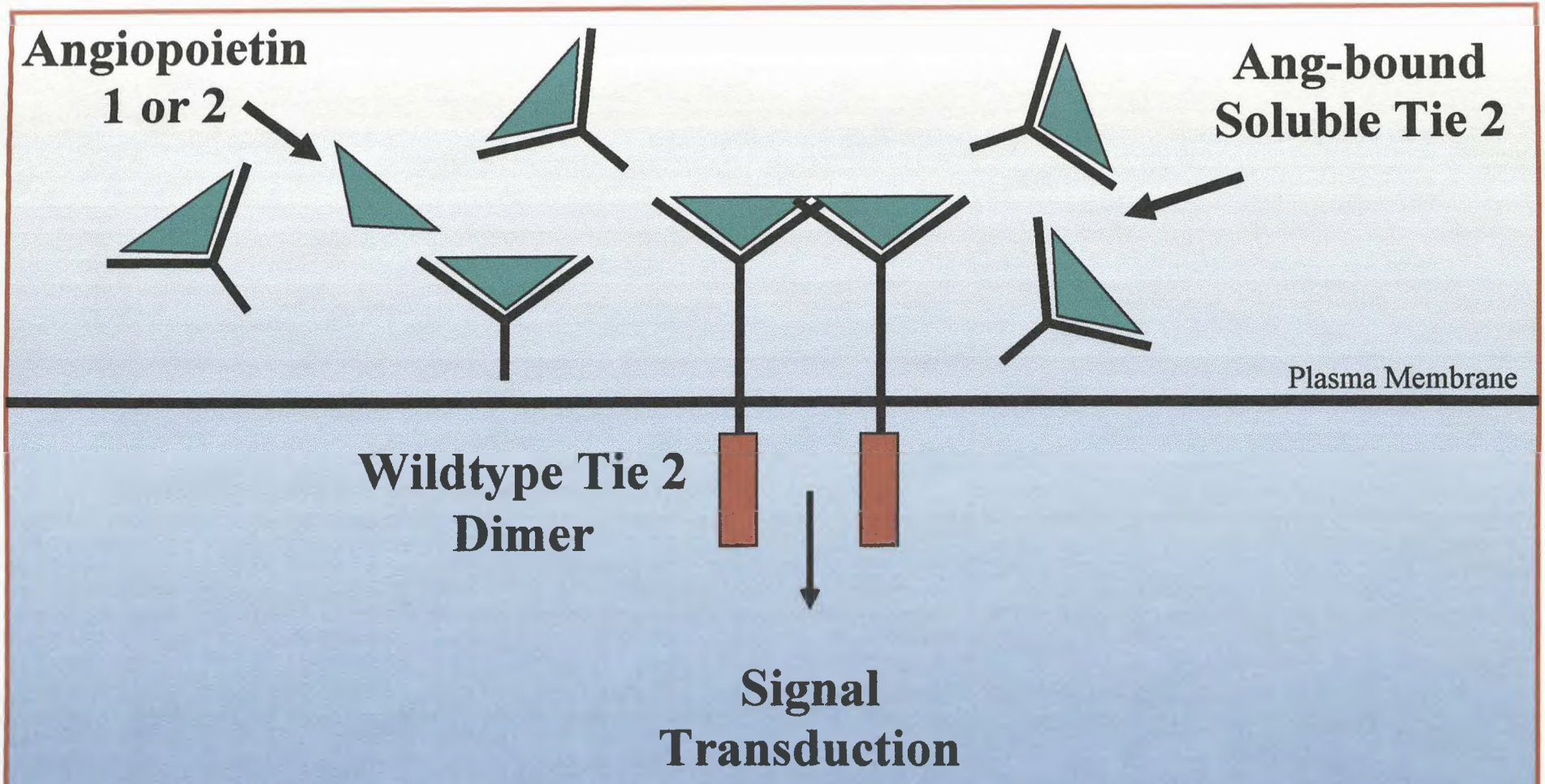


Fig 2.2 RAdExTie2 and RAdExTek block angiopoietin signalling by expressing the soluble extracellular domain of Tie 2, which binds and sequesters angiopoietins before they can bind their native cell surface receptor thus preventing angiopoietin signalling. RAd-sVEGFR1 and RAd-sVEGFR2 use the same mechanism to prevent VEGF signalling.

CHAPTER 3

Known Regulators of Adipocyte Differentiation and Their Effects on Different Parameters of Adipocyte Growth and Differentiation

3.1 Introduction

Adipocyte differentiation is regulated by hormones, growth factors and cytokines (MacDougald and Lane 1995; Ailhaud 1996; Gregoire *et al.* 1998; Sorisky 1999). Essential components include cAMP (Russell and Ho 1976), insulin (Rubin *et al.* 1978), and glucocorticoids (Hauner *et al.* 1989). Differentiation can be characterized by chronological changes in the expression of many genes. These include LPL (Ailhaud 1996), PPARγ (Chawla *et al.* 1994), GLUT4 (Kaestner *et al.* 1990), G3PDH (Spiegelman *et al.* 1983), leptin (MacDougald *et al.* 1995), resistin (Steppan *et al.* 2001) and adiponectin (Scherer *et al.* 1995; Hu *et al.* 1996b) which appear during different stages of differentiation, leading to the characteristic changes in morphology and the accumulation of triglyceride in the cytoplasm.

Several different cell lines of rodent origin such as 3T3-L1 (Green and Meuth 1974) have been used to study adipocyte differentiation *in vitro*. No human preadipocyte cells have been immortalised to cell lines but primary cultures of preadipocytes from humans, mice and rats from different anatomical sites are used. A wide variety of protocols have been developed to induce adipocyte differentiation but the effects of these components on different aspects of adipocyte growth and function (e.g. lipogenesis, leptin production, lipolysis *et al*) are unknown.

More recently the importance of the transcription factor PPAR-γ has been demonstrated in adipocyte differentiation (Chawla *et al.* 1994). In gain-of-function studies where non-adipogenic fibroblastic cells were made to express PPAR-γ, a

strong differentiation response was obtained (Tontonoz *et al.* 1994; Rosen *et al.* 2000). The addition of synthetic ligands for PPAR-γ such as rosiglitazone has shown promotion of adipogenesis *in vitro* in human adipocytes (Adams *et al.* 1997) although the requirement for insulin, dexamethasone and cAMP *in vitro* remains. Adams *et al* (1997) showed that there is enhanced lipid accumulation and G3PDH activity with rosiglitazone-induced differentiation. However other markers of adipocyte growth and differentiation were not examined (Adams *et al.* 1997).

Before embarking on the studies described in the subsequent chapters, the roles of the most frequently used supplements added to induce human adipocyte differentiation i.e. insulin, dexamethasone, T3 and IBMX, were examined in studies on lipid accumulation, ^{14}C glucose incorporation into lipid (lipogenesis), protein content, DNA content, and leptin production. The effects of these agonists alone and in combination at inducing maximal differentiation were examined. Since PPAR-γ activation has been shown to increase adipocyte differentiation, the thiazolidinedione, rosiglitazone which activates PPAR-γ, was examined. Its effects on lipid accumulation, ^{14}C glucose incorporation into lipid (lipogenesis), protein content, DNA content, and leptin production were examined individually and in combination with insulin, dexamethasone, T3 and IBMX.

3.2 Results

3.2.1 Effects of Insulin, Dexamethasone, T3, and IBMX on Protein Synthesis in Preadipocytes Incubated in DMEM/Ham's F-12 Medium

Following isolation of preadipocytes, cells were grown to confluence in DMEM/Ham's F-12 medium containing 15% foetal bovine calf serum. This takes between 2-4 days, depending on isolate. The four compounds, 100 nM insulin, 100 nM dexamethasone, 0.2 nM T3, and 0.25 mM IBMX, referred to hereafter as normal differentiation medium (Petruschke and Hauner 1993), were added individually and in combination to confluent preadipocytes in the absence of serum. IBMX was added for the first 4 days of differentiation (Russell and Ho 1976). Medium additions were maintained throughout the 20 day period and medium and compounds were changed every 2-4 days. After this 20 day period, the cell layer was removed and the amount of protein present was determined. Results of treated cells are given as percentage mean values ± SEM compared to DMEM/Ham's F-12 medium untreated control cells and are shown in **Fig 3.1**. We compared the data of the effects of the individual supplements with those control medium (no supplements) and those in normal differentiation medium. Dexamethasone and T3 both apparently reduced protein content but statistical analysis showed no significant effect when using $p<0.05$. A comparison of the effects of the individual supplements with those in normal differentiation medium was carried out. This is shown in **Fig 3.1** with * referring to a significant difference between normally differentiated cells and the cells containing individual compounds. Only dexamethasone-treated cells showed a significant

reduction in protein compared to normally differentiated cells while the other components showed no significant change from control.

3.2.2 Effects of Insulin, Dexamethasone, T3, and IBMX on Lipid Accumulation in Preadipocytes Incubated in DMEM/Ham's F-12 Medium

As an assay of differentiation, lipid accumulation in treated cells was measured. To quantify this effect, the 20 day old preadipocytes were stained with the dye Oil Red O, and the amount of stain taken up was quantified. Results of treated cells are given as percentage mean values ± SEM compared to DMEM/Ham's F-12 medium untreated control cells and corrected for protein content. Normal differentiation medium significantly increased lipid accumulation compared to control cells, but none of the compounds alone had a significant stimulatory effect on the total amount of lipid present after culture for 20 days (**Fig 3.2**). We compared the data of the effects of the individual supplements with those in normal differentiation medium. This is shown in **Fig 3.2** with * referring to a significant difference between normally differentiated cells and the cells containing individual compounds. There was significantly less lipid accumulation in all individual treatments compared to normally differentiated cells.

3.2.3 Effects of Insulin, Dexamethasone, T3, and IBMX on Lipogenesis in Preadipocytes Incubated in DMEM/Ham's F-12 Medium

To determine lipogenesis, the uptake of ^{14}C-glucose into ethanol extractable material in 20 day old, treated, preadipocytes was assessed. Results of treated cells are given as percentage mean values ± SEM compared to DMEM/Ham's F-12 medium untreated control cells. Data are corrected for protein content. Normal differentiation medium significantly increased the uptake of ^{14}C-glucose into lipid, but insulin, dexamethasone, and IBMX, when used alone had no significant stimulatory effect (**Fig 3.3**). T3 when used alone however, significantly increased lipogenesis. We compared the data of the effects of the individual supplements with those in normal differentiation medium. This is shown in **Fig 3.3** with * referring to a significant difference between normally differentiated cells and the cells containing individual compounds. There was a significantly less lipogenesis in all individual treatments compared to normally differentiated cells.

3.2.4 Effects of Insulin, Dexamethasone, T3, and IBMX on Leptin Secretion in Preadipocytes Incubated in DMEM/Ham's F-12 Medium

Preadipocytes were incubated in DMEM/Ham's F-12 medium and treated with insulin, dexamethasone, T3, and IBMX, individually and in combination over 20 days. To determine the total amount of leptin secreted, an ELISA was carried out on the conditioned medium collected over the last 4 days of the 20 day. Results of treated cells are given as percentage mean values ± SEM compared to DMEM/Ham's

F-12 medium untreated control cells (**Fig 3.4**). Secreted leptin was detectable in the medium in all conditions even the control (control leptin secretion = 0.30 ± 0.08 ng/ml). However none of the compounds when used alone had a significant effect on leptin secreted over the last 4 days of the 20 day treatment period. The combination of factors however, in normal differentiation medium (**Fig 3.4**), significantly increased leptin secreted into the medium almost 3 fold.

3.2.5 Effects of Insulin, Dexamethasone, T3, and IBMX on DNA Content in Preadipocytes Incubated in DMEM/Ham's F-12 Medium

Confluent preadipocytes were treated with insulin, dexamethasone, T3, and IBMX, for 20 days. After this 20 day period, the cell layer was removed and the amount of DNA present was determined. Results of treated cells are given as percentage mean values ± SEM compared to DMEM/Ham's F-12 medium untreated control cells. None of the compounds individually and in combination had a significant effect on DNA content (**Fig 3.5**).

3.2.6 Effects of Rosiglitazone on Preadipocyte Morphology Assessed by Changes in Cell Morphology

Preadipocytes were induced to differentiate for 20 days in medium containing insulin, dexamethasone, T3, and, for the first four days of culture, IBMX. **Fig 3.6A** shows preadipocytes before differentiation is induced. The preadipocytes have a

characteristic fibroblastic appearance. **Fig 3.6B** shows preadipocytes cultured in DMEM/Ham's F-12 medium for 20 days. Even without any additions a low level of differentiation was observed. When differentiation is induced, morphological changes occur with cells becoming spherical with cytoplasmic lipid droplets accumulating as shown in **Fig 3.6C**. The addition of rosiglitazone (10^{-6} M) to the differentiation medium markedly increased the number of visible lipid droplets as shown in **Fig 3.6D** compared with preadipocytes differentiated in differentiation medium alone shown in **Fig 3.6C**.

3.2.7 Effects of Rosiglitazone on Cell Survival, Leptin Secretion, Lipid Accumulation and Lipogenesis

To assess the effect of rosiglitazone on preadipocytes, 5 different parameters were examined. These were protein content, total leptin secretion over 20 days of differentiation, DNA content, accumulated lipid, and lipogenesis. The final two parameters have been corrected for protein content. **Table 3.1** shows the effect of the addition of rosiglitazone (10^{-6} M) to cells cultured in DMEM/Ham's F-12 medium for 20 days. Results of cells are given as percentage mean values ± SEM compared to their DMEM/Ham's F-12 medium untreated control cells. Rosiglitazone alone had no significant effect on protein content, total leptin secretion over 20 days of differentiation, DNA content, accumulated lipid, or lipogenesis.

To assess the effect of rosiglitazone on differentiation, five different parameters which were protein content, total leptin secretion over 12 days of differentiation, total DNA

content, the amount of accumulated lipid, and lipogenesis were measured. The final two parameters have been corrected for protein content. **Table 3.2** shows the effect of the addition of rosiglitazone (10^{-6} M) to cells cultured in normal differentiation medium for 20 days. Results are given as percentage mean values ± SEM compared to their control cells cultured in normal differentiation medium. The addition of rosiglitazone to the differentiation medium caused a small but significant increase of 20% in protein content of the cells ($p<0.01$). DNA content was increased by a similar amount although the effect was not statistically significant. Rosiglitazone increased the amount of lipid accumulated two-fold ($p<0.0001$) and the level of de novo lipogenesis was increased seven-fold ($p<0.0001$) when compared with cells cultured for 20 days in normal differentiation medium alone. Rosiglitazone had no effect on the total amount of leptin secreted over 20 days of preadipocyte differentiation compared with cells cultured for 20 days in normal differentiation medium alone.

3.2.8 Expression of PPAR-γ in Preadipocytes Differentiated in Normal and Rosiglitazone-Containing Differentiation Medium

Preadipocytes were induced to differentiate over 10 days in differentiation medium with and without 10^{-6} M rosiglitazone. Cell layer proteins were isolated daily from preadipocytes (Day 0) and for the subsequent 10 days throughout their differentiation (Day 10) and were analysed by Western blotting. 100μg protein was loaded in each lane of the gel, and a monoclonal antibody specific to the C-terminus of PPAR-γ was used. **Fig 3.7A** shows the daily time course of PPAR-γ expression in preadipocytes

differentiated in normal differentiation medium (Day 0-10). A band of 48 kDa was detectable in Day 3 preadipocytes and the level of PPAR-γ expression increased until Day 8 where expression of PPAR-γ fell. **Fig 3.7B** shows the time course of PPAR-γ expression in preadipocytes differentiated in differentiation medium containing rosiglitazone (Day 0-10). A band of 48 kDa was detectable in Day 1 preadipocytes and the level of PPAR-γ expression increased until Day 5 where expression of PPAR-γ fell to control levels.

3.2.9 Effects of Normal and Rosiglitazone-Containing Differentiation Medium on Protein Synthesis in Differentiating Preadipocytes Over 12 Days

Preadipocytes were induced to differentiate over 12 days in normal and rosiglitazone-containing differentiation medium. Cell layer proteins were isolated daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12) and the protein content was measured daily. Results of cells are given as percentage mean values ± SEM compared to their respective control Day 0 preadipocytes (**Fig 3.8**). As adipocyte differentiation progressed, preadipocytes differentiated in normal differentiation medium showed a significant reduction in protein, levels falling to 50% of control Day 0 levels ($p<0.01$). In preadipocytes differentiated in rosiglitazone-containing differentiation medium, a reduction in protein was seen but this was not as pronounced as in preadipocytes differentiated in normal differentiation medium, with levels reaching 68% of control Day 0 levels ($p<0.01$) at Day 12.

3.2.10 Effects of Normal and Rosiglitazone-Containing Differentiation Medium on Lipid Accumulation in Differentiating Preadipocytes Over 12 Days

Preadipocytes were induced to differentiate over 12 days in normal and rosiglitazone-containing differentiation medium. Lipid accumulation was measured as described in the Materials and Methods section daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12). Results of cells are given as percentage mean values ± SEM corrected for protein content and compared to their respective control Day 0 preadipocytes (**Fig 3.9**). As adipocyte differentiation progressed, preadipocytes differentiated in normal differentiation medium showed a significant increase in lipid accumulation at Day 7 (263% of control, $p<0.05$). Lipid content increased until day 12, with levels reaching 494% of control ($p<0.01$). In preadipocytes differentiated in rosiglitazone-containing differentiation medium there was a significant increase in lipid accumulation seen at Day 6 (363% of control, $p<0.01$) with lipid levels increasing to 696% of control ($p<0.01$) at Day 12.

3.2.11 Effects of Normal and Rosiglitazone-Containing Differentiation Medium on Lipogenesis in Differentiating Preadipocytes Over 12 Days

Preadipocytes were induced to differentiate over 12 days in normal and rosiglitazone-containing differentiation medium. Lipogenesis was measured as described in the Materials and Methods section daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12). Results of cells are given as percentage mean values ± SEM corrected for protein content and compared to their respective control Day 0

preadipocytes (**Fig 3.10**). As adipocyte differentiation progressed, preadipocytes differentiated in normal differentiation medium showed a significant increase in lipogenesis at Day 10 (3361% of control, $p<0.01$). Lipogenesis increased until day 12 with levels reaching 4893% of control ($p<0.01$). In preadipocytes differentiated in rosiglitazone-containing differentiation medium there was a significant increase in lipogenesis seen at Day 7 (8183% of control, $p<0.01$) with lipogenesis levels increasing to 14558% of control ($p<0.01$) at day 11. By Day 12 lipogenesis fell although it was still higher than control.

3.2.12 Effects of Normal and Rosiglitazone-Containing Differentiation Medium on Leptin Secretion in Differentiating Preadipocytes Over 12 Days

Preadipocytes were induced to differentiate over 12 days in normal and rosiglitazone-containing differentiation medium. Secreted leptin in the conditioned medium was measured as described in the Materials and Methods section daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12). Results are given as the daily secreted amount of leptin (ng/ml) ± SEM of 3 independent samples (n=3) (**Fig 3.11**). Leptin was initially detected on Day 4 in cells incubated in either normal or rosiglitazone-containing medium. This steadily increased as differentiation progressed with the largest level of leptin secretion seen in Day 12 differentiated preadipocytes. Unlike the other parameters measured, there was no difference in leptin secretion between normal and rosiglitazone-treated cells in differentiation medium (Normal differentiation medium leptin secretion = 4.6 ± 2.07 ng/ml, rosiglitazone containing differentiation medium = 4.5 ± 1.42 ng/ml).

3.2.13 Effects of Normal and Rosiglitazone-Containing Differentiation Medium on DNA Content in Differentiating Preadipocytes Over 12 Days

Preadipocytes were induced to differentiate over 12 days in normal and rosiglitazone-containing differentiation medium. DNA content was measured as described in the Materials and Methods section daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12). Results of cells are given as percentage mean values ± SEM and compared to their respective control Day 0 preadipocytes (**Fig 3.12**). As adipocyte differentiation progressed, preadipocytes differentiated in normal differentiation medium showed no significant change in DNA content until Day 11 where there was a significant decrease. Likewise in preadipocytes differentiated in rosiglitazone-containing differentiation medium, there was no significant change in DNA content until Day 11 when a significant decrease was seen.

3.2.14 Effects of Differing Concentrations of Insulin on Protein Content During Preadipocyte Differentiation in Normal and Rosiglitazone Differentiated Preadipocytes

Preadipocytes were induced to differentiate over 20 days in normal and rosiglitazone-containing differentiation medium with different concentrations of insulin. After this 20 day period, the cell layer was removed and the amount of protein present was determined. Results of treated cells are given as percentage mean values ± SEM compared control cells differentiated in normal and rosiglitazone-containing differentiation medium but in the absence of insulin (**Fig 3.13**). Insulin at a dose of

100nM and 1000nM caused a significant increase in protein content in normally differentiated preadipocytes. In rosiglitazone differentiated cells, insulin at all doses caused a significant increase in protein content with insulin at 100nM showing the greatest increase though this was not significantly different from other doses of insulin.

3.2.15 Effects of Insulin on Lipid Content During Preadipocyte Differentiation in Normal and Rosiglitazone Differentiated Preadipocytes

To assess the degree of differentiation, lipid accumulation in insulin treated cells was measured. To quantify this effect on lipid accumulation, the 20 day old preadipocytes were stained with the dye Oil Red O, and the amount of stain taken up and quantified by colorimetry. Results of treated cells are given as percentage mean values ± SEM compared with control cells differentiated in normal and rosiglitazone-containing differentiation medium but in the absence of insulin and corrected for protein content (**Fig 3.14**). Insulin (100nM) significantly increased lipid content in normally differentiated preadipocytes. Higher insulin concentrations had no further effects. In rosiglitazone- differentiated cells, 10nM insulin produced an increase in lipid content although this was effect not significant. At 100nM insulin, the stimulatory effects were significant and greater in the rosiglitazone-treated cells with no further effects at higher doses.

3.2.16 Effects of Insulin on Lipogenesis During Preadipocyte Differentiation in Normal and Rosiglitazone Differentiated Preadipocytes

Lipogenesis was measured in 20 day old insulin-treated, differentiated preadipocytes as described in the Materials and Methods section. Results of treated cells are given as percentage mean values ± SEM compared with control cells differentiated in normal and rosiglitazone-containing differentiation medium but in the absence of insulin and corrected for protein content (**Fig 3.15**). Insulin (100nM) significantly increased lipogenesis in normally differentiated preadipocytes with higher insulin concentrations having no further effects. In rosiglitazone-containing medium cells were more insulin-sensitive and 10nM insulin produced a significant increase in lipogenesis. There was no further increase and levels fell at a dose of 1000nM though this reduction was not significantly different from that at 10nM insulin.

3.2.17 Effects of Differing Concentrations of Rosiglitazone on Protein Content During Preadipocyte Differentiation

Preadipocytes were induced to differentiate over 20 days in normal differentiation medium with different concentrations of rosiglitazone added. After this 20 day period, the cell layer was removed and the amount of protein present was determined. Results of treated cells are given as percentage mean values ± SEM compared control cells differentiated in normal differentiation medium (**Fig 3.16**). Rosiglitazone at all concentrations caused a significant increase in protein content. Rosiglitazone at

1000nM produced the largest increase (178% of control, $p<0.01$), though there was no significant difference in protein content between doses of rosiglitazone.

3.2.18 Effects of Rosiglitazone on Lipid Content During Preadipocyte Differentiation

To assess the degree of differentiation, lipid accumulation in rosiglitazone treated cells was measured. To quantify this effect on lipid accumulation, the 20 day old preadipocytes were stained with the dye Oil Red O, and the amount of stain taken up quantified by colorimetry. Results of treated cells are given as percentage mean values ± SEM compared control cells differentiated in normal differentiation medium and corrected for protein content (**Fig 3.17**). We also analysed whether different doses of rosiglitazone caused a significant effect on lipid content. This is also shown in **Fig 3.17** with * referring to a significant difference between different doses of rosiglitazone. Rosiglitazone at all concentrations caused a significant increase in lipid content compared to control with rosiglitazone at a dose of 1000 nM (217% of control, $p<0.01$) inducing the largest increase.

3.2.19 Effects of Differing Concentrations of Rosiglitazone on Lipogenesis During Preadipocyte Differentiation

Lipogenesis was measured in 20 day old rosiglitazone treated differentiated preadipocytes as described in the Materials and Methods section. Results of treated

cells are given as percentage mean values ± SEM compared control cells differentiated in normal differentiation medium and corrected for protein content (**Fig 3.18**). We also analysed whether different doses of rosiglitazone caused a significant effect on lipogenesis. This is also shown in **Fig 3.18** with * referring to a significant difference between different doses of rosiglitazone. Rosiglitazone at all concentrations caused a significant increase in lipogenesis compared to control with rosiglitazone at a dose of 1000nM (1241% of control, $p<0.01$) causing the largest increase.

3.3 Discussion

The first observation was that the level of differentiaton observed between different patient samples varied greatly. This is due to differentiation capacity being donor-dependent and decreasing with age (Bjorntorp *et al.* 1982; Deslex *et al.* 1987b; Kirkland *et al.* 1990; Gregoire *et al.* 1995).

The effect of insulin, dexamethasone, T3, and IBMX on the differentiation of human preadipocytes was examined. Preadipocytes cultured for 20 days in DMEM/Ham's F-12 medium alone show a low level of spontaneous differentiation. This may be because preadipocytes secrete autocrine factors that can induce differentiation. When examined individually, none of the compounds except T3 was found to have any effect on protein and DNA content or on markers of differentiation including lipogenesis, lipid and leptin content. T3 had a stimulatory effect on lipogenesis when used alone and early studies have shown that T3 stimulates lipogenesis as well as

lipolysis in adipocytes (Flores-Delgado *et al.* 1987; Oppenheimer *et al.* 1991). The effects of T3 did not however approach the effects of the combination of all 4 supplements i.e. insulin, dexamethasone, T3, and IBMX confirming the requirement of these compounds in combination to induce human adipocyte differentiation.

Activation of the insulin signalling pathway causes the activation of downstream molecules, which play a role in adipocyte differentiation such as PI3K and the GLUT4 transporter protein leading to glucose uptake (Gustafson *et al.* 1999), Ras (Benito *et al.* 1991), and PKB (Magun *et al.* 1996) which have been implicated in adipocyte differentiation (Sorisky 1999). Dexamethasone has been shown to be required in number of adipocyte cell lines and primary culture systems for differentiation to occur (Deslex *et al.* 1987a; Deslex *et al.* 1987b; Hauner *et al.* 1989). Dexamethasone signals through the glucocorticoid receptor, inducing genes involved in adipocyte differentiation such as C/EBP transcription factors, which play a major role in adipocyte differentiation (Wu *et al.* 1996). IBMX inhibits phosphodiesterases thus increasing intracellular levels of cAMP (Parsons *et al.* 1988; Gregoire *et al.* 1998). Elevations in cAMP activate C/EBP-β in 30A5 preadipocytes which activates PPAR-γ, which is required for differentiation (Tae *et al.* 1995). Collectively all these signalling pathways synergise to stimulate differentiation.

Rosiglitazone alone had no effect on any of the parameters measured i.e. lipid accumulation, ^{14}C glucose incorporation into lipid (lipogenesis), DNA content, protein content, and leptin production. The addition of rosiglitazone to medium used to initiate differentiation increases adipocyte differentiation having a significant

stimulatory effect on several parameters. Rosiglitazone activates the transcription factor PPAR-γ whose expression increases when differentiation is induced. This in turn activates many genes downstream such as C/EBP-α (Grimaldi 2001), PI3K (Rieusset *et al.* 1999), and other genes as yet unknown which are required for differentiation. Our study shows that PPAR-γ expression increases when differentiation is induced. Levels of PPAR-γ do however fall later in the differentiation process. Expression of PPAR-γ was seen earlier in rosiglitazone-differentiated preadipocytes and levels were maximum when lipid droplets first appeared and fell as more lipid accumulated. In normally differentiated preadipocytes expression of PPAR-γ was detected at a later stage and for a longer period, coincidental with the later appearance and slower rate of accumulation of lipid droplets in these cells. The fall in PPAR-γ is surprising considering that it is expressed in mature adipocytes (Auboeuf *et al.* 1997; Vidal-Puig *et al.* 1997) and is required to maintain the characteristics of mature 3T3-L1 adipocytes (Tamori *et al.* 2002). However TNF-α, which is expressed late in differentiation, is known to inhibit PPAR-γ mRNA and protein expression (Zhang *et al.* 1996a; Xing *et al.* 1997) which could explain the fall in PPAR-γ expression. Furthermore PPAR-γ has been shown to antagonise C/EBP-α upregulation of leptin mRNA, which may explain why levels of leptin are reduced with rosiglitazone treatment (Hollenberg *et al.* 1997).

We found that protein levels decrease as differentiation progresses in both normal and rosiglitazone-differentiated preadipocytes with a larger drop in protein levels seen in normally differentiated preadipocytes. This agrees with our data showing that rosiglitazone-differentiated preadipocytes show increased protein levels compared to

normally differentiated preadipocytes. DNA levels decreased slightly as differentiation progressed while levels of lipogenesis, lipid, and leptin increase as differentiation progresses in normal and rosiglitazone-differentiated preadipocytes. Levels of lipogenesis and lipid were markedly higher in rosiglitazone-differentiated preadipocytes than in normally differentiated preadipocytes but the increase in leptin levels were similar in both normal and rosiglitazone-differentiated preadipocytes.

When comparing normal and rosiglitazone differentiated preadipocytes the protein and DNA content of the cell layer were modestly increased. Lipid content of cells was increased two-fold but lipogenesis was increased seven-fold compared with cells differentiated in differentiation medium without rosiglitazone. In contrast leptin secretion was not increased with rosiglitazone treatment consistent with a preferential inhibition of this marker of differentiation compared with lipogenesis.

Studies with 3T3 cells (Kallen and Lazar 1996) and short-term *in vitro* studies in mature human adipocytes showed that PPAR-γ activation reduces leptin mRNA. The data show that throughout differentiation PPAR-γ activation increases the extent and rate of differentiation into mature fat cells while having no such stimulatory effect on leptin synthesis. Consistent with its role as an insulin-sensitizer, PPAR-γ activation in human adipocytes was found to increase the transcription and insulin-induced activation of the p85 alpha subunit of PI3K (Rieusset *et al.* 1999). Since insulin is reported to be a repressor of glucocorticoid-induced leptin synthesis (Reul *et al.* 1997), as well as having antilipolytic functions (Rieusset *et al.* 2001), our study

showing no increase in leptin secretion compared with elevated lipid stores following PPAR-γ activation, is compatible with the data from other studies.

There was a significant increase in protein content when preadipocytes were differentiated in differentiation medium containing rosiglitazone but not such an increase in the DNA content. This implies that rosiglitazone has caused an increase in protein expression without an increase in cell number. The activation of PPAR-γ by rosiglitazone, the genes induced by its activation and their respective translated products may explain this apparent increase in protein levels (Hamm *et al.* 1999; Rieusset *et al.* 1999; Grimaldi 2001). Genes involved in glucose uptake and lipid accumulation are upregulated in response to PPAR-γ activation (Hamm *et al.* 1999; Rosen and Spiegelman 2000) which explains the increases seen in lipid content and lipogenesis with the addition of rosiglitazone to the differentiation-inducing medium

In summary, insulin, dexamethasone, T3, and IBMX are required for differentiation of human subcutaneous preadipocytes. The addition of the PPAR-γ activating compound rosiglitazone increases both the rate and level of preadipocyte differentiation however leptin secretion is not increased by rosiglitazone suggesting that this pathway is inhibited by PPAR-γ activation.

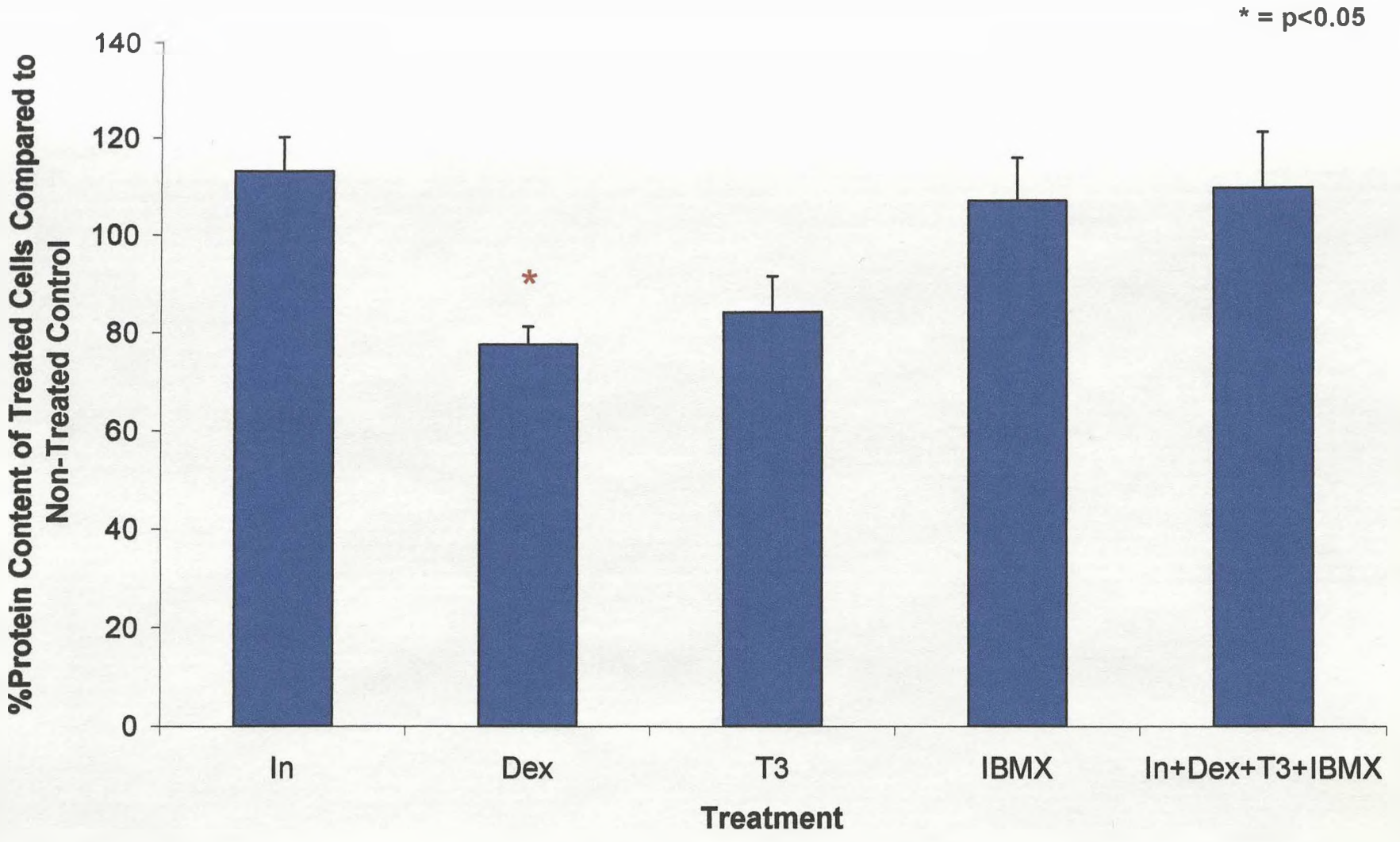


Fig 3.1 The effect of insulin, dexamethasone, T3, IBMX, and all four in combination on the protein content of preadipocytes incubated in DMEM/Ham's F-12 medium. After 20 days, protein content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to preadipocytes incubated in DMEM/Ham's F-12 medium ± SEM of 3 independent samples (n=3). * relates to statistical analysis compared to DMEM/Ham's F-12 medium control cells, while * relates to statistical analysis to normal differentiated cells compared to individual treatments.

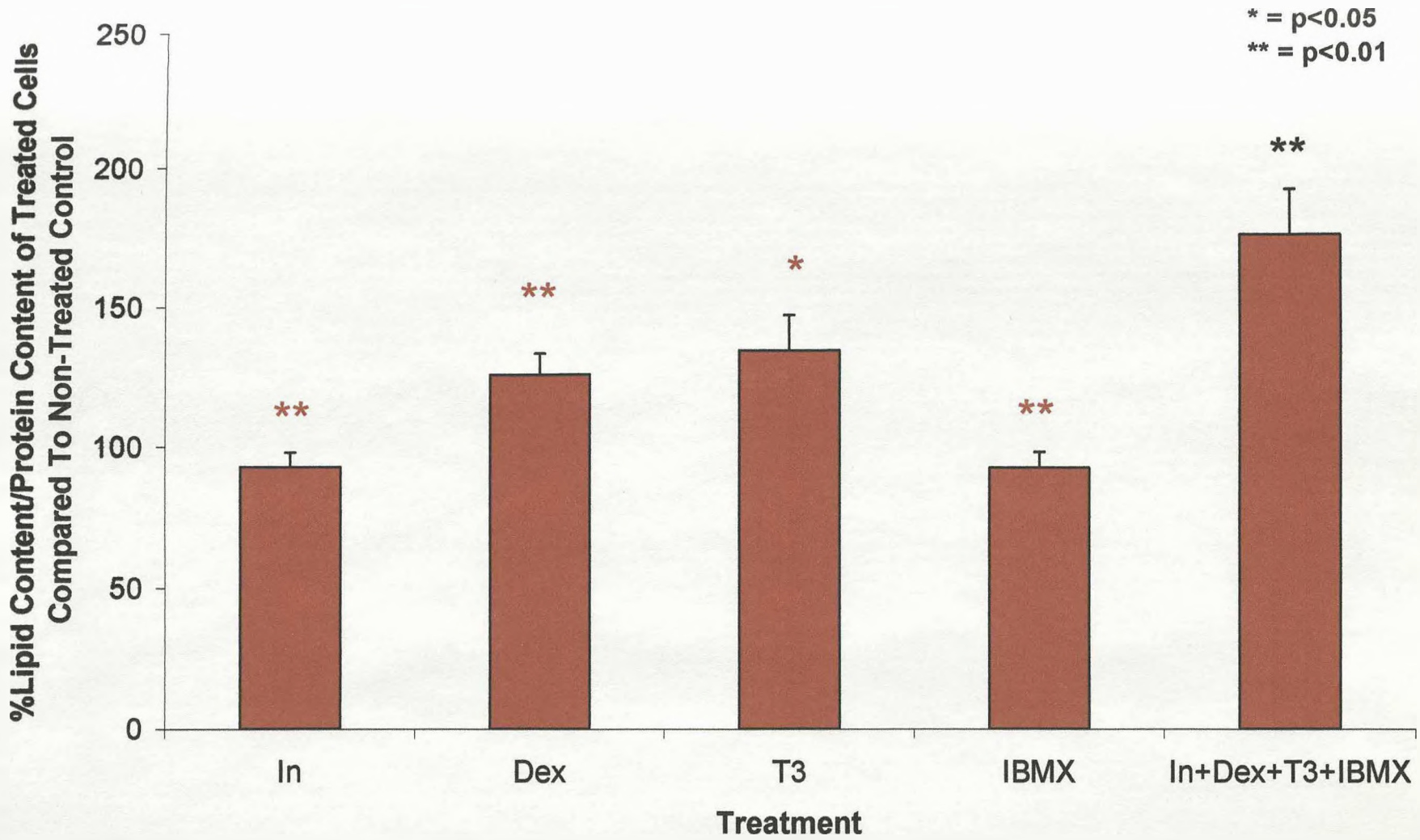


Fig 3.2 The effect of insulin, dexamethasone, T3, IBMX, and all four in combination on lipid content of preadipocytes incubated in DMEM/Ham's F-12 medium. After 20 days, lipid content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to preadipocytes incubated in DMEM/Ham's F-12 medium and corrected for protein content ± SEM of 3 independent samples (n=3). * relates to statistical analysis compared to DMEM/Ham's F-12 medium control cells, while * relates to statistical analysis to normal differentiated cells compared to individual treatments.

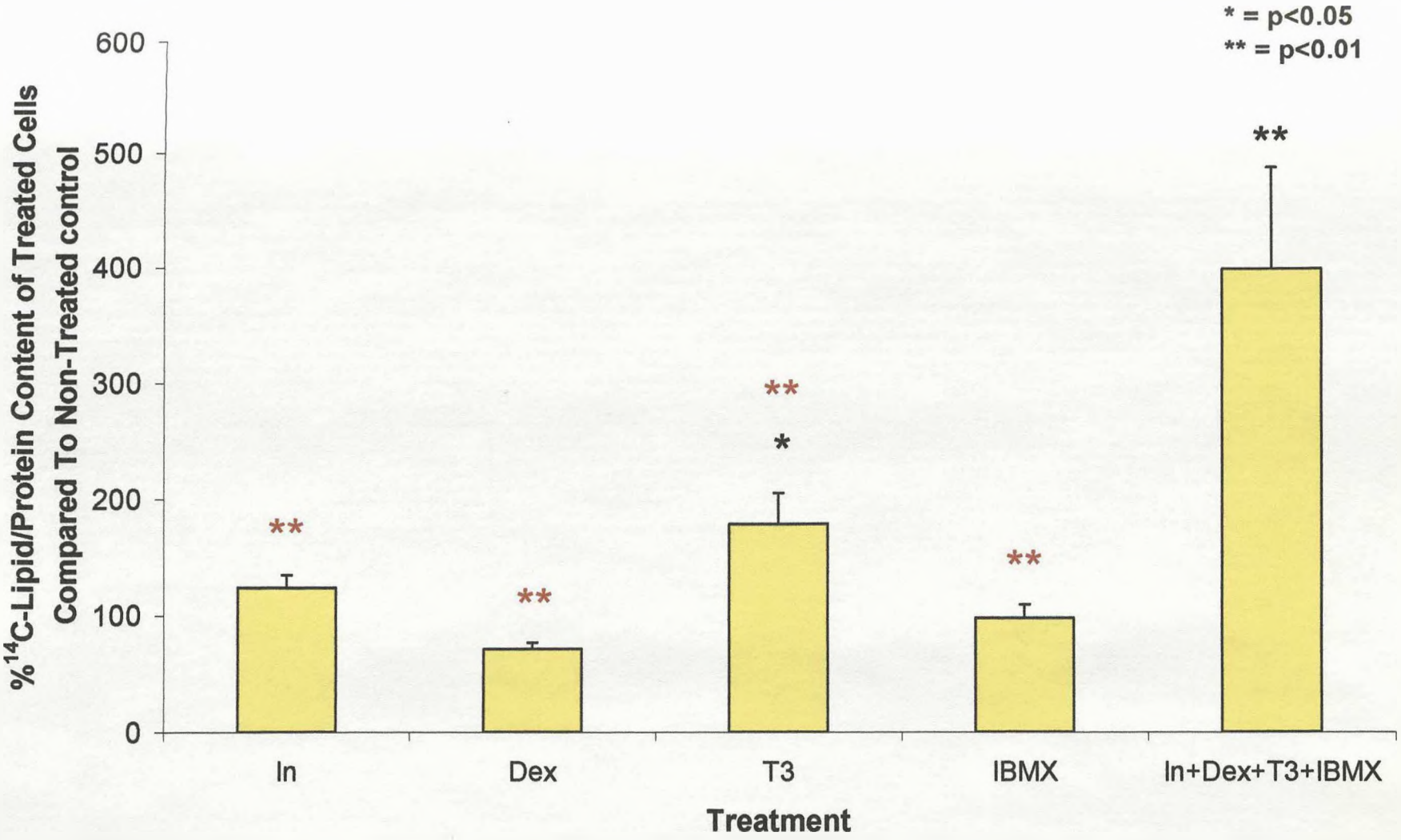


Fig 3.3 The effect of insulin, dexamethasone, T3, IBMX, and all four in combination on lipogenesis of preadipocytes incubated in DMEM/Ham's F-12 medium. After 20 days, lipogenesis was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to preadipocytes incubated in DMEM/Ham's F-12 medium and corrected for protein content ± SEM of 3 independent samples (n=3). * relates to statistical analysis compared to DMEM/Ham's F-12 medium control cells, while * relates to statistical analysis to normal differentiated cells compared to individual treatments.

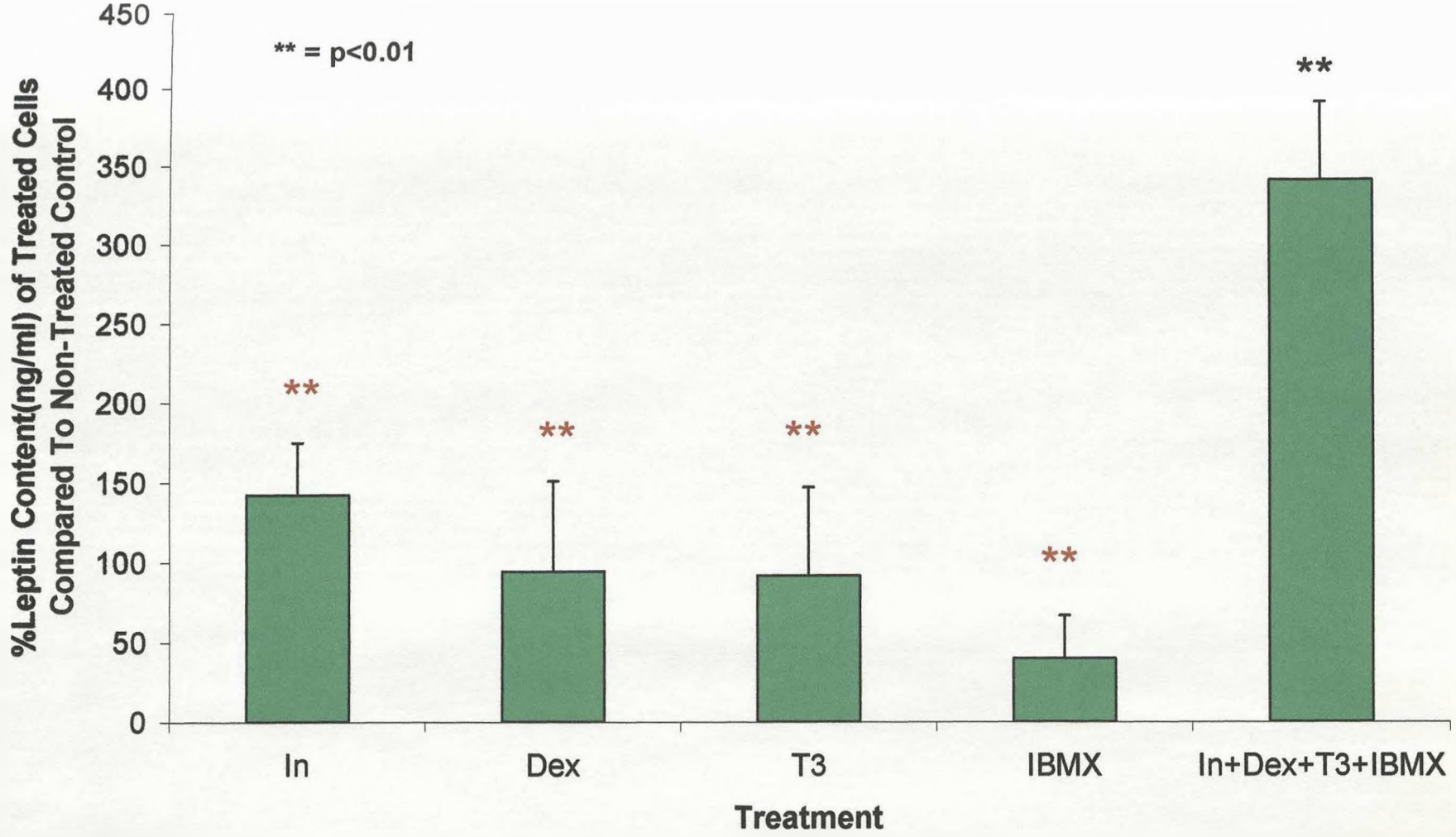


Fig 3.4 The effect of insulin, dexamethasone, T3, IBMX, and all four in combination on the leptin content of preadipocytes incubated in DMEM/Ham's F-12 medium. After 20 days, leptin content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to preadipocytes incubated in DMEM/Ham's F-12 medium ± SEM of 3 independent samples (n=3). * relates to statistical analysis compared to DMEM/Ham's F-12 medium control cells, while * relates to statistical analysis to normal differentiated cells compared to individual treatments.

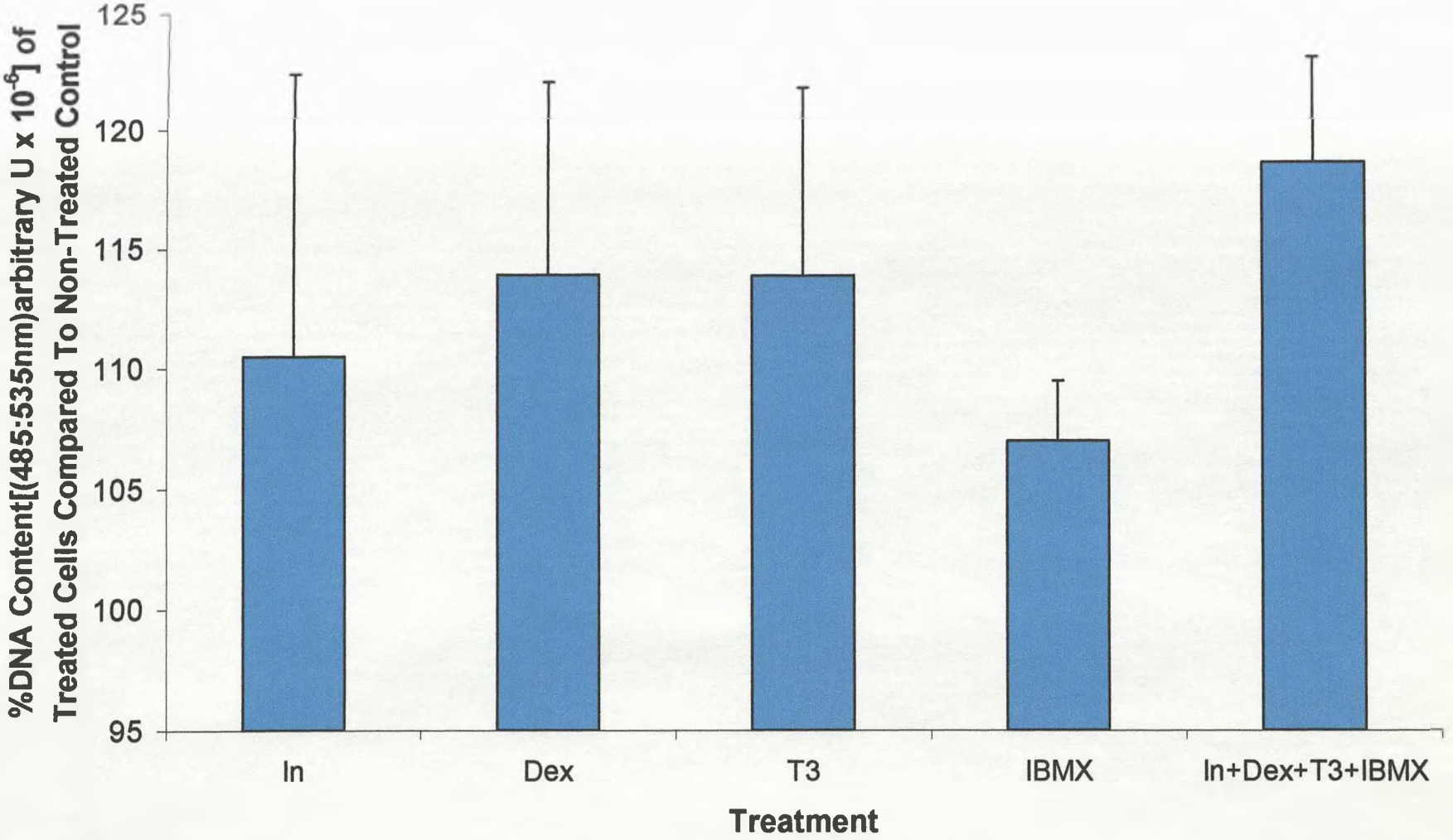


Fig 3.5 The effect of insulin, dexamethasone, T3, IBMX, and all four in combination on the DNA content of preadipocytes incubated in DMEM/Ham's F-12 medium. After 20 days, DNA content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to preadipocytes incubated in DMEM/Ham's F-12 medium ± SEM of 3 independent samples (n=3).

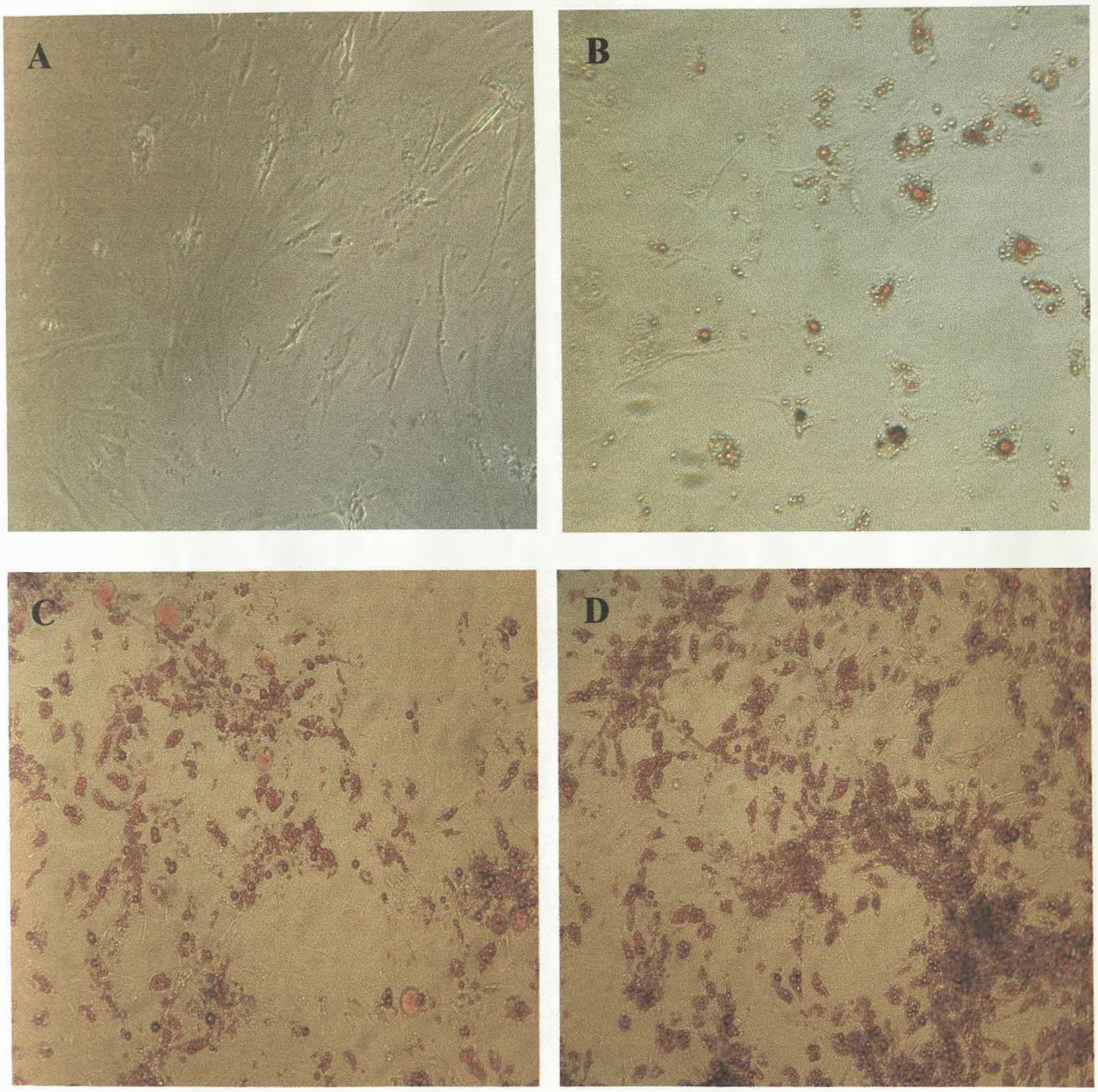


Fig 3.6 Human adipocyte differentiation. Cells have been stained with the lipid specific dye Oil Red O. (A) Day 0 human preadipocytes. (B) 20-day culture of preadipocytes in DMEM/Ham's F-12 medium. (C) 20-day culture of preadipocytes in differentiation medium. (C) 20-day culture of preadipocytes in differentiation medium with rosiglitazone (10^{-6}M). Magnification x100.

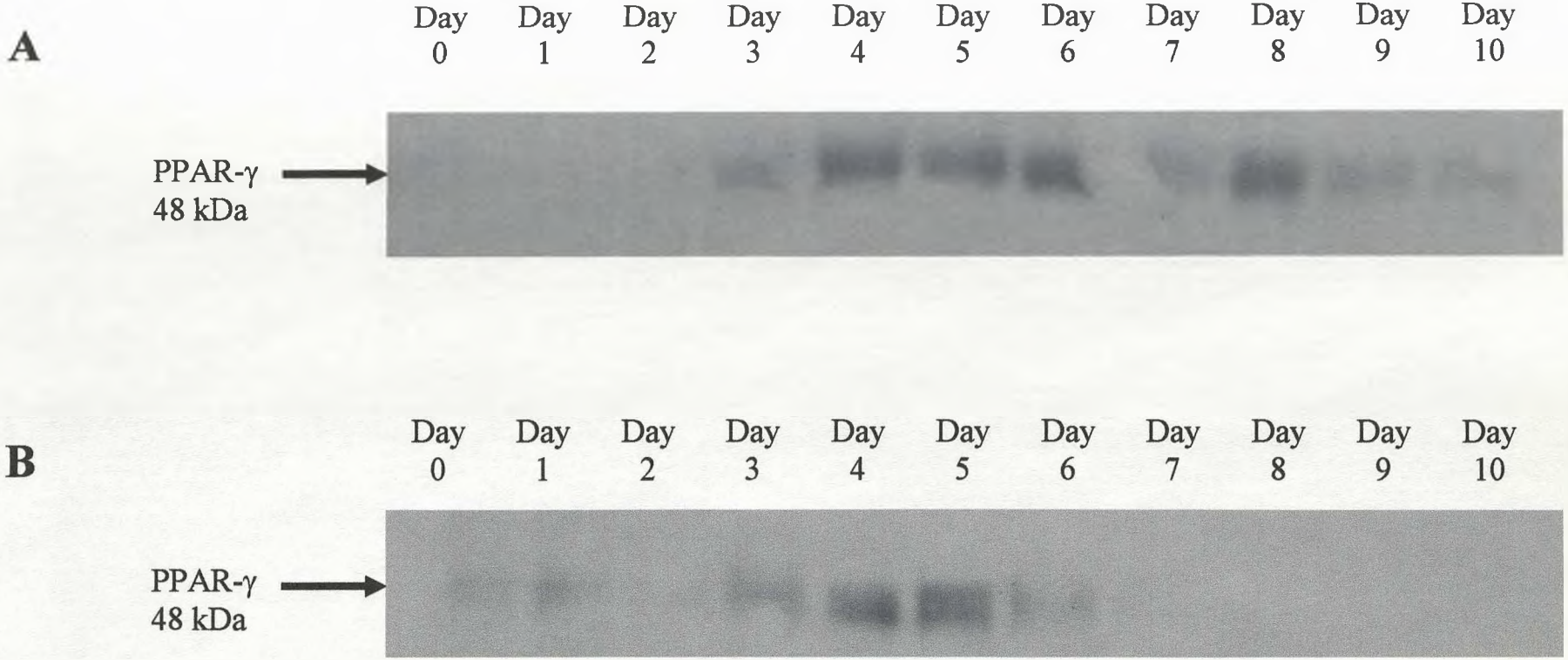


Figure 3.7 Western blot analysis of PPAR-γ protein expression over the course of preadipocyte differentiation. 100μg of protein was loaded per lane. (A) Preadipocytes differentiated over the course of 10 days in differentiation medium. (B) Preadipocytes differentiated over the course of 10 days in differentiation medium containing rosiglitazone (10^{-6}M) (n=4).

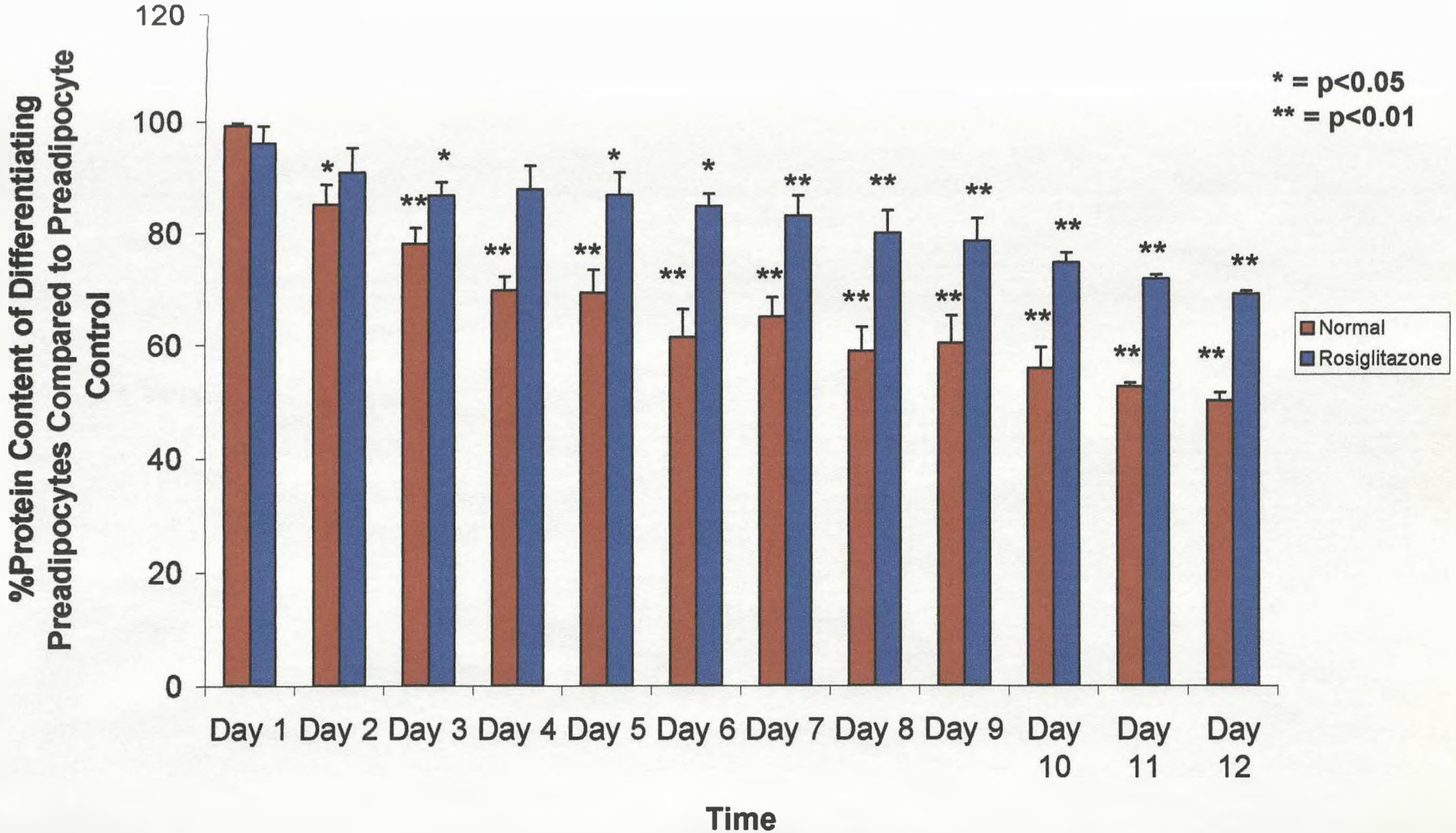


Fig 3.8 The effect on the protein content of differentiating preadipocytes with and without rosiglitazone (10^{-6}M) over 12 days. Protein content was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes ± SEM of 3 independent samples (n=3).

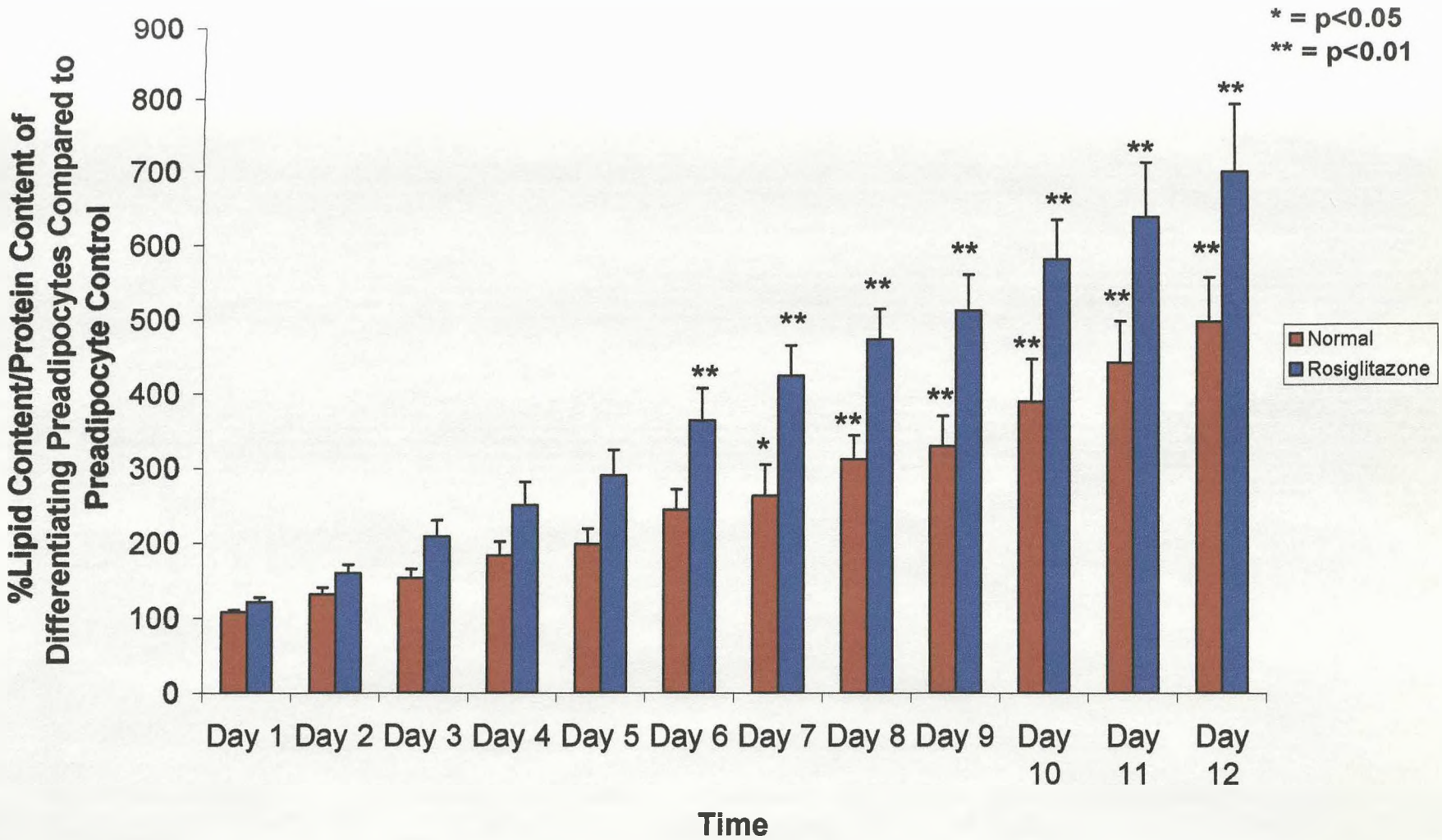


Fig 3.9 The effect on the lipid content of differentiating preadipocytes with and without rosiglitazone (10^{-6}M) over 12 days. Lipid content was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes and corrected for protein content ± SEM of 3 independent samples (n=3).

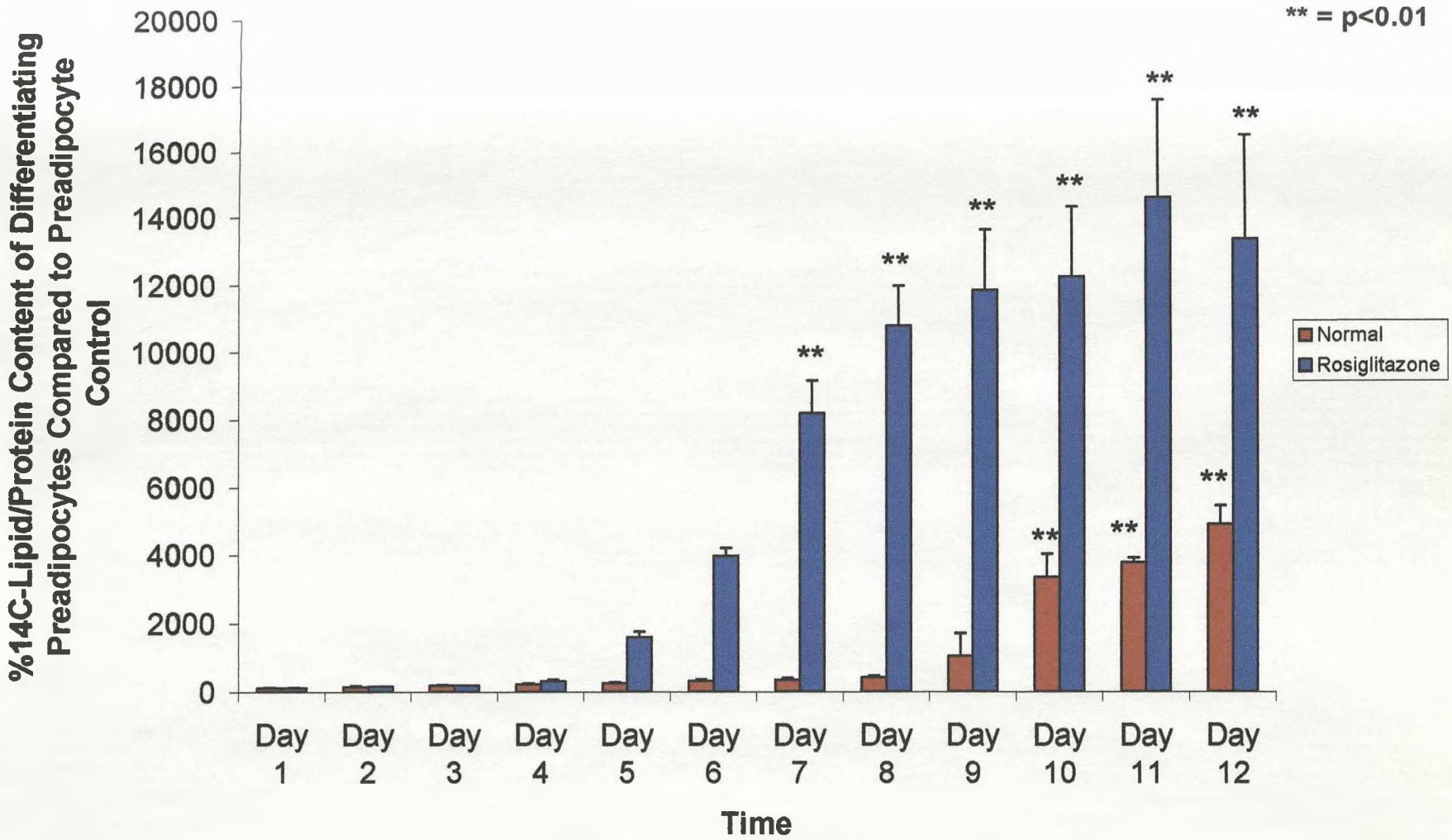


Fig 3.10 The effect on lipogenesis of differentiating preadipocytes with and without rosiglitazone (10^{-6}M) over 12 days. Lipogenesis was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes and corrected for protein content ± SEM of 3 independent samples (n=3).

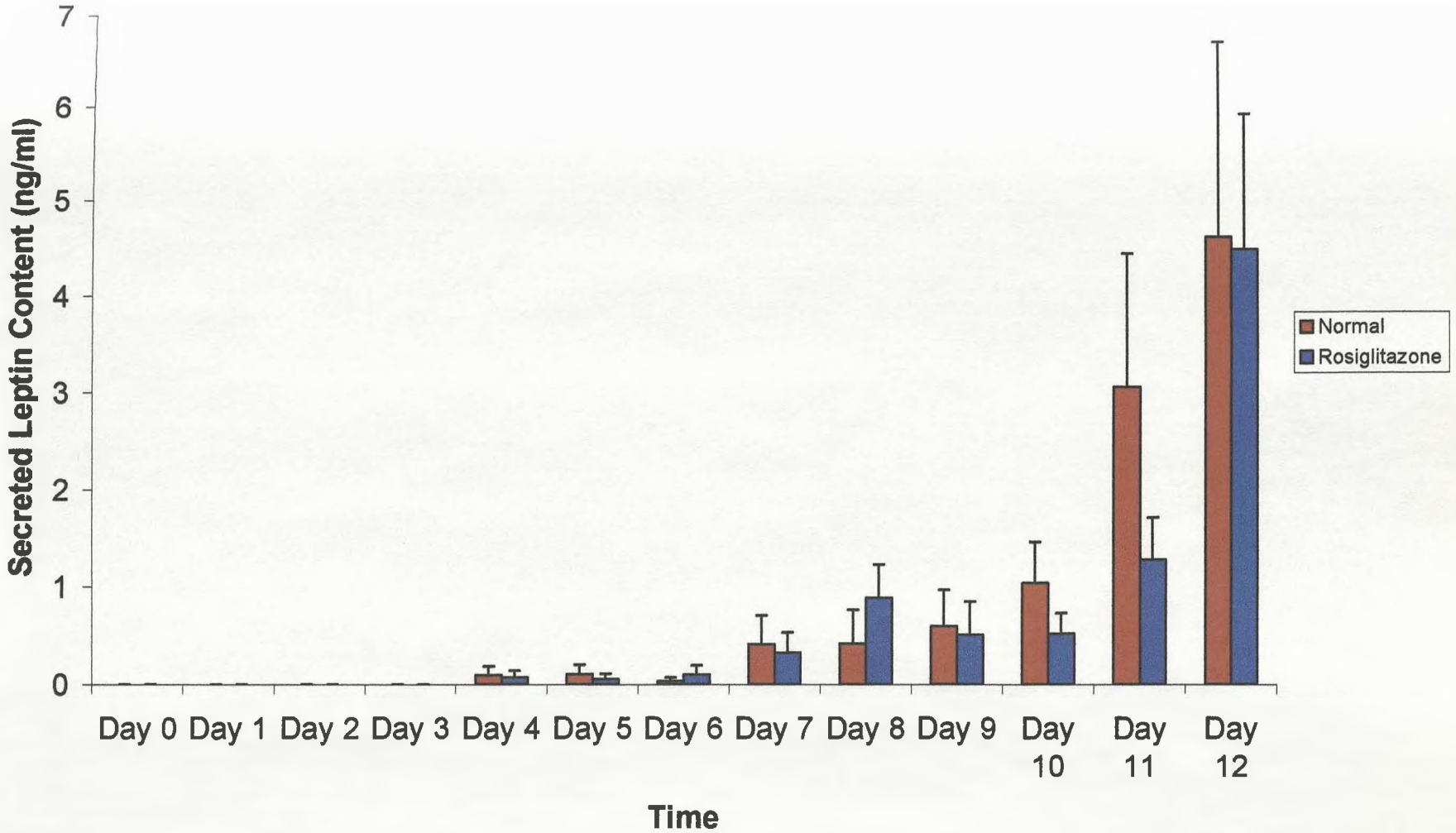


Fig 3.10 The effect on the leptin content of differentiating preadipocytes with and without rosiglitazone (10^{-6}M) over 12 days. Leptin content was measured daily as described in the Materials and Methods chapter. Results are given as the daily secreted amount of leptin (ng/ml) ± SEM of 3 independent samples (n=3).

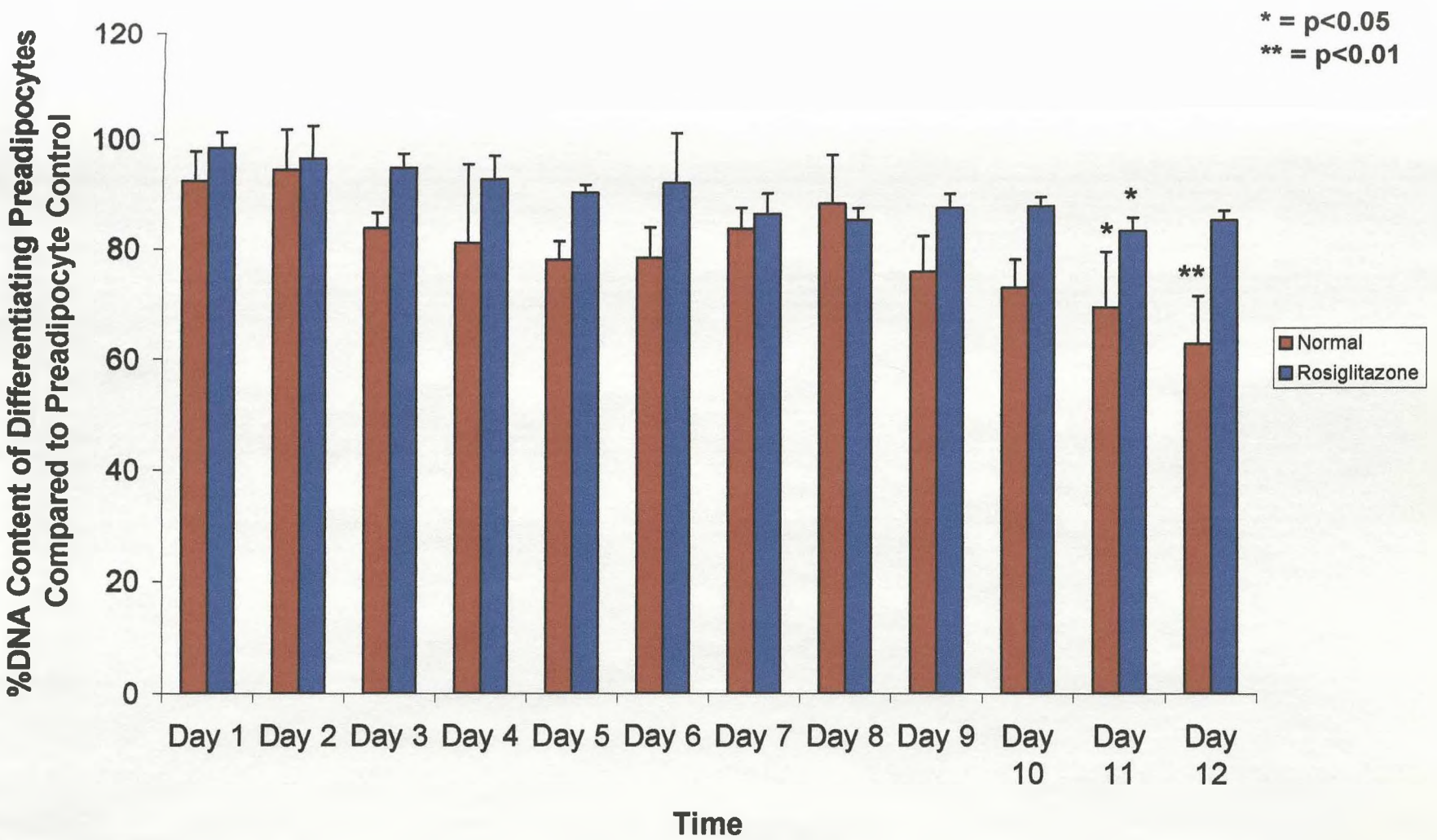


Fig 3.12 The effect on DNA content of differentiating preadipocytes with and without rosiglitazone (10^{-6}M) over 12 days. DNA content was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes ± SEM of 3 independent samples (n=3).

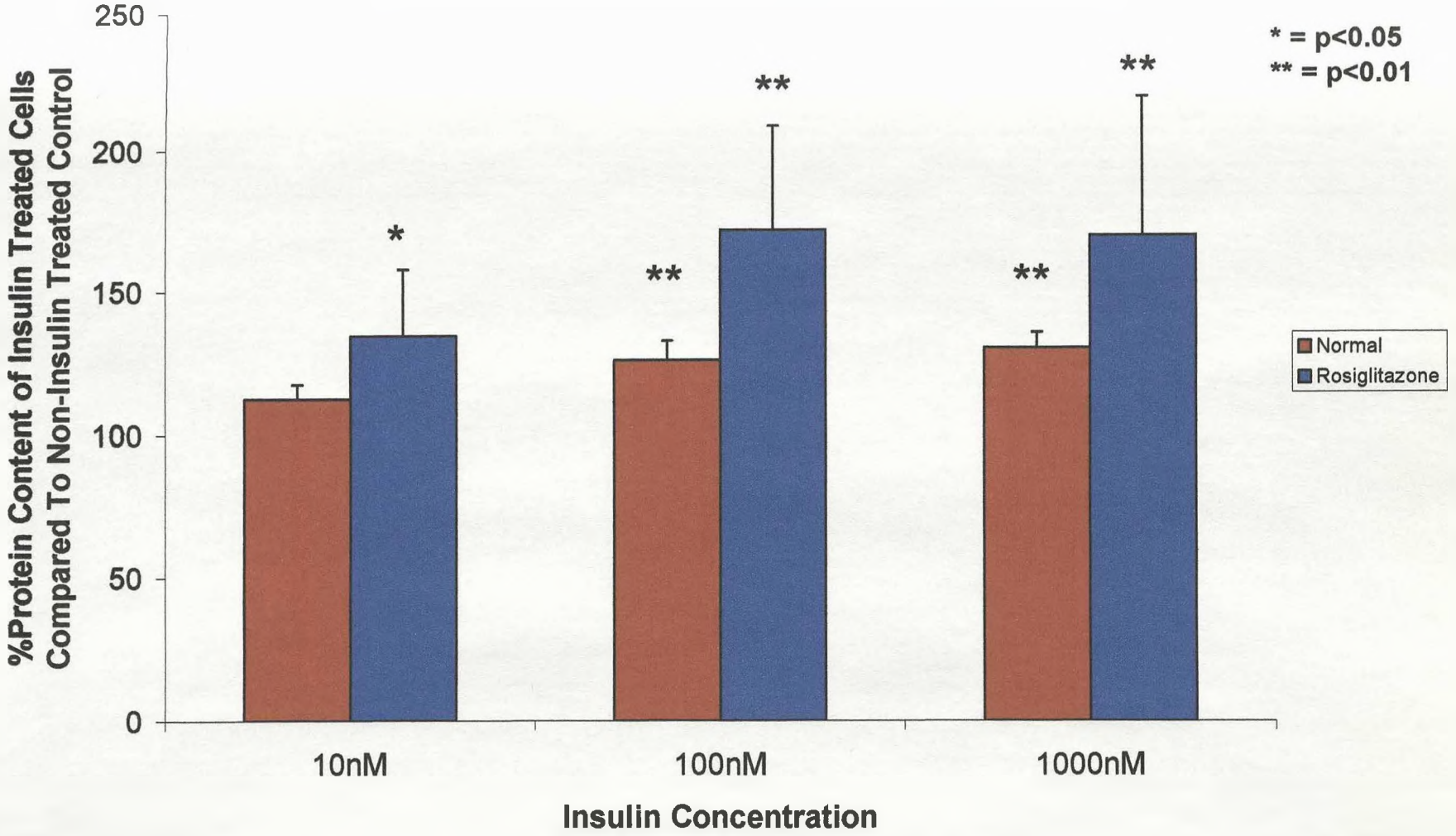


Fig 3.13 The effect of different concentrations of insulin on the protein content of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 20 days, protein content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to non-insulin treated control differentiated preadipocytes ± SEM of 5 independent samples (n=5).

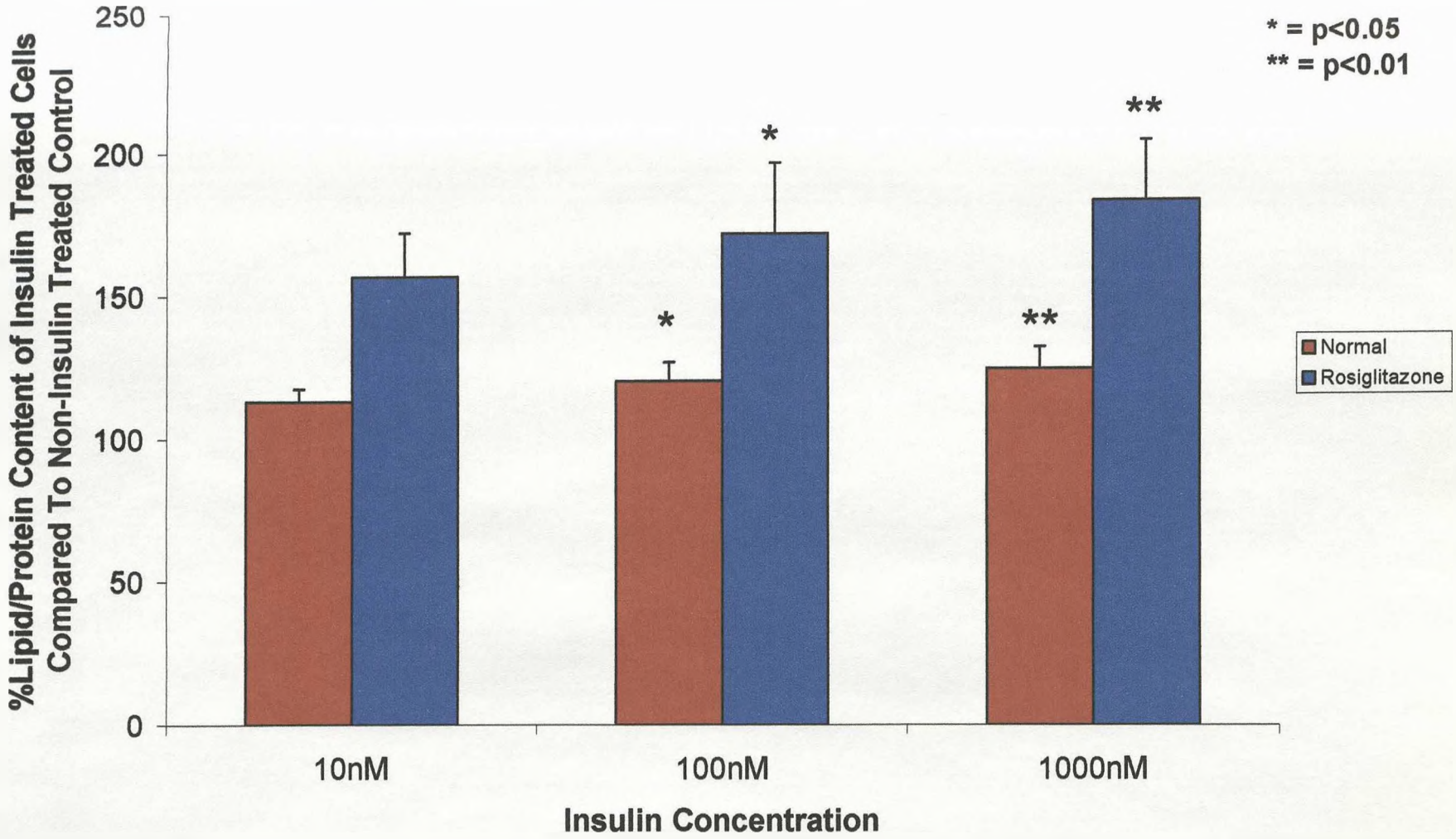


Fig 3.14 The effect of different concentrations of insulin on the lipid content of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 20 days, lipid content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to non-insulin treated control differentiated preadipocytes and corrected for protein content ± SEM of 5 independent samples (n=5).

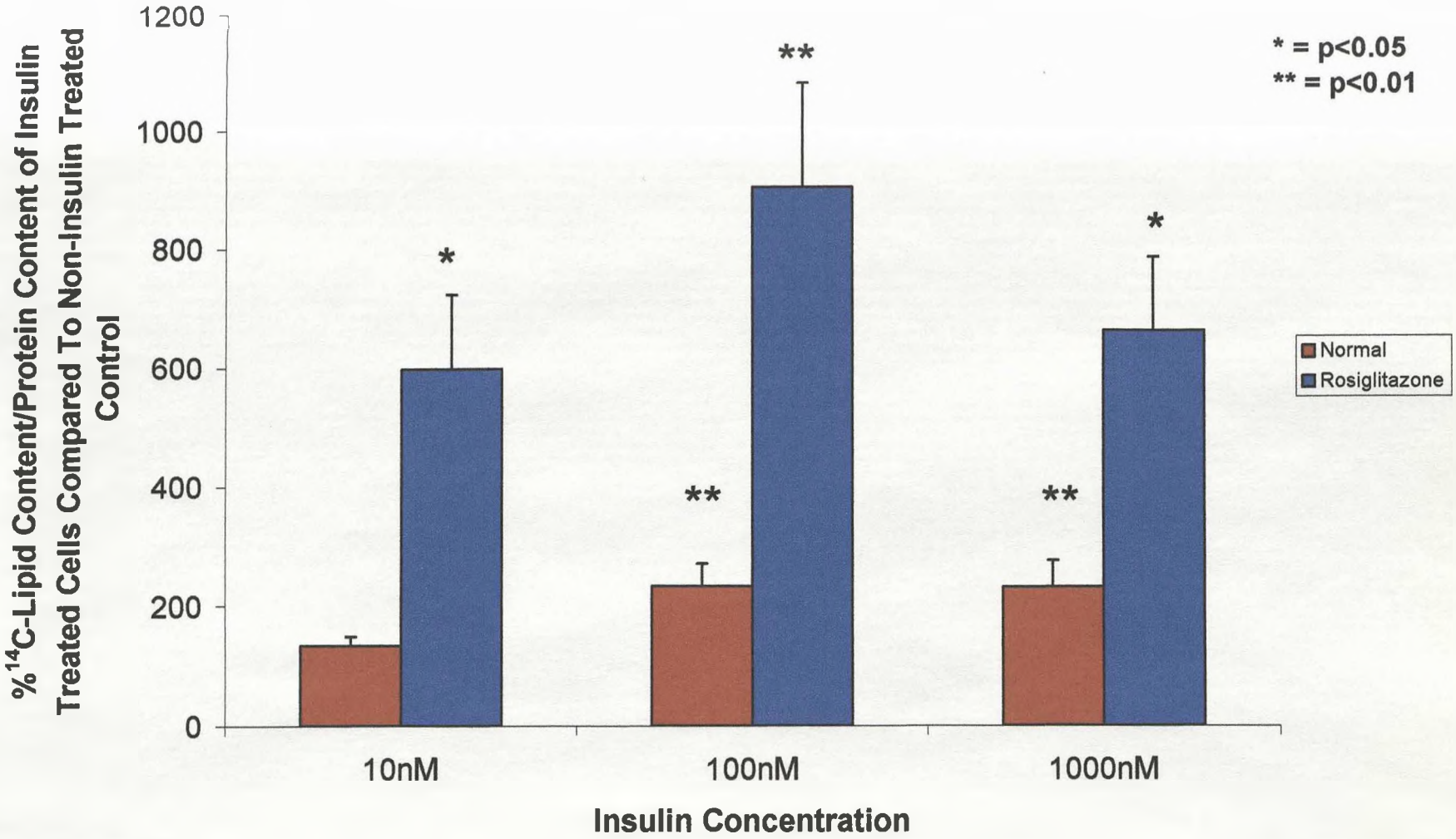


Fig 3.15 The effect of different concentrations of insulin on lipogenesis of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 20 days, lipogenesis was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to non-insulin treated control differentiated preadipocytes and corrected for protein content ± SEM of 5 independent samples (n=5).

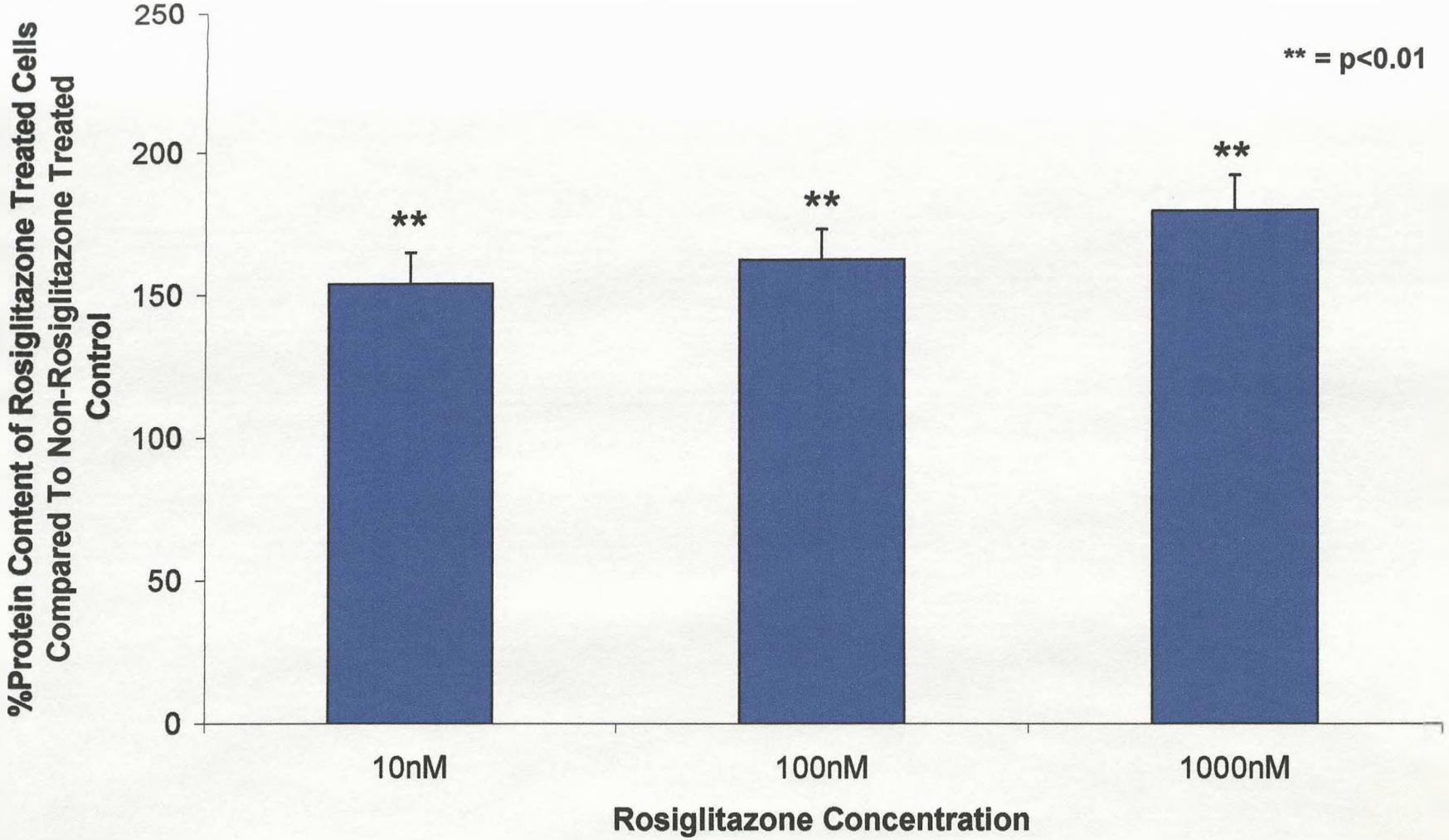


Fig 3.16 The effect of different concentrations of rosiglitazone on the protein content of differentiated preadipocytes. After 20 days, protein content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to non-rosiglitazone treated control differentiated preadipocytes ± SEM of 3 independent samples (n=3).

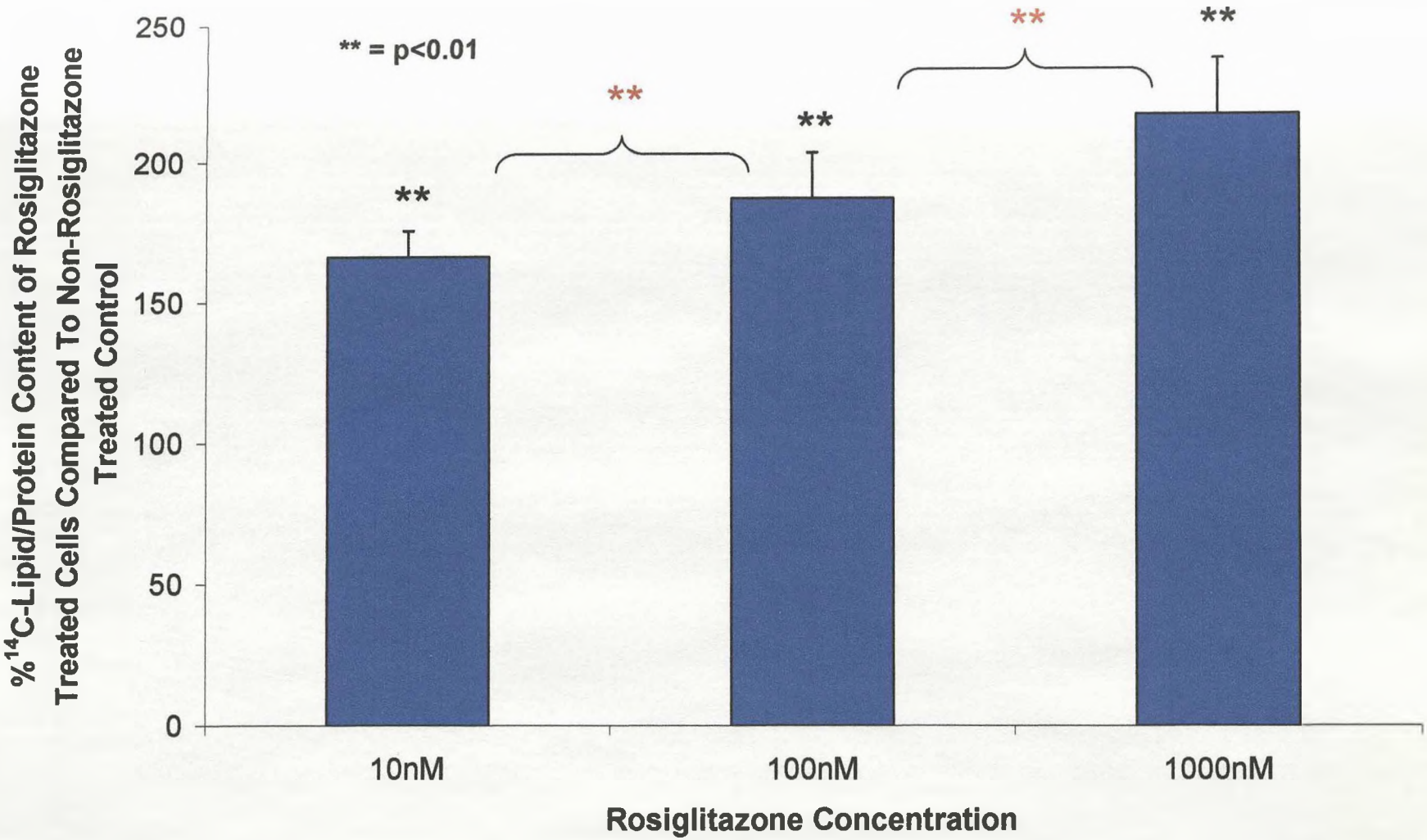


Fig 3.17 The effect of different concentrations of rosiglitazone on the lipid content of differentiated preadipocytes. After 20 days, lipid content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to non-rosiglitazone treated control differentiated preadipocytes and corrected for protein content ± SEM of 3 independent samples (n=3). * relates to statistical analysis compared to non-rosiglitazone treated control differentiated preadipocytes, while * relates to statistical analysis comparing different concentrations of rosiglitazone.

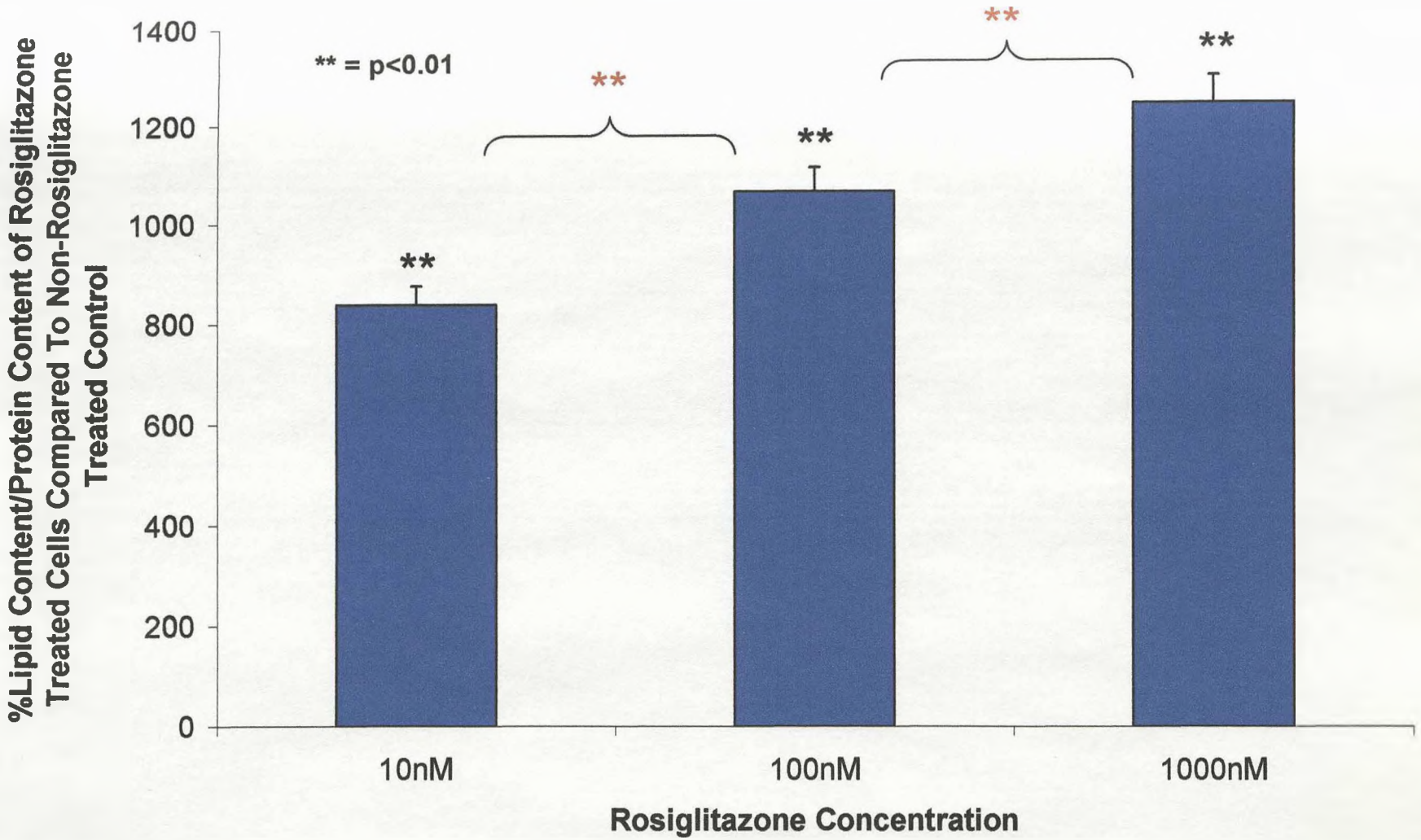


Fig 3.18 The effect of different concentrations of rosiglitazone on lipogenesis of differentiated preadipocytes. After 20 days, lipogenesis was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to non-rosiglitazone treated control differentiated preadipocytes and corrected for protein content ± SEM of 3 independent samples (n=3). * relates to statistical analysis compared to non-rosiglitazone treated control differentiated preadipocytes, while * relates to statistical analysis comparing different concentrations of rosiglitazone.

Assay	Mean ± SEM	Significance
	DMEM/Ham's F-12 Medium + Rosiglitazone	
Protein Content (mg)	124.44% ± 20.58%	p>0.05
Leptin Content (ng/ml)	97.56% ± 37.17%	p>0.05
DNA content (485:535nm) arbitrary units	99.60% ± 7.01%	p>0.05
Lipid Content (Abs 520nm) per mg protein	94.66% ± 10.89%	p>0.05
^{14}C-Lipid (DPM) per mg protein	79.81% ± 8.93%	p>0.05

Table 3.1 The effect of rosiglitazone alone on adipocyte differentiation. Preadipocytes were cultured in DMEM/Ham's F-12 medium containing rosiglitazone (10^{-6} M) for 20 days. After this period, the protein content, leptin content, cell count, lipid content and the level of lipogenesis was assessed to determine the level of differentiation. Results are given as the percentage mean value compared to preadipocytes cultured in DMEM/Ham's F-12 medium for 20 days ± SEM of 3 independent samples (n=3).

Assay	Mean ± SEM Normal Differentiation Medium + Rosiglitazone	Significance
Protein Content (mg)	120.18% ± 2.25%	p<0.01
Leptin Content (ng/ml)	78.14% ± 29.37%	p>0.05
DNA content (485:535nm) arbitrary units	115.69% ± 15.26%	p>0.05
Lipid Content (Abs 520nm) per mg protein	201.67% ± 8.26%	p<0.0001
^{14}C-Lipid (DPM) per mg protein	722.00% ± 19.42%	p<0.0001

Table 3.2 The effect of rosiglitazone on adipocyte differentiation. Preadipocytes were cultured in normal differentiation medium and differentiation medium containing 10^{-6} M rosiglitazone for 20 days. After this period, the protein content, leptin content, cell count, lipid content and the level of lipogenesis was assessed to determine the level of differentiation. Results are given as the percentage mean value compared to preadipocytes cultured in DMEM/Ham's F-12 medium for 20 days ± SEM of 3 independent samples (n=4).

CHAPTER 4

Delineating Signalling Pathways Important To Adipocyte Differentiation

4.1 Introduction

In the previous chapter, the effects on adipocyte differentiation of individual components of the differentiation-inducing medium were examined and compared with that induced by differentiation medium containing all supplements. The data indicated that the collective effects of many signalling pathways were necessary to produce a synergistic effect to induce adipocyte differentiation. Alone none of the components was effective.

This study aims to determine whether the differentiation program in adipocytes is differentially susceptible to inhibition of signalling pathways. Previous approaches to study signalling pathways include; overexpression of signalling proteins such as Ras, to determine its effect on adipocyte differentiation (Benito *et al.* 1991); the use of antisense oligonucleotides directed against specific proteins such as p42/44 MAPK (Sale *et al.* 1995); the expression of dominant-negative constructs which interfere with signalling pathways (Kotani *et al.* 1998). The major disadvantage with these methods is the expense and complexity of using these methods. For example, overexpression of proteins may be toxic to the cell and have nonspecific effects. Also the fidelity of signalling can break down giving misleading results. Antisense oligonucleotides need to be optimised to ensure the specificity of action and can also be toxic.

Recent approaches have used chemical compounds which can be used *in vitro* to inhibit specific signalling proteins. The main advantages of these compounds are the

ease of use in different cell culture systems although their efficiency of inhibition may vary in different systems due to issues with membrane permeability and the metabolism of these compounds to more or less active forms (Cohen 2003). Inhibitor compounds can enter cells within minutes so that indirect effects caused, for example, by changes in gene expression are excluded. Inhibitors are relatively inexpensive but the major drawback is with specificity. These compounds inhibit other proteins and not just the intended target protein so care must be taken when interpreting results (Borsch-Haubold *et al.* 1998). Another advantage is that inhibitor administration can be regulated but continuous administration is required to maintain the inhibitory effects.

In this study, to accomplish inhibition of signalling pathways, differentiating human preadipocytes were incubated with 4 different inhibitor compounds: rapamycin, PD98059, wortmannin, and SB203580, which inhibit $p70^{S6K}$, p42/44 MAPK, PI3K, and p38 MAPK respectively. Rapamycin inhibits the insulin-responsive kinase, $p70^{S6}$ kinase by binding with FK-506-binding protein-12 (FKBP12) and this complex interacts with mTOR causing the inhibition of $p70^{S6K}$ (Bell *et al.* 2000). PD98059 has been shown to inhibit MEK1, an enzyme, which activates p42/44 MAPK (Alessi *et al.* 1995). p42/44 MAPK plays a major role in the regulation of cell growth and differentiation in adipocytes and elsewhere (Aubert *et al.* 1999). SB203580 is a pyridinyl imidazole, which inhibits p38 MAPK (Ono and Han 2000) while wortmannin inhibits PI3K, an enzyme that selectively phosphorylates the 3-position of the inositol ring and is acutely activated by insulin and other growth factors (Evans *et al.* 1995). These compounds inhibit pathways known to be involved in cell

differentiation. We have also examined the effect of PPAR-γ activation by rosiglitazone on the effects of the inhibitors.

4.2 Results

4.2.1 Effects of Rapamycin, SB203580, PD98059 and Wortmannin on Protein Synthesis in Differentiated Preadipocytes

Preadipocytes were induced to differentiate over 20 days in differentiation medium with and without 10^{-6} M rosiglitazone. Cells were treated with 4 different inhibitor compounds at cocnentrations previously shown to be effective in other cell types; 10^{-7} M rapamycin (IC_{50} 50 pM) (Price *et al.* 1992), 10^{-5} M PD98059 (IC_{50} 2 μM) (Alessi *et al.* 1995), 10^{-7} M wortmannin (IC_{50} 5 nM) (Powis *et al.* 1994), 10^{-6} M SB203580 (IC_{50} 100 nM) (Cuenda *et al.* 1995). Stock solutions were made up in dimethyl sulfoxide (DMSO) and control cultures were treated with differentiation medium and the vehicle, DMSO. The 4 inhibitors were added to cells when differentiation was initiated and were maintained in the medium throughout the 20 day period. After this 20 day period, the cell layer was removed and the amount of protein present was determined. Results of inhibitor-treated cells are given as percentage mean values ± SEM compared to untreated control differentiated preadipocytes. None of the inhibitors had a significant effect on the total amount of protein present after 20 days of differentiation (**Fig 4.1**).

4.2.2 Effects of Rapamycin, SB203580, PD98059 and Wortmannin on Lipid Accumulation of Differentiated Preadipocytes

To assess the degree of differentiation, lipid accumulation in differentiated cells was measured. **Fig 4.2A** shows 20 day old differentiated preadipocytes, while Fig 4.2B-E shows 20 day old differentiated preadipocytes treated with rapamycin, SB203580, PD98059, and wortmannin respectively. The addition of rapamycin and SB203580 (**Fig 4.2B & 4.2C**) significantly reduced the amount of spherical lipid droplets observed while the addition of PD98059 and wortmannin (**Fig 4.2D & 4.2E**) had little effect on lipid accumulation compared to differentiated preadipocytes without inhibitor (**Fig 4.2A**).

To quantify this effect on lipid accumulation, the 20 day old preadipocytes were stained with the dye Oil Red O, and the amount of stain taken up, quantified. **Fig 4.3** shows the effect of the inhibitors on lipid accumulation of differentiated cells in the presence and absence of rosiglitazone in the differentiation medium. This was corrected for protein content. Rapamycin (74% of control, $p<0.05$) and SB203580 (77% of control, $p<0.05$) significantly reduced the level of lipid accumulation in cells, while PD98059 and wortmannin had no significant effect (**Fig 4.3**). In preadipocytes differentiated in the presence of rosiglitazone, rapamycin (63% of control, $p<0.01$) and SB203580 (52% of control, $p<0.01$) again significantly inhibited lipid accumulation, while PD98059 and wortmannin had no effect (**Fig 4.3**).

4.2.3 Effects of Rapamycin, SB203580, PD98059 and Wortmannin Inhibitors on Lipogenesis of Differentiated Preadipocytes

To determine lipogenesis, the uptake of ^{14}C-glucose in 20 day old differentiated preadipocytes was assessed. Cells had been differentiated with and without rosiglitazone in the differentiation medium and the inhibitors had been added to the cells throughout the 20 day period. This was corrected for protein content. The results are shown in **Fig 4.4**. Rapamycin (75% of control, $p<0.05$) and SB203580 (53% control, $p<0.01$) significantly reduced the uptake of ^{14}C-glucose into lipid, while PD98059 and wortmannin had no significant effect. In preadipocytes differentiated in the presence of rosiglitazone, rapamycin (67% of control, $p<0.01$) and SB203580 (53% of control, $p<0.01$) again significantly reduced the incorporation of ^{14}C-glucose into lipid, while PD98059 and wortmannin had no effect. The inhibitory effects were enhanced in the presence of rosiglitazone.

4.2.4 Effects of Rapamycin, SB203580, PD98059 and Wortmannin on Leptin Secretion from Differentiated Preadipocytes

Preadipocytes were differentiated in the presence of the 4 inhibitors (rapamycin, SB203580, PD98059, and wortmannin) over 12 days and the conditioned medium was collected daily. To determine the total amount of leptin secreted, an ELISA was performed on the conditioned medium collected daily over 12 days. Daily leptin secretion values were pooled, allowing the determination of total leptin secreted after 12 days of differentiation.

Fig 4.5 shows the effect of the 4 inhibitors on leptin secretion during differentiation over a 12 day period. The addition of SB203580 (6% of control, $p<0.01$) markedly inhibited leptin secretion while the addition of wortmannin (22% of control, $p<0.05$), rapamycin (34% of control, $p<0.05$), and PD98059 (34% of control, $p<0.05$) also significantly inhibited leptin secretion compared with control.

4.2.5 Effect of TNF-α Treatment on Phospho-p38 MAPK

Preadipocytes and differentiated preadipocytes were treated with TNF-α (100ng/ml) for different time periods. The total cellular protein of TNF-α treated preadipocytes and differentiated preadipocytes was analysed by Western Blotting. 100μg protein was loaded in each lane of the gel, and an antibody to phospho-p38 MAPK was used. Fig 4.6A shows the time course of phospho-p38 MAPK activation in preadipocytes, while Fig 4.6B shows the time course of phospho-p38 MAPK activation in differentiated preadipocytes. Equal amounts (100 ug) of protein were loaded in each lane following assessment of protein content. A single band of 43 kDa was detectable after 5 minutes incubation with TNF-α in preadipocytes (**Fig 4.6A**) and 15 minutes TNF-α incubation in differentiated preadipocytes (**Fig 4.6B**). After 1h, phospho-p38 MAPK levels reduced to undetectable levels.

4.3 Discussion

We used specific inhibitors of different signalling pathways and assessed the effect on markers of adipocyte differentiation. Inhibition of $p70^{S6K}$ and p38 MAPK inhibited adipocyte differentiation, assessed by the lipogenic activity of differentiated cells and the level of lipid accumulation within the cells. The inhibitory effects were greater for p38 MAPK inhibition. Inhibition of PI3K and p42/44 MAPK however had no inhibitory effect on lipid storage, but all 4 inhibitors markedly inhibited leptin secretion, a late marker for adipocyte differentiation.

Rapamycin has been shown to be an inhibitor of differentiation of 3T3-L1 cells (Yeh *et al.* 1995b) and also differentiation of human adipocytes (Bell *et al.* 2000). $p70^{S6K}$ has been implicated in protein synthesis especially in the transcriptional and translational regulation of insulin effects (Berven and Crouch 2000). We did not find inhibitory effects on protein synthesis over 20 days in culture suggesting that rapamycin is not cytotoxic. However new protein synthesis, which would be necessary to initiate a program of differentiation, may be limited.

In agreement with studies in 3T3-L1 cells (Engelman *et al.* 1998) we found that p38 MAPK inhibition inhibits human adipocyte differentiation. Its essential roles in inflammation, growth, differentiation, the cell cycle, and cell death have been shown in other cell types (Ono and Han 2000). In 3T3-L1 cells, the active phosphorylated form of the transcription factor C/EBP-β was reduced by SB203580 (Engelman *et al.* 1998). C/EBP-β is an activator of C/EBP-α and PPAR-γ, two potent adipogenic

transcription factors (Cowherd *et al.* 1999). Inhibition of p38 MAPK may thus inhibit C/EBP-α and PPAR-γ activation and block adipocyte differentiation (Engelman *et al.* 1998). p38 MAPK is phosphorylated in response to insulin leading to the activation of glucose membrane transporters but not translocation (Sweeney *et al.* 1999). Sweeney *et al* (1999) have proposed that the p38 MAPK pathway is required for GLUT4 transporter activation leading to insulin-stimulated uptake of glucose hence being important in lipogenesis and differentaition (Sweeney *et al.* 1999). However another study has contradicted the involvement of p38 MAPK in glucose uptake (Kayali *et al.* 2000).

We show here that TNF-α activates the p38 MAPK pathway in human preadipocytes and adipocytes consistent with data from 3T3-L1 cells (Engelman *et al.* 1998) and foetal brown adipocytes (Valladares *et al.* 2000). Although the molecular weight of p38 MAPK was slightly higher than predicted, this was the only band labelled and we conclude that the discrepancy in molecular weight is likely due to inaccuracies in the prestained markers. Since our data show that the p38 MAPK pathway is essential for differentiation, we conclude that TNF-α's inhibitory effects on differentiation are not mediated by activating the p38 MAPK signalling pathway.

The non-involvement of the p42/44 MAPK signalling pathway in adipocyte lipogenesis has been shown in some, but not all studies with 3T3-L1 cells. One report has shown that 3T3-L1 cells treated with antisense oligonucleotides to p42/44 MAPK depleted p42/44 MAPK and severely blocked adipocyte differentiation (Sale *et al.* 1995). Other reports have shown that p42/44 MAPK activation blocks adipocyte

differentiation and inhibition with PD98059, results in increased adipocyte differentiation (Hu *et al.* 1996a; Font de Mora *et al.* 1997; Porras *et al.* 1998). In human cells, PPARγ is a substrate for p42/44 MAPK and its phosphorylation leads to decreased adipocyte differentiation (Hu *et al.* 1996a; Zhang *et al.* 1996b; Camp and Tafuri 1997). A genetic mutation in PPAR-γ at this phosphorylation site has been found in obese individuals (Ristow *et al.* 1998). Blocking p42/44 MAPK should therefore increase differentiation but our studies in human adipocytes induced to differentiate in long term culture, show that other pathways downstream of p42/44 MAPK must mitigate this stimulation.

PI3K plays an essential role in the regulation of various cellular activities including proliferation, differentiation, and the prevention of apoptosis (Xia and Serrero 1999). In this study, we show that the inhibition of PI3K by wortmannin had no effect on human adipocyte lipogenesis. This contradicts the results of other studies, which have shown that wortmannin inhibits adipocyte differentiation (Tomiyama *et al.* 1995; Xia and Serrero 1999). However those studies have been carried out in cell lines such as 3T3-L1 cells where postconfluent mitoses are thought to be required early on for successful differentiation of this cell line (clonal expansion phase) (Yeh *et al.* 1995a). PI3K inhibition is likely to interfere with this phase whereas this staging is not required in human adipocytes (Entenmann and Hauner 1996). Studies looking at insulin treatment on GLUT4 levels have shown differences between 3T3-F442A and primary rat adipocytes thus reinforcing the apparent differences between cell lines and primary culture (Buren *et al.* 2002) and Ryden *et al* (2002) found constitutively active p42/44 MAPK in these cells (Ryden *et al.* 2002). However the presence of a PI3K-

independent pathway involved in glucose uptake may explain why there is no inhibition in lipogenesis (Chiang *et al.* 2001). It is surprising that wortmannin is less potent than rapamycin in our assays, since PI3K is upstream of the rapamycin target, $p70^{s6}$ kinase. Our data suggest that the pathways are not directly linked.

In contrast to the disparate effects on lipogenesis, all four inhibitors profoundly inhibited leptin secretion. Rapamycin and SB203580 inhibit adipocyte differentiation and as expected, leptin secretion is inhibited as leptin is expressed in differentiated adipocytes and the inhibitors are inhibiting differentiation (Zhang *et al.* 2000). A recent study has shown that a rapamycin-sensitive pathway is involved in the translation of leptin mRNA in response to nutrients (Roh *et al.* 2003). This may explain why leptin production is decreased in response to rapamycin. PD98059 and wortmannin, which inhibit pathways, involved in insulin signalling, inhibited leptin secretion without inhibition of lipogenesis and lipid storage. Since insulin stimulates secretion of stores of leptin and does not increase leptin expression (Bradley and Cheatham 1999), it seems unlikely that its effects on leptin gene expression are mediated through these pathways. It should however be remembered that the design of this study differs from the acute, short-term studies showing insulin-mediated inhibition of leptin mRNA (Gettys *et al.* 1996; Hardie *et al.* 1996). Indirect effects of insulin on genes important in leptin gene transcription may occur. These could include autocrine growth factors such as FGFs, IGFs, and angiopoietins, which will use some of these signalling pathways. Furthermore dexamethasone and cortisol are known to increase leptin expression and to interact with the p42/44 MAPK signalling pathway. Steroid receptors recruit p160 family members whose activity is regulated

by p42/44 MAPK (Lopez *et al.* 2001). Inhibition of p42/44 MAPK may thus inhibit glucocorticoid receptor signalling and inhibit leptin production (Bradley and Cheatham 1999).

Serum leptin has been shown to be an independent risk factor in cardiovascular disease (Soderberg *et al.* 1999; Wallace *et al.* 2001). Both insulin and rosiglitazone increase differentiation of preadipocytes and increase adipose mass. Inhibiting different signalling pathways resulted in inhibition of leptin secretion, but not always inhibition of adipocyte differentiation. This suggests that there is a redundancy in signalling as far as differentiation is concerned, but not in the regulation of leptin secretion. Rosiglitazone treatment is associated with favourable changes in adipose tissue cytokines including leptin, adiponectin and TNF-α (Shimizu *et al.* 1998; Combs *et al.* 2002; McTernan *et al.* 2002). Our data indicate that this is due to differential effects of PPAR-γ agonists on differentiation as opposed to leptin secretion.

In summary, leptin secretion, a late marker for adipocyte differentiation is inhibited by all four inhibitors suggesting that the pathways they inhibit are essential for leptin secretion. In contrast, our results show that in human adipocytes only the $p70^{S6K}$ and p38 MAPK pathways are important for induction of lipogenesis, while PI3K and p42/44 MAPK pathways are not.

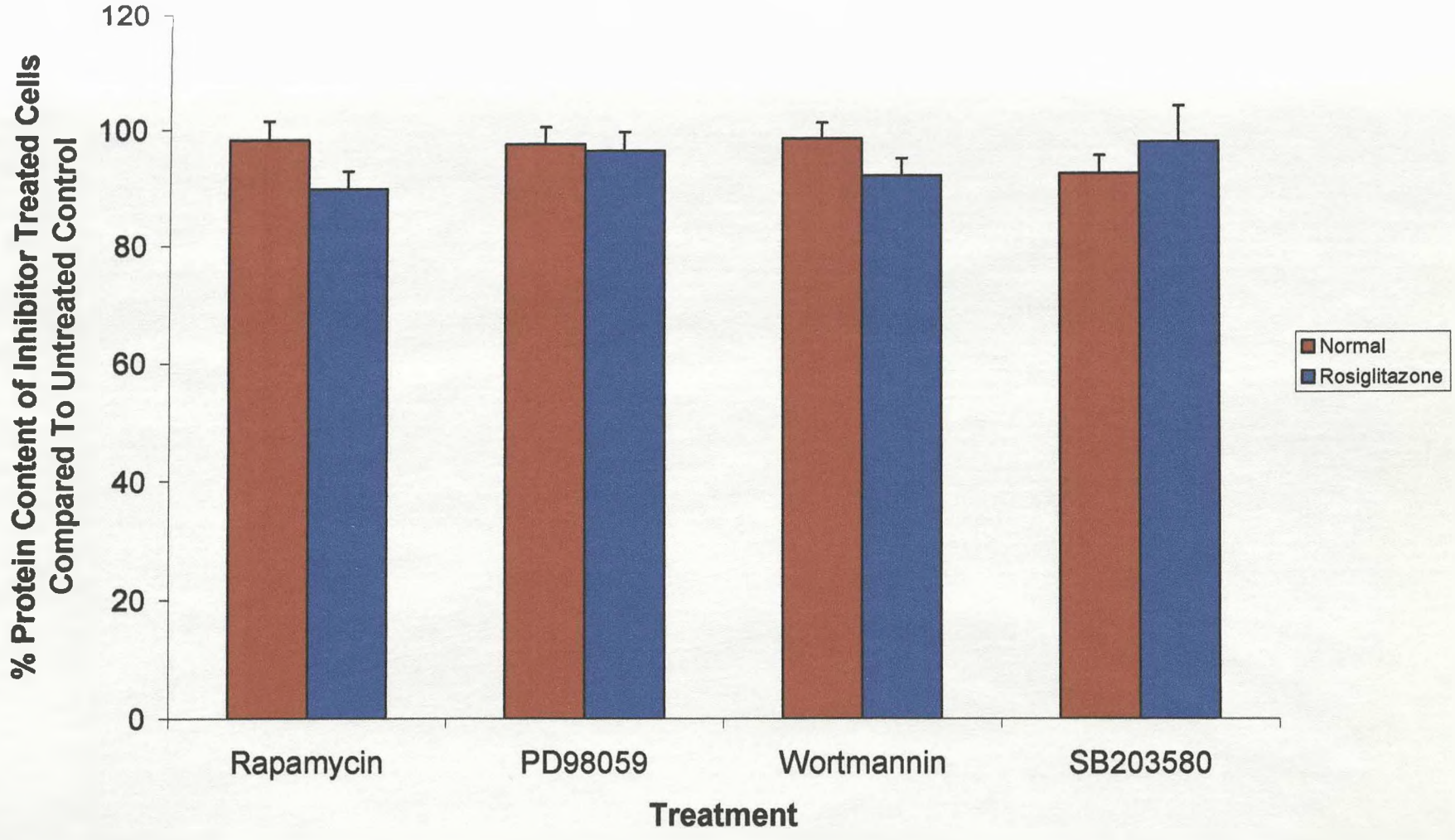


Fig 4.1 The effect of the 4 inhibitors on the protein content of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 20 days, protein content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to untreated control differentiated preadipocytes ± SEM of 4 independent samples (n=4).

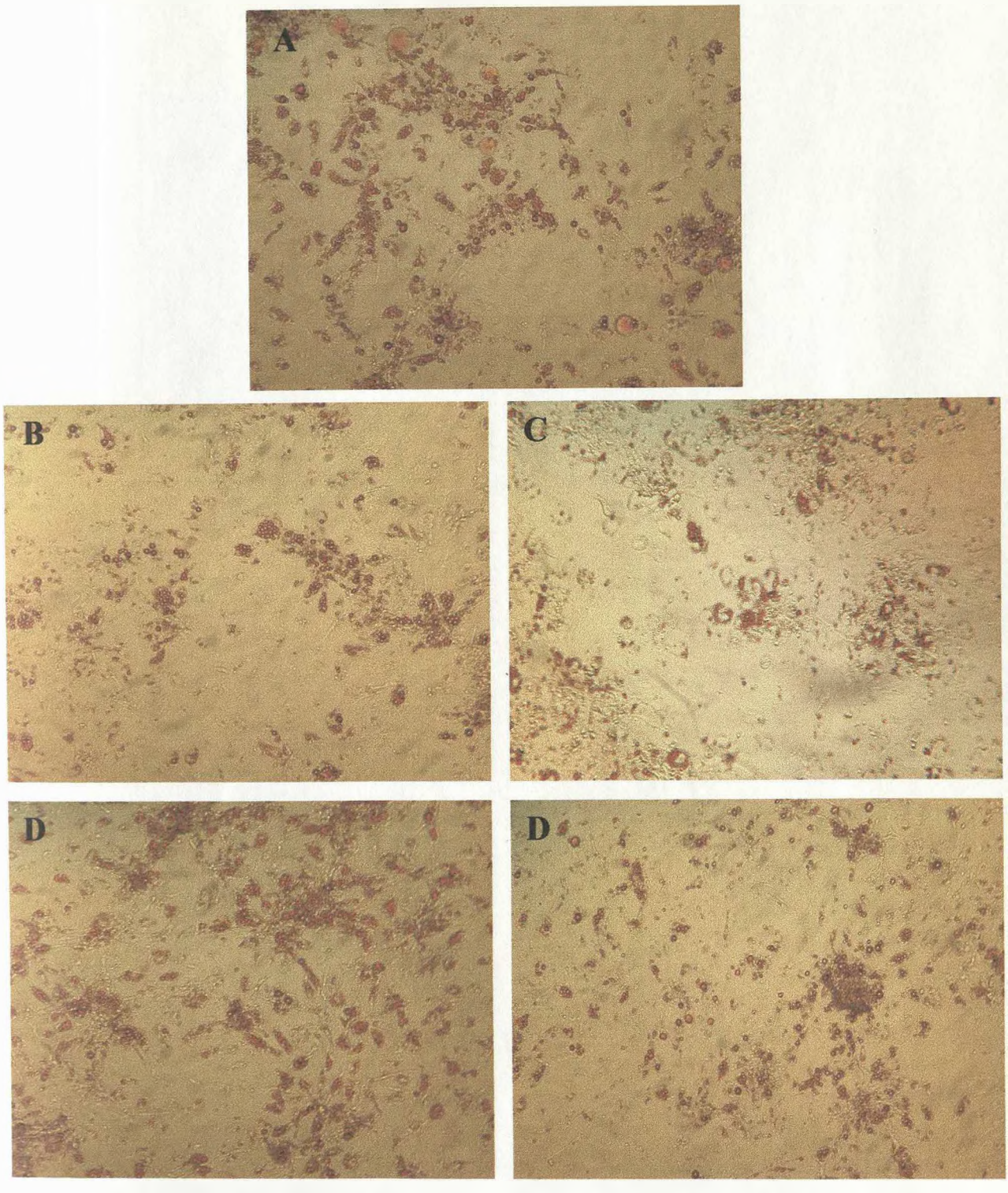


Fig 4.2 The effect of the inhibitors on adipocyte differentiation. Cells have been stained with the lipid specific dye Oil Red O. (A) 20-day culture of preadipocytes in differentiation medium. (B) 20-day culture of preadipocytes in differentiation medium and rapamycin (10^{-7}M). (C) 20-day culture of preadipocytes in differentiation medium and SB203580 (10^{-6}M). (D) 20-day culture of preadipocytes in differentiation medium and PD98059 (10^{-5}M). (E) 20-day culture of preadipocytes in differentiation medium and wortmannin (10^{-7}M). Magnification x100.

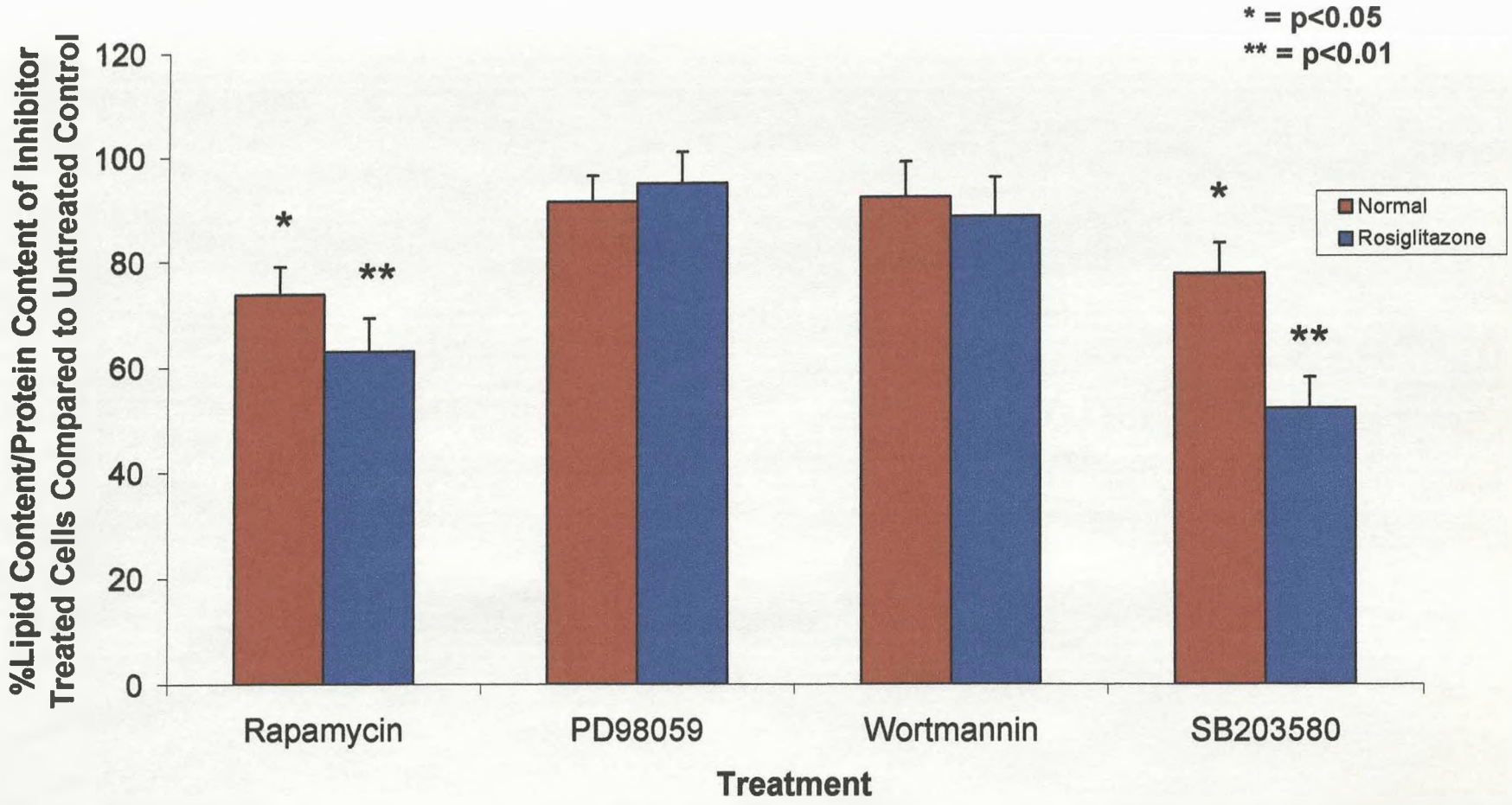


Fig 4.3 The effect of the 4 inhibitors on the lipid content of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 20 days, lipid content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to untreated control differentiated preadipocytes and corrected for protein content ± SEM of 4 independent samples (n=4).

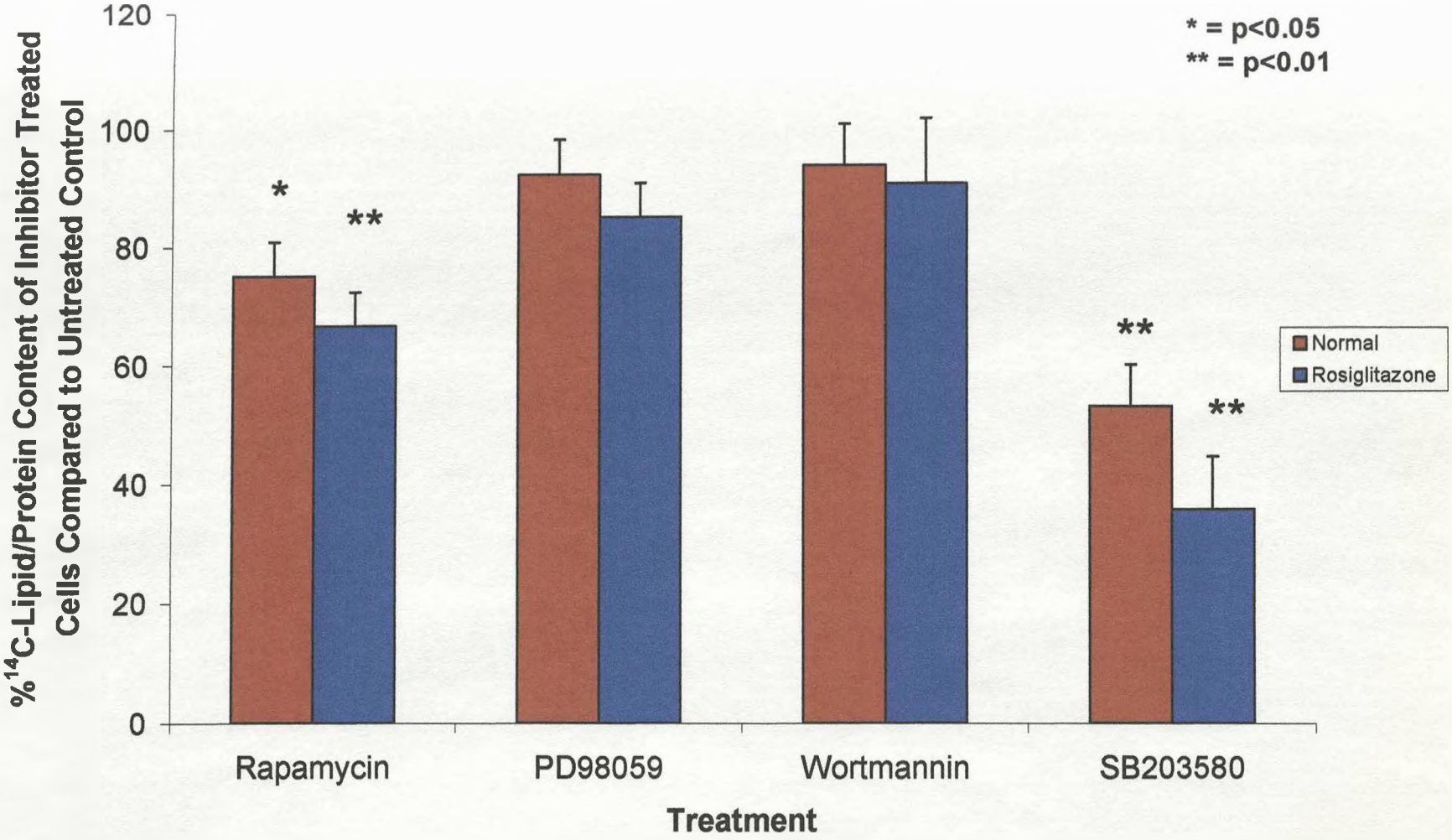


Fig 4.4 The effect of the 4 inhibitors on lipogenesis of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 20 days, lipogenesis was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to untreated control differentiated preadipocytes and corrected for protein content ± SEM of 4 independent samples (n=4).

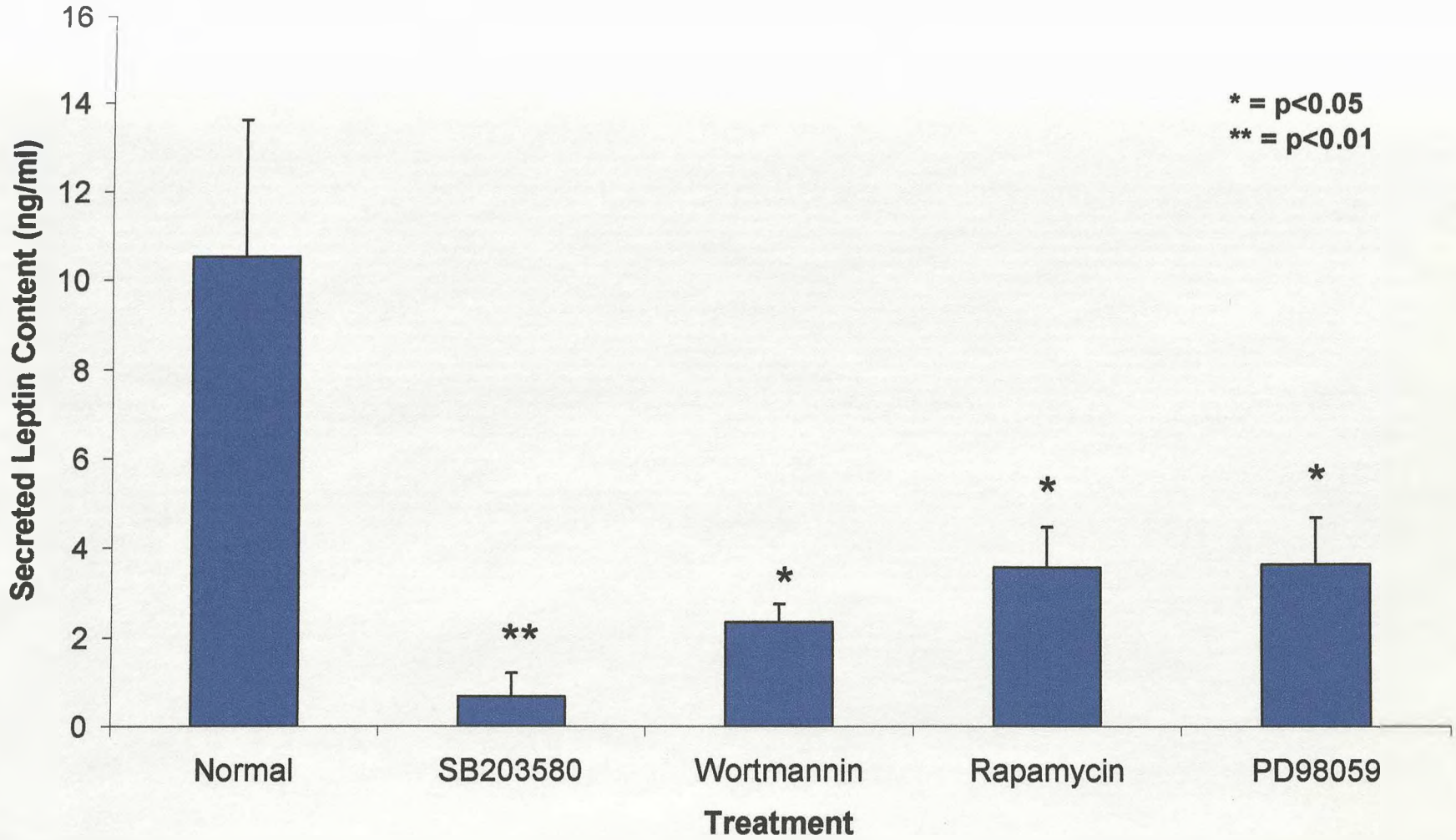


Fig 4.5 The effect of the 4 inhibitors on the secreted leptin content of differentiated preadipocytes. Preadipocytes were differentiated over a 12 day period with the inclusion of the inhibitors. Conditioned medium was removed daily and the secreted leptin content was measured using an ELISA kit allowing the total secreted leptin content of cells differentiated over 12 days to be determined. Results are given as the mean ± SEM of 3 independent samples (n=3).

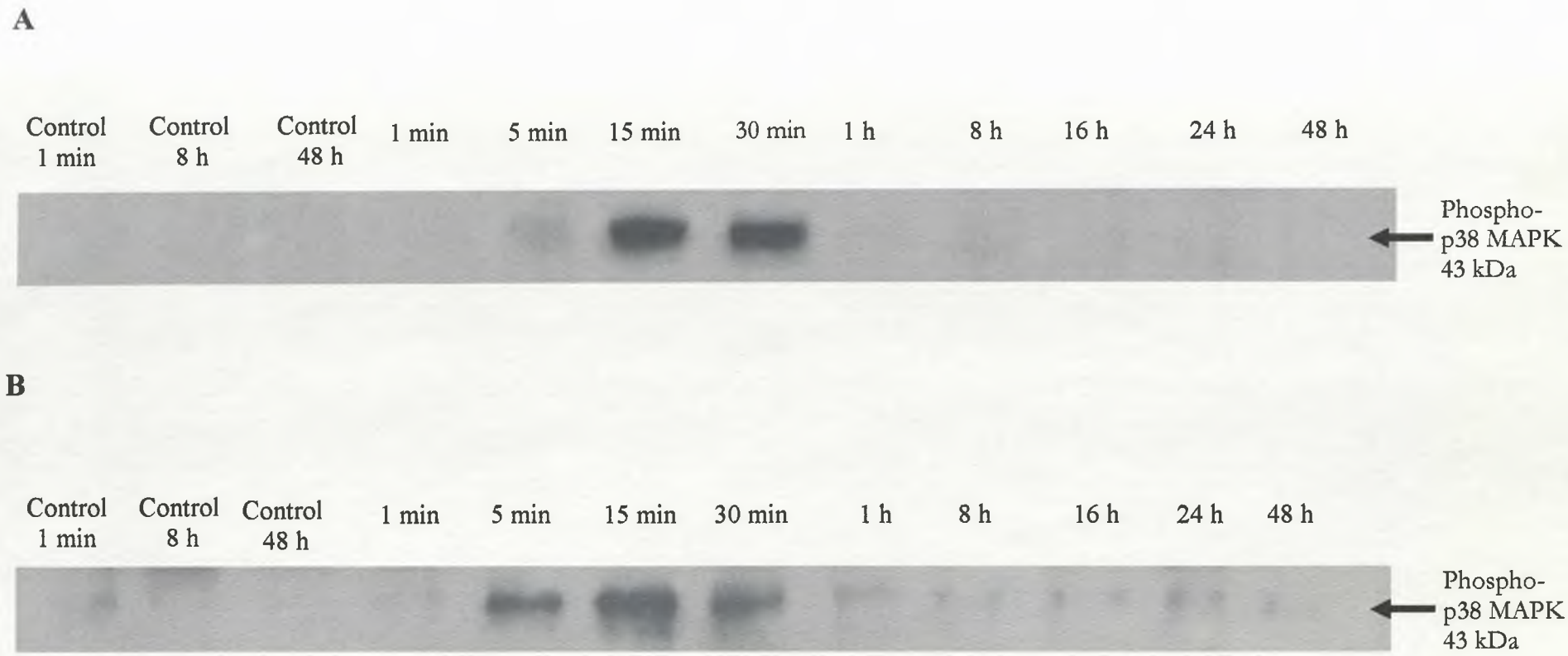


Fig 4.6 The effects of TNF-α treatment on phospho-p38 MAPK activation. (A) Preadipocytes were cultured in DMEM:F12 with TNF-α (100ng/ml) for indicated incubation periods. Control lanes contain no TNF-α treatment. Equal amounts (100 ug) of protein were loaded in each lane following assessment of protein content. Phospho-p38 MAPK activation was detected in the cell layer by Western blotting. (B) 20 day old differentiated preadipocytes were cultured in DMEM:F12 with TNF-α (100ng/ml) for indicated incubation periods. Control lanes contain no TNF-α treatment. Equal amounts (100 ug) of protein were loaded in each lane following assessment of protein content. Phospho-p38 MAPK activation was detected in the cell layer by Western blotting (n=8).

CHAPTER 5

Nongenomic Activation of p42/44 MAPK by Regulators of Adipocyte Growth and Differentiation

5.1 Introduction

Previously we have shown that a combination of different inducing agents such as insulin, glucocorticoids and T3 are essential for adipocyte differentiation. These agents are known to have effects on different aspects of the adipocyte growth and function. Clinically thyroid hormones are known to increase basal metabolic rate and to stimulate lipogenesis and lipolysis (Oppenheimer *et al.* 1991; Krotkiewski 2000; Pucci *et al.* 2000) as well as being required for adipocyte differentiation. The steroid hormone oestrogen has been shown to promote preadipocyte replication in both human and rats (Dieudonne *et al.* 2000; Anderson *et al.* 2001) and to regulate lipogenesis and lipolysis in human adipocytes (Palin *et al.* 2003). Oestrogen plays an important role in the deposition of fat in a site-specific and sex-specific manner in both humans and rats (Bjorntorp 1996; Dieudonne *et al.* 2000; Anderson *et al.* 2001; Pedersen *et al.* 2001). The glucocorticoids such as the steroid hormones, cortisol and dexamethasone, are required for adipocyte differentiation (Hauner *et al.* 1989). Glucocorticoids are known to affect fat storage clearly seen in Cushing's syndrome where central body obesity is a feature (Lamberts and Birkenhager 1976; Leal-Cerro *et al.* 2001). Rosiglitazone is known to promote adipocyte differentiation (Adams *et al.* 1997; Patel *et al.* 2003) and is known to affect fat storage (Martens *et al.* 2002).

T3, rosiglitazone and the steroid hormones, signal through the classical genomic pathway whereby the signalling molecule enters the nucleus bound to a receptor protein and binds DNA to modulate gene transcription (Losel and Wehling 2003). There are several reports showing that steroid hormones and thyroid hormones can

activate the p42/44 MAPK pathway via a nongenomic mechanism (Lin *et al.* 1999a). This nongenomic mechanism results in the rapid activation of signalling cascades such as the p42/44 MAPK pathway and does not involve the classical genomic pathway. For example, oestrogen can nongenomically activate p42/44 MAPK through a membrane receptor (Endoh *et al.* 1997), while the glucocorticoid, corticosterone nongenomically causes changes in the electrophysiological excitability and sensory responsiveness of hindbrain neurons in the roughskin newt by a membrane receptor (Moore and Orchinik 1994). High-affinity T3 binding sites have been identified in membranes from rat liver, (Pliam and Goldfine 1977), thymocytes (Segal 1989), human placenta (Alderson *et al.* 1985), and vascular smooth muscle cells (VSMC) (Ojamaa *et al.* 1996) which may facilitate the nongenomic actions of thyroid hormone. The p42/44 MAPK pathway is involved in a number of cellular processes such as embryogenesis, differentiation, proliferation and cell death (Pearson *et al.* 2001). The activation of this pathway may contribute to some of the effects of these compounds in the adipocyte.

The aim of this study was to determine whether ligands for nuclear receptors (dexamethasone, cortisol, oestrogen, T3 and the PPARγ activator, rosiglitazone) activate p42/44 MAPK in human adipocytes and preadipocytes. Effects were compared with those of insulin, a bona fide activator of p42/44 MAPK, which has known effects in adipocytes, and is able to activate the p42/44 MAPK pathway.

5.2 Results

Preadipocytes and differentiated preadipocytes were cultured in DMEM:F12 alone for 24 h and treated with either insulin, T3, oestrogen, rosiglitazone, dexamethasone or cortisol at different concentrations for 30 min. Time course studies were also performed for periods up to 24 h. Treatments were carried out at least 3 times on different adipocyte preparations (n=3). The total cellular protein of treated preadipocytes and differentiated preadipocytes was analysed by Western blotting. 100μg protein was loaded in each lane of the gel, and an antibody to phospho-p42/44 MAPK was used. Membranes were stripped and reprobed for p42/44 MAPK to show equal loading of protein.

5.2.1 Effects of Insulin Treatment on Phospho-p42/44 MAPK and p42/44 MAPK Expression

Preadipocytes and 20 day old differentiated preadipocytes were treated with insulin for 30 min at different concentrations (10^{-6}-10^{-10}M). Control lanes contain no insulin treatment but were treated with the same volume of vehicle (25mM glycine, 130mM NaCl, pH 2.5). **Fig 5.1A** shows the effect of different insulin concentrations on preadipocytes and differentiated preadipocytes on phospho-p42/44 MAPK expression. A band corresponding to 44 kDa was detected. Activation of phospho-p42/44 MAPK was seen at concentrations ranging from 10^{-6}-10^{-8}M in both preadipocytes and differentiated preadipocytes compared to the control. **Fig 5.1B** shows the time course of the effects of insulin when used at a concentration of 10^{-7}M for 10 min, 30 min, 1

h, 8 h, and 24 h. Control lanes contain no insulin treatment. In preadipocytes detectable changes in p42/44 MAPK phosphorylation were seen 10 min following challenge with insulin. The highest level of activation was seen at 30 min before declining at 24 h in preadipocytes. In differentiated preadipocytes p42/44 MAPK phosphorylation was detected at 30 min. Activation was sustained with time compared to the control and there was no decrease at 24h.

5.2.2 Effects of T3 Treatment on Phospho-p42/44 MAPK and p42/44 MAPK Expression

Preadipocytes and 20 day old differentiated preadipocytes were treated with T3 for 30 min at different concentrations (10^{-7}-10^{-11}M). Control lanes contain no T3 treatment but were treated with the same volume of vehicle which was 0.1 M NaOH. **Fig 5.2A** shows the effect of varying T3 concentrations on phospho-p42/44 MAPK expression in preadipocytes and differentiated preadipocytes. Phospho-p42/44 MAPK was seen at all concentrations of T3 (10^{-7}-10^{-11}M) in both preadipocytes and differentiated preadipocytes. Preadipocytes and 20 day old differentiated preadipocytes were treated with T3 (10^{-10}M) for 10 min, 30 min, 1 h, 8 h, and 24 h. Control lanes contain no T3 treatment. **Fig 5.2B** shows the effect on phospho-p42/44 MAPK expression at different time periods after T3 treatment (10^{-10}M) in preadipocytes and differentiated preadipocytes. Phospho-p42/44 MAPK was seen at 30 min post T3 addition in both preadipocytes and differentiated preadipocytes compared to the untreated control. In preadipocytes phospho-p42/44 MAPK was reduced at 8 h following T3 addition and returned to control levels at 24 h. Phospho-p42/44 MAPK levels in differentiated

preadipocytes declined between 1-8 h post T3 treatment and continued to fall to control levels at 24 h.

5.2.3 Effects of Rosiglitazone Treatment on Phospho-p42/44 MAPK and p42/44 MAPK Expression

Preadipocytes and 20 day old differentiated preadipocytes were treated with rosiglitazone for 30 min at different concentrations (10^{-6}-10^{-10}M). Control lanes were treated with the same volume of vehicle which was DMSO. Fig **5.3A** shows the effect on phospho-p42/44 MAPK expression at different rosiglitazone concentrations on preadipocytes and differentiated preadipocytes. No activation of p42/44 MAPK was seen at any concentration of rosiglitazone compared with control. **Fig 5.3B** shows the effect on phospho-p42/44 MAPK expression at different time periods after rosiglitazone treatment (10^{-6}M). No activation of p42/44 MAPK was seen at any time point or any concentration of rosiglitazone compared to control. Although the figure showing the time course of phospho-p42/44 MAPK expression in preadipocytes was variable, further experiments did not show consistent effects.

5.2.4 Effects of Oestrogen Treatment on Phospho-p42/44 MAPK and p42/44 MAPK Expression

Preadipocytes and 20 day old differentiated preadipocytes were treated with water-soluble, cyclodextrin-encapsulated 17β oestradiol (E2) for 30 min at different concentrations (10^{-7}-10^{-11}M). Control lanes were treated with the same volume of

vehicle which was 2-hydroxypropyl-cyclodextrin. **Fig 5.3A** shows the effect of E2 at varying concentrations on phospho-p42/44 MAPK expression and **Fig 5.3B** phospho-p42/44 MAPK expression at varying times post challenge with E2 (10^{-8}M) on preadipocytes and differentiated preadipocytes. No phospho-p42/44 MAPK was seen at any concentration of E2 or at any time point compared with the control in either cell type.

5.2.5 Effects of Dexamethasone Treatment on Phospho-p42/44 MAPK and p42/44 MAPK Expression

Preadipocytes and 20 day old differentiated preadipocytes were treated with dexamethasone for 30 min at different concentrations (10^{-6}-10^{-10}M). Control lanes were treated with the same volume of vehicle which was DMSO. **Fig 5.5** shows the effect on phospho-p42/44 MAPK of different dexamethasone concentrations on preadipocytes and differentiated preadipocytes. No phospho-p42/44 MAPK was seen at any concentration of dexamethasone compared with the control.

5.2.6 Effects of Cortisol Treatment on Phospho-p42/44 MAPK and p42/44 MAPK Expression

Preadipocytes and 20 day old differentiated preadipocytes were treated with cortisol for 30 min at different concentrations (10^{-6}-10^{-10}M). Control lanes were treated with the same volume of vehicle which was HBSS. Fig 5.5 shows the effect on phospho-p42/44 MAPK expression of different cortisol concentrations on preadipocytes and

differentiated preadipocytes. No phosphorylation of p42/44 MAPK was seen at any concentration of cortisol compared with the control.

5.3 Discussion

T3 at low, physiological concentrations (10^{-11}M) was a potent stimulator of p42/44 MAPK phosphorylation in both preadipocytes and differentiating preadipocytes with a very rapid and sustained activation. Insulin, although not as potent as T3, stimulated p42/44 MAPK phosphorylation at low doses, rapidly, and its effects were even more long lasting than those of T3. The steroid hormones, cortisol, dexamethasone and oestrogen as well as the PPARγ activator and insulin sensitizer, rosiglitazone had no effect on p42/44 MAPK phosphorylation.

The rapid and sustained activation of p42/44 MAPK in both preadipocytes and differentiated preadipocytes by T3 is a novel finding. Previous studies have shown p42/44 MAPK to be activated by the thyroid hormones, T3 or T4, in other cultured cells such as HeLa and CV-1 cells (Lin et al 1999). However supraphysiological concentrations (10^{-7}M) of T3 were used to activate p42/44 MAPK unlike our study where physiological concentrations of T3 activated p42/44 MAPK. IN these earlier studies cells were cultured in serum which contains thyroid hormone binding proteins (thyroxine binding globulin, albumin) which may affect the sensitivity whereas the adipocytes used in our study were cultured in serum-free medium. The role of p42/44 MAPK activation by thyroid hormones was postulated to potentiate the cellular actions of certain cytokines and growth factors such as IFN-γ and EGF (Lin *et al.*

1999a; Lin *et al.* 1999b). Conceivably p42/44 MAPK activation by T3 may mediate some of its actions as well as potentiating the actions of other factors, such as insulin, in the adipocyte.

Activation of p42/44 MAPK by T3 may be important in potentiating the transcriptional activity of the receptor for thyroid hormones, TR. TRβ contains a p42/44 MAPK binding region which undergoes serine phosphorylation by p42/44 MAPK (Davis *et al.* 2000). p42/44 MAPK phosphorylation has been implicated in increasing TRβ activity and preventing its degradation. This was demonstrated in CV-1 cells where inhibition of p42/44 MAPK by PD98059 decreased TRβ expression and activity (Chen *et al.* 2003). Nongenomic activation of p42/44 MAPK by T3 may increase T3 binding to its receptor, TRβ and increase transcriptional activity.

Our study and other studies have shown thyroid hormones to promote adipocyte differentiation in both human (Hauner *et al.* 1989) and murine models (Levacher *et al.* 1984; Darimont *et al.* 1993). Hyperthyroidism and hypothyroidism have been shown to enhance and decrease lipolysis respectively (Lafontan *et al.* 2000) leading to the conclusion that thyroid hormone is important in lipolysis. Thyroid hormones can amplify the lipolytic actions of catecholamines by increasing β_2-adrenoreceptor expression thus increasing cAMP and PKA activation. PKA phosphorylates HSL leading to increased lipolysis in the hyperthyroid state (Hellstrom *et al.* 1997). Alternatively recent work has shown that catecholamines, increasing cAMP levels, can activate p42/44 MAPK which also phosphorylates HSL and stimulates lipolysis. Greenberg *et al* (2001) showed that blocking this pathway with U0126 or PD98059,

inhibitors of p42/44 MAPK, partially inhibited catecholamine-induced lipolysis in 3T3-L1 cells (Greenberg *et al.* 2001) suggesting that the p42/44 MAPK pathway is important in stimulating lipolysis. Studies have also shown that p42/44 MAPK activation by TNF-α stimulates lipolysis in both 3T3-L1 and human adipocytes (Zhang *et al.* 2002). Conversely blocking this pathway with U0126 or PD98059, inhibited lipolysis (Zhang *et al.* 2002; Souza *et al.* 2003). p42/44 MAPK phosphorylation may therefore be important in the mechanism by which thyroid hormone induces its lipolytic effect in adipocytes possibly mediated by catecholamines. To address this, inhibition of p42/44 MAPK by PD98059 or U0126, is needed to determine whether T3 effects on lipolysis in adipocytes are mediated through this pathway.

Many studies have shown that insulin activates the p42/44 MAPK signalling pathway in cells including 3T3-L1 adipocytes (Suga *et al.* 1997) and rat adipocytes (Yang and Farese 1993). This is the first time however that insulin effects have been examined in human adipocytes and preadipocytes. In other cells, insulin-stimulated activation of the p42/44 MAPK pathway has been shown to be involved in growth and differentiation (Boulton *et al.* 1991; Saltiel and Kahn 2001). However using PD98059 we showed that p42/44 MAPK activation was not essential for induction of the programme of differentiation leading to lipogenesis although leptin synthesis was decreased (Patel *et al.* 2003). Activation of the p42/44 MAPK pathway may however be important in other functions of the adipocyte, but not in differentiation.

Insulin caused a more sustained activation of p42/44 MAPK rather than the acute stimulation by T3. This may possibly lead to differential effects with short-term activation of p42/44 MAPK activating different pathways to more sustained activation. This may be a potential mechanism by which p42/44 MAPK activation by different activators can have differential effects.

Oestrogen is known to affect adipose tissue function and distribution (Bjorntorp 1996; Dieudonne *et al.* 2000; Anderson *et al.* 2001; Pedersen *et al.* 2001; Palin *et al.* 2003). There are two identified oestrogen receptors (ER), ERα and ERβ. Both are expressed in adipose tissue (Pedersen *et al.* 1996; Crandall *et al.* 1998), but expression varies depending on adipose depot (Anwar *et al.* 2001; Pedersen *et al.* 2001) and stage of differentiation (Joyner *et al.* 2001; Pedersen *et al.* 2001). Oestrogen is known to activate p42/44 MAPK via a nongenomic mechanism (Collins and Webb 1999) in a variety of cells types such as breast cancer (Migliaccio *et al.* 1996), endothelial (Shaul 1999), osteoblastic (Endoh *et al.* 1997), and neuroblastoma cells (Watters *et al.* 1997) through a membrane receptor for oestrogen. In this study on human preadipocytes and adipocytes, oestrogen did not rapidly activate p42/44 MAPK. This is in direct contrast to a study in rat adipocytes where oestrogen was found to activate p42/44 MAPK (Dos Santos *et al.* 2002). Using parametrial adipose tissue, oestrogen activated p42/44 MAPK in differentiated preadipocytes but not in proliferating preadipocytes and early differentiated preadipocytes. Their study showed the expression of ERα in membrane fractions of adipocytes but not preadipocytes. Membrane ERα enables rapid p42/44 MAPK activation (Endoh *et al.* 1997) and explains why p42/44 MAPK is rapidly activated in rat adipocytes but not

preadipocytes with oestrogen treatment. Activation of p42/44 MAPK in human preadipocytes or adipocytes by oestrogen may therefore depend on the expression of the appropriate ER. Although Anwar *et al* (2001) found both ERα and ERβ in the membrane of human preadipocytes and adipocytes, it was not determined whether these isoforms activate p42/44 MAPK (Anwar *et al.* 2001). Alternatively species differences between rats and humans may explain p42/44 MAPK activation by oestrogen in rats and not humans. Further studies will have to be carried out looking at different adipose depots and at different stages of differentiation to determine whether there is expression of an ER enabling p42/44 MAPK activation by oestrogen in human adipose.

The glucocorticoids, cortisol and the synthetic compound dexamethasone, have been shown to inhibit p42/44 MAPK in a variety of cells such as osteoblasts (Hulley *et al.* 1998), airway smooth muscle cells (Fernandes *et al.* 1999), and pulmonary endothelial cells (Pelaia *et al.* 2001). One proposed mechanism of p42/44 MAPK inactivation is by the decreased degradation of MAPK phosphatase-1 (MKP-1), an enzyme which inactivates p42/44 MAPK. MKP-1 is upregulated by glucocticoids leading to increased inactivation of p42/44 MAPK (Kassel *et al.* 2001). Glucocorticoid excess in humans leads to visceral obesity leading to insulin resistance, dyslipidemia and hypertension. Glucocorticoids are known to regulate a number of processes in adipose tissue such as metabolism and fat cell size as well as promoting the differentiation of preadipocytes to adipocytes (Bjorntorp 1991; Bjorntorp 1996; Bujalska *et al.* 1999; Joyner *et al.* 2001; Zhang *et al.* 2001). Both cortisol and dexamethasone had no effect on p42/44 MAPK phosphorylation in both

preadipocytes and differentiated preadipocytes, thus the activation of the p42/44 MAPK pathway may not be directly involved in the short term effects of glucocorticoids on adipose tissue.

The insulin sensitizer rosiglitazone has been shown to activate p42/44 MAPK in VSMC. Rosiglitazone treatment was proapoptotic and activation of p42/44 MAPK was seen 3 minutes post treatment but was independent of the proapototic effects of rosiglitazone (Gouni-Berthold *et al.* 2001). We did not find activation of p42/44 MAPK by rosiglitazone at any time point or at any dose. We conclude that rosiglitazone does not directly mediate any of its effects through the activation of p42/44 MAPK in preadipocytes or differentiated preadipocytes.

To summarize, T3 activates p42/44 MAPK phosphorylation in adipocytes and this may contribute to its lipolytic effects. Insulin also activates the p42/44 MAPK pathway and this may contribute to growth and proliferation of adipocytes. Steroid hormones and the insulin sensitizer, rosiglitazone do not however use this pathway to mediate their effects in human adipose tissue.

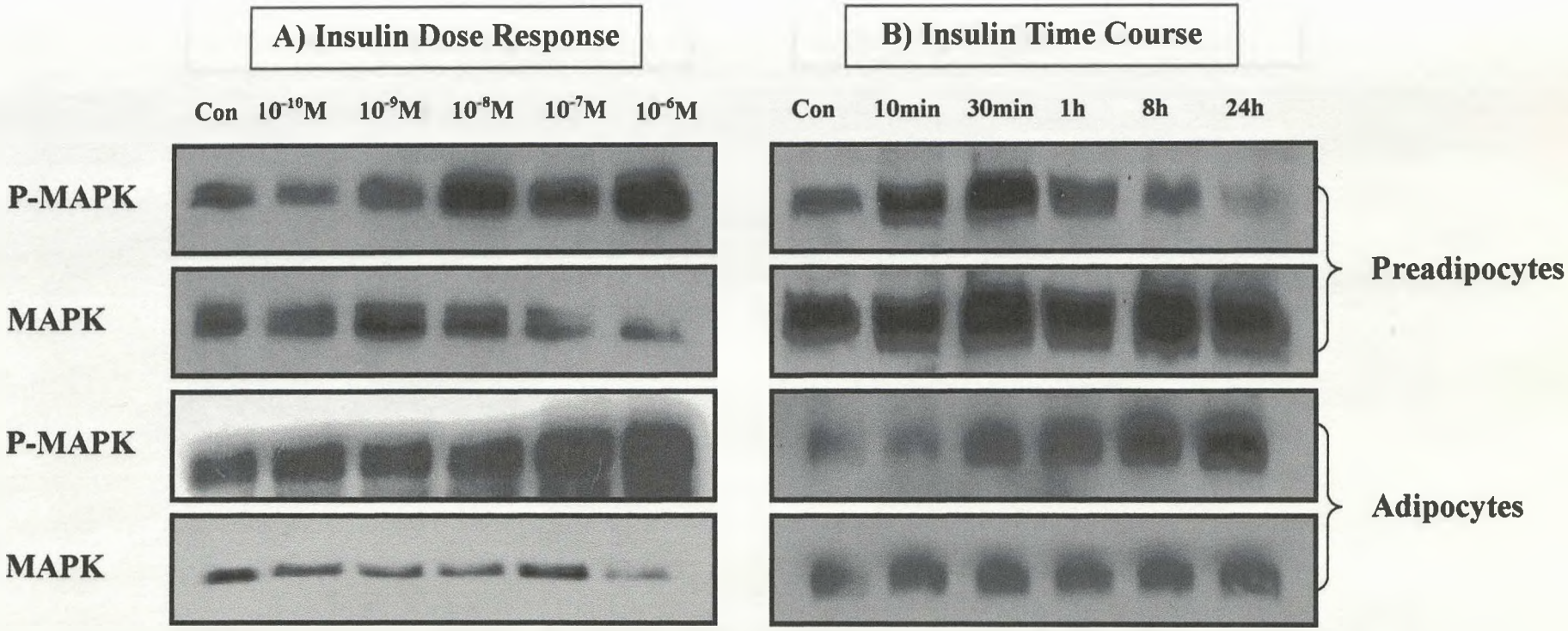


Fig 5.1 The effects of insulin treatment on phospho-p42/44 MAPK and p42/44 MAPK activation. (A) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with insulin for indicated concentrations for 30 min. Control lanes contain no insulin treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting. (B) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with insulin (10^{-7}M) for indicated incubation periods. Control lanes contain no insulin treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting.

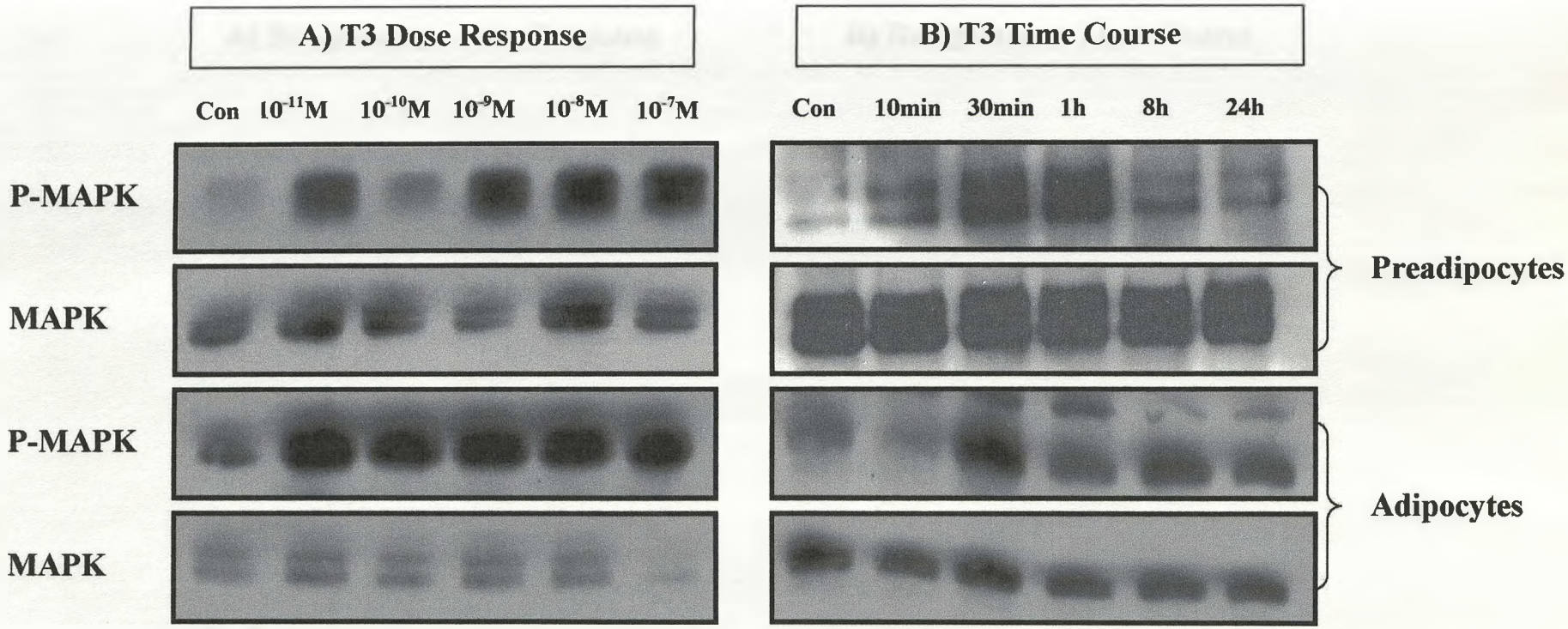


Fig 5.2 The effects of T3 treatment on phospho-p42/44 MAPK and p42/44 MAPK activation. (A) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with insulin for indicated concentrations for 30 min. Control lanes contain no T3 treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting. (B) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with T3 (10^{-10}M) for indicated incubation periods. Control lanes contain no T3 treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting.

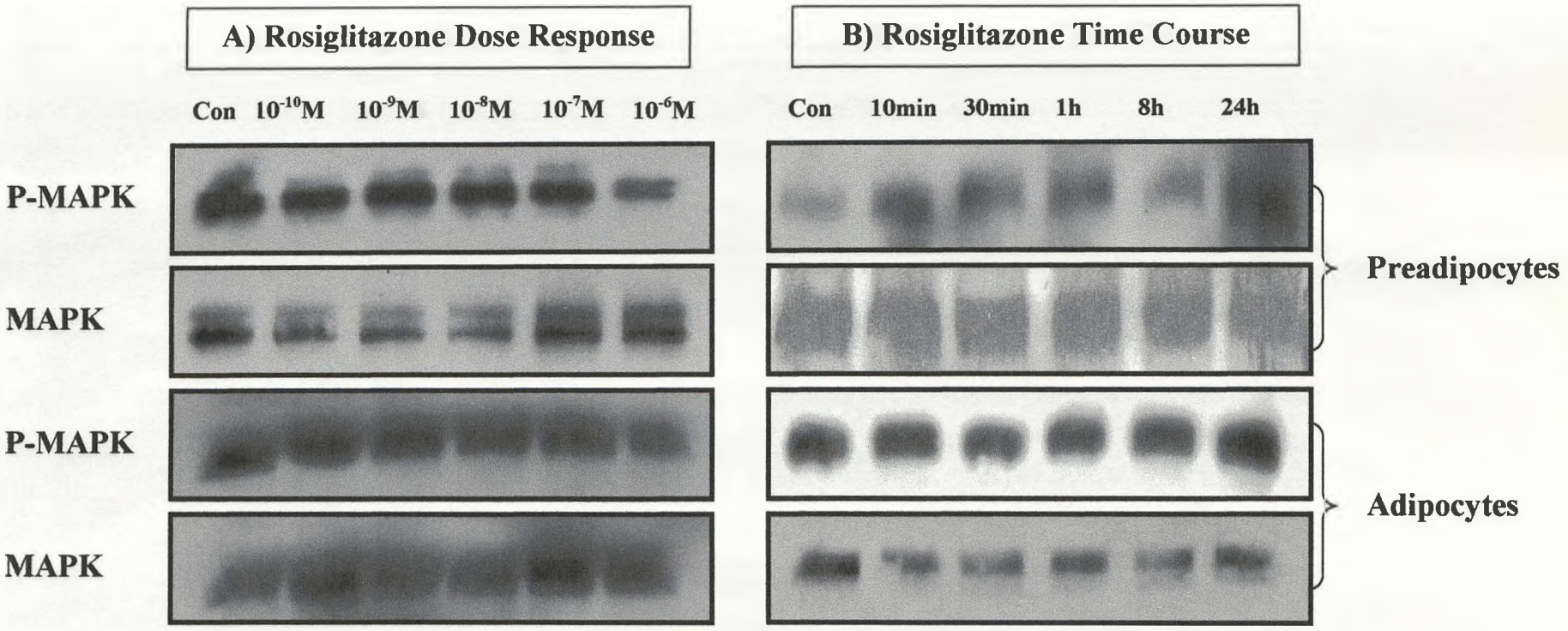


Fig 5.3 The effects of rosiglitazone treatment on phospho-p42/44 MAPK and p42/44 MAPK activation. (A) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with rosiglitazone for indicated concentrations for 30 min. Control lanes contain no rosiglitazone treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting. (B) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with rosiglitazone (10^{-6}M) for indicated incubation periods. Control lanes contain no rosiglitazone treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting.

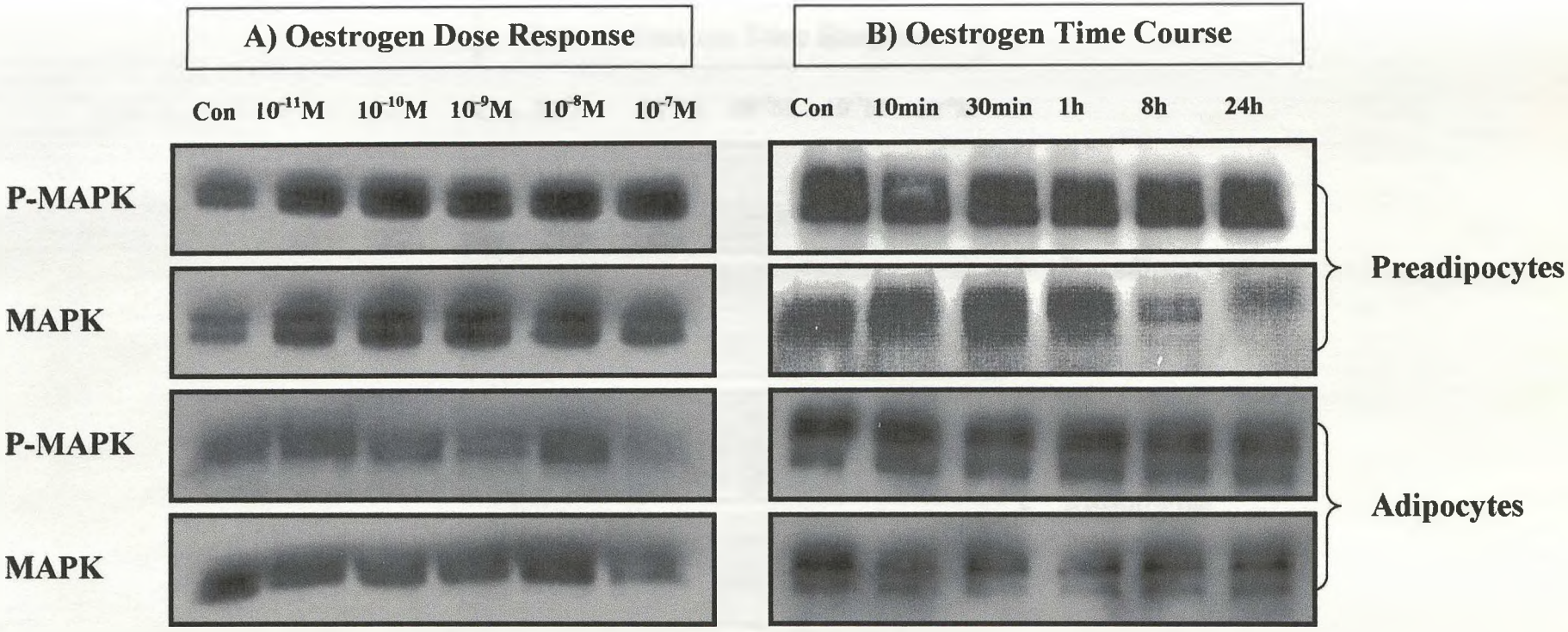


Fig 5.4 The effects of oestrogen treatment on phospho-p42/44 MAPK and p42/44 MAPK activation. (A) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with oestrogen for indicated concentrations for 30 min. Control lanes contain no oestrogen treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting. (B) Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with oestrogen (10^{-8}M) for indicated incubation periods. Control lanes contain no oestrogen treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting.

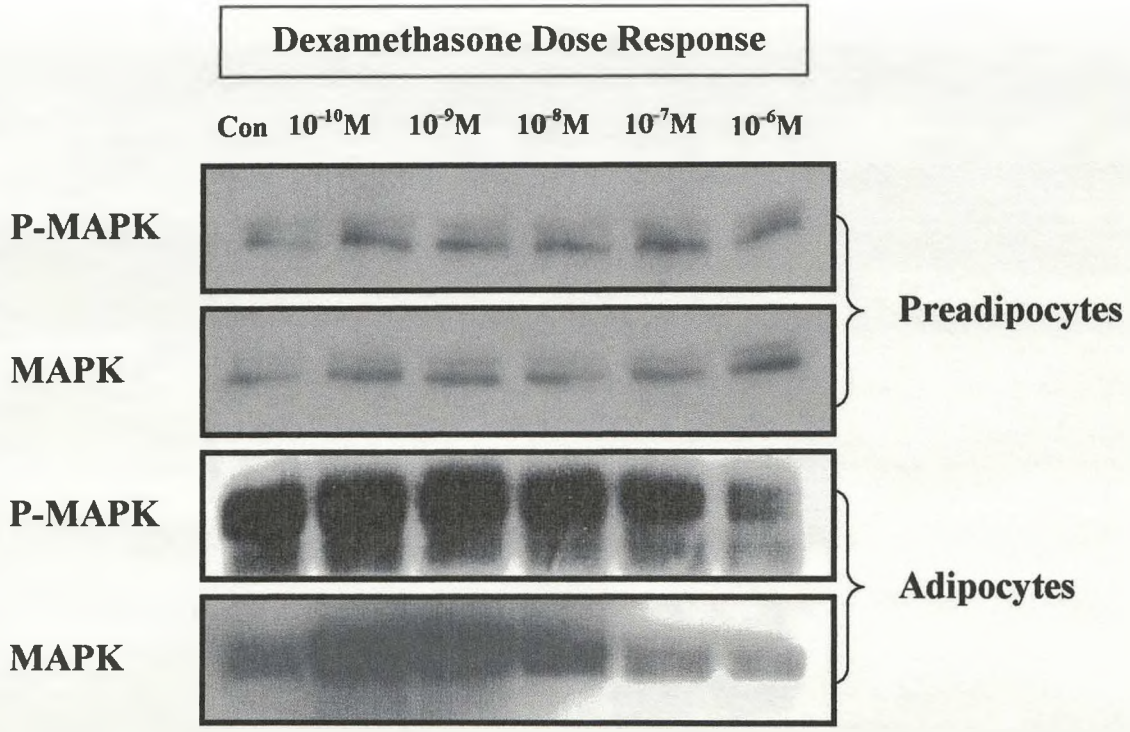


Fig 5.5 The effects of dexamethasone treatment on phospho-p42/44 MAPK and p42/44 MAPK activation. Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with dexamethasone for indicated concentrations for 30 min. Control lanes contain no dexamethasone treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting

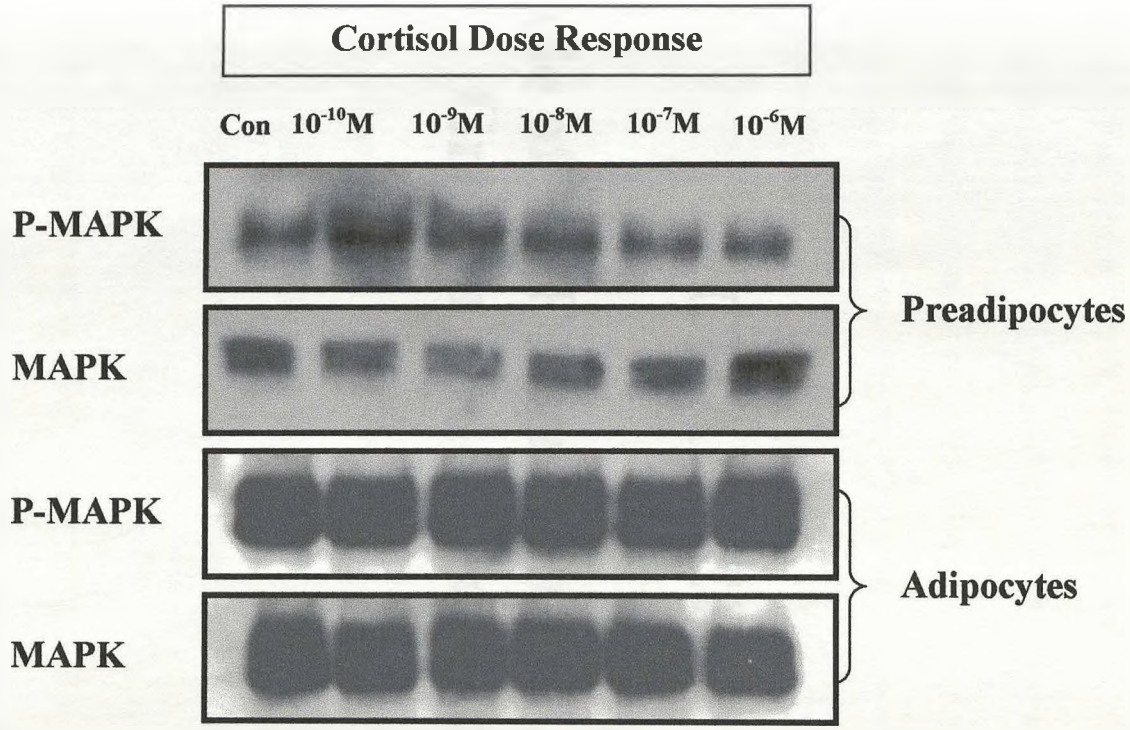


Fig 5.6 The effects of cortisol treatment on phospho-p42/44 MAPK and p42/44 MAPK activation. Preadipocytes and 20 day old differentiated preadipocytes were cultured in DMEM:F12 with cortisol for indicated concentrations for 30 min. Control lanes contain no cortisol treatment. Phospho-p42/44 MAPK and p42/44 MAPK activation was detected in the cell layer by Western blotting

CHAPTER 6

Role of FGF Signalling in Adipocyte Growth and Differentiation

6.1 Introduction

In the previous chapters, we showed that adipogenesis requires the cooperative effects of several signalling pathways. Although differentiation is dependent on these exogenously added factors, factors secreted by the adipocyte are known to influence adipocyte differentiation. These include TNF-α (Petruschke and Hauner 1993; Zhang *et al.* 2000), leptin (Zhang *et al.* 1994; Zhang *et al.* 1999; Zhang *et al.* 2000) interleukin-6 (IL-6) (Fried *et al.* 1998), IGF-I (Nougues *et al.* 1993), agouti (Mynatt and Stephens 2001), and prostaglandins (Hopkins and Gorman 1981), all of which are secreted by adipocytes, and all have been shown to have autocrine effects.

The potential autocrine role of the FGF family on adipocytes has been largely unexplored. This is surprising since FGFs are known to affect both growth and differentiation in other systems. Similar to the ligands, very little work has been done on the expression of FGFRs in adipose tissue with only one reported study looking at the mRNA expression of FGFRs (Gabrielsson *et al.* 2002). The current knowledge of FGFs, their receptors and their signalling in adipocytes is therefore limited.

The aims of this study were to determine whether autocrine FGFs (FGF-2 and FGF-10) exist in human adipose and whether signalling through FGFRs affects preadipocyte growth and differentiation. To examine the expression of FGFs and the cognate receptor, FGFR1, RT-PCR and Western blotting were used to assay mRNA and protein expression throughout adipocyte differentiation. To establish whether FGFs play an autocrine role in the adipocyte, FGFR signalling was inhibited by two

methods: PD166866 is a compound which specifically inhibits FGFR1 signalling in a number of cells including NIH 3T3 cells, L6 muscle cells, and human placenta by inhibiting tyrosine kinase activity of FGFR1 (Panek *et al.* 1998). Dominant negative versions of FGFR have been shown to inhibit FGF signalling (Amaya *et al.* 1991; Ueno *et al.* 1992). Adenovirus vectors were used to introduce these truncated forms of dominant negative FGFR1 into cells. This has been shown to inhibit all forms of FGFR signalling (Ueno *et al.* 1992).

6.2 Results

6.2.1 Expression of FGF-2 in Human Differentiating Preadipocytes

Preadipocytes were induced to differentiate over 12 days in differentiation medium with and without 10^{-6} M rosiglitazone. Cell layer proteins were isolated daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12) and were analysed by Western blotting. 100μg protein was loaded in each lane of the gel, and an antibody to FGF-2 was used. **Fig 6.1A** shows the time course of FGF-2 expression in preadipocytes differentiated in differentiation medium (Day 0-12). A band of 24 kDa was detectable in Day 0 preadipocytes and was present throughout the 12 days of differentiation. The level of FGF-2 expression began to fall at Day 8 and continued to fall. **Fig 6.1B** shows the time course of FGF-2 expression in preadipocytes differentiated in differentiation medium containing rosiglitazone (Day 0-12). A band

of 24 kDa was detectable in Day 0 preadipocytes and was present throughout the 12 days of differentiation. FGF-2 expression fell at Day 6 and continued to fall.

Conditioned medium was collected from Day 0 preadipocytes and Day 12 differentiated preadipocytes and the level of FGF-2 was measured using an ELISA kit. Data are given as the mean ± SEM of 4 independent samples. Day 12 differentiated preadipocytes secreted more FGF-2 than Day 0 preadipocytes (16.57 ± 0.48 pg/ml vs 6.58 ± 0.09 pg/ml n=4). This difference was statistically significant to $p<0.0001$.

6.2.2 Expression of FGF-10 in Human Differentiating Preadipocytes

Preadipocytes were induced to differentiate in normal differentiation medium. RNA was isolated from day 0 preadipocytes, day 6 differentiated preadipocytes and mature adipocytes (supplied by Alison Harte) and RT-PCR was carried out to identify whether FGF-10 mRNA was expressed. FGF-10 mRNA was expressed as a 313 bp product in day 0 preadipocytes, day 6 differentiated preadipocytes and mature adipocytes (**Fig 6.2**).

6.2.3 Expression of FGFR1 in Human Differentiating Preadipocytes

To examine the expression of FGFR1, immunohistochemistry was carried out on preadipocytes, day 12 normally differentiated preadipocytes, and day 12

rosiglitazone-differentiated preadipocytes using specific antiserum to the C-terminus of FGFR1. Preadipocytes were cultured on glass coverslips and induced to differentiate in differentiation medium with and without rosiglitazone. Control incubations had no primary antibody present. There was strong staining for FGFR1 in preadipocytes, day 12 normally differentiated preadipocytes and day 12 rosiglitazone differentiated preadipocytes compared to control incubations (**Fig 6.3**).

To quantify FGFR1 expression throughout differentiation, Western blotting was used. Preadipocytes were induced to differentiate over 9 days in normal differentiation medium. Cell layer proteins were isolated daily from preadipocytes to differentiated preadipocytes and were analysed by Western blotting. 100μg protein was loaded in each lane of the gel, and an antibody to C-terminus of FGFR1 was used. Fig **6.4A** shows the time course of the C-terminus of FGFR1 expression in preadipocytes differentiated in differentiation medium (Day 0-9). A band of 125 kDa was detectable in Day 0 preadipocytes and was present throughout the 9 days of differentiation. The level of FGFR1 expression however began to fall at Day 5 and continued to fall.

6.2.4 Expression of Virally-transduced, Truncated FGFR1 in Preadipocytes

To show that truncated FGFR1 which inhibits FGFR signalling is expressed by cells transduced with RAdDN-FGFR1, preadipocytes were infected with RAdDN-FGFR1 virus at a MOI of 20 pfu/cell and induced to differentiate over 4 days in differentiation medium. Cell layer proteins were isolated daily from preadipocytes and RAdDN-FGFR1 infected differentiated preadipocytes and Western blotting was used to show

that cells were infected by RAdDN-FGFR1 virus. 100μg protein was loaded in each lane of the gel, and an antibody to N-terminus of FGFR1 was used to detect truncated FGFR1. Day 0 preadipocytes not infected with RAdDN-FGFR1 virus showed no expression of the truncated form of FGFR1. Day 1 infected cells showed a low level of expression of N-terminus of FGFR1 (79 kDa), which increased over the next few days (**Fig 6.4B**). Truncated FGFR1 was present for 8 days following adenoviral transduction (data not shown).

6.2.5 Effects of Inhibition of FGFR on Protein Content in Differentiated Preadipocytes Using Recombinant Adenovirus Expressing Truncated FGFR1 and PD166866, Specific Inhibitors of FGFR Signalling

Preadipocytes were infected with RAdDN-FGFR1 virus at a MOI of 20 pfu/cell. Control cultures were infected with RAd-β-galactosidase virus at a MOI of 20 pfu/cell. Preadipocytes were treated with the FGFR1 inhibitor PD166866 (10^{-7} M). Preadipocytes were induced to differentiate over 12 days in differentiation medium with and without 10^{-6} M rosiglitazone. PD166866 was added to cells when differentiation was initiated and was maintained in the medium throughout the 12 day period. **Fig 6.5** shows the effect of the RAdDN-FGFR1 and the PD166866 compound on adipocyte differentiation. **Fig 6.5** shows light micrographs of 12 day old RAd-β-galactosidase treated differentiated preadipocytes (**A**), cells treated with RAdDN-FGFR1 (**B**), and cells treated with PD166866 (**C**). Transduction with RAdDN-FGFR1 virus and addition of PD166866 (**Fig 6.5B&C**) visibly reduced the number of cells compared to the control. After the 12 day differentiation period, the cell layer

was removed and the amount of protein present was determined. Results of treated cells are given as percentage mean values ± SEM compared to RAd-β-galactosidase treated control differentiated preadipocytes (**Fig 6.6**). RAdDN-FGFR1 virus and PD166866 significantly reduced the level of protein in cells. In preadipocytes differentiated in the presence of rosiglitazone, RAdDN-FGFR1 virus and PD166866 again significantly reduced protein in cells.

To examine the protein content in the cell layer over 12 days of differentiation, preadipocytes were treated with RAd-β-galactosidase virus, RAdDN-FGFR1 virus, and PD166866. They were induced to differentiate over 12 days in differentiation medium. Cell layer proteins were isolated daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12) and the protein content was measured. Results of treated cells are given as percentage mean values ± SEM compared to their respective control Day 0 preadipocytes (**Fig 6.7**). Although there was a reduction in protein content as differentiation progressed, cells treated with PD166866 and particularly RAdDN-FGFR1 virus showed a marked and significant reduction in protein content throughout differentiation. This reduction was much more pronounced than that seen with RAd-β-galactosidase.

6.2.6 Effects of Inhibition of FGFR Signalling on Glucose Uptake in Differentiated Preadipocytes Using Recombinant Adenovirus Expressing Truncated FGFR1 and PD166866, Specific Inhibitors of FGFR Signalling

The uptake of ^{14}C-glucose in 12 day old differentiated preadipocytes was determined. Cells had been differentiated with and without rosiglitazone in the differentiation medium and the viruses and PD166866 were added to the cells throughout the 12 day period. Data were corrected for protein content. ^{14}C-glucose uptake into cells was significantly reduced by RAdDN-FGFR1 virus and PD166866 (**Fig 6.8**). In preadipocytes differentiated in the presence of rosiglitazone, RAdDN-FGFR1 virus and PD166866 again significantly reduced the level of ^{14}C-glucose uptake in cells (**Fig 6.8**).

The time course of ^{14}C-glucose uptake into cells over 12 days of differentiation with and without RAd-β-galactosidase virus, RAdDN-FGFR1 virus, and PD166866 was examined. The uptake of ^{14}C-glucose was measured daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12) and corrected for protein content. Results of treated cells are given as percentage mean values ± SEM compared to respective control Day 0 preadipocytes (**Fig 6.9**). Cells treated with RAd-β-galactosidase virus showed a time-dependent increase in uptake of ^{14}C-glucose as differentiation progressed. This was not markedly different to that seen in cells which had not been treated with RAd-β-galactosidase virus. Cells treated with RAdDN-FGFR1 or PD166866 showed a significant, time-dependent reduction in the uptake of ^{14}C-glucose as differentiation progressed. The increased uptake seen with differentiation

was abolished by this treatment. In cells treated with RAdDN-FGFR1, ^{14}C-glucose uptake was reduced to 9% of control while PD166866 treated cells showed a reduction in ^{14}C-glucose to 47% of control.

6.3 Discussion

FGFR1 was strongly expressed in human preadipocytes, with levels of FGFR1 declining as differentiation progressed. The function of FGFR1 signalling was examined using a specific inhibitor of FGFR1, PD166866. We inhibited signalling through all forms of FGFR using recombinant adenovirus to transduce a dominant negative construct of FGFR1. There were large reductions in both growth and adipogenesis with PD166866 treatment. These effects were even greater when RAdDN-FGFR1 was used, suggesting that isoforms of FGF which do not act through FGFR1, were also important in regulating adipocyte growth and function. Although the reduction in protein content with transduction with RAdDN-FGFR1 virus could be due to toxic effects caused by viral transduction, transduction with RAd-β-galactosidase virus did not have the same effect on protein content. Therefore we conclude that the effects observed with RAdDN-FGFR1 virus transduction are due to effects on FGF signalling. In human subcutaneous preadipocytes and differentiating preadipocytes, we found strong expression of FGF-2 in the cell layer with the predominant expression of the high molecular weight 24 kDa form of FGF-2, but not the 18 kDa form. As preadipocytes were induced to differentiate, levels of FGF-2 dropped when lipid droplets began to appear. This was also seen when using preadipocytes differentiated in rosiglitazone. These showed an earlier decrease in

FGF-2 levels coincidental with the earlier appearance of lipid droplets in these cells. Secretion of FGF-2 however was found to be higher in differentiated preadipocytes than preadipocytes.

Studies looking at the expression of FGF-2 in adipose tissue have shown conflicting data. One study looking at the mRNA expression of FGF-2 in adipose tissue in humans, showed FGF-2 to be present only in adipocytes and not in preadipocytes (Gabrielsson *et al.* 2002). Another human study found an mRNA corresponding to FGF-2 related protein in isolated preadipocytes, differentiated preadipocytes, and mature adipocytes of omental adipose tissue (Teichert-Kuliszewska *et al.* 1992; Teichert-Kuliszewska *et al.* 1994). Our study looking at the protein expression clearly shows that FGF-2 is expressed in preadipocytes and is regulated through differentiation.

The expression of the 24 kDa form of FGF-2 rather than the 18 kDa form of FGF-2 in preadipocytes and differentiating preadipocytes is interesting. All forms of FGF-2 are mitogenic but FGF-2 may have roles other than a mitogen (Bikfalvi *et al.* 1997) and it is reported to regulate transcription directly in the nucleus (Nakanishi *et al.* 1992). The 18 kDa form of FGF-2 is found mainly in the cytosol and is a known mitogen inducing cell motility and proliferation through interaction with its cell surface receptors (Bikfalvi *et al.* 1995). The high molecular weight forms of FGF-2 are thought to be nuclear and are mitogenic being inducers of anchorage dependent growth and growth in low serum (Bikfalvi *et al.* 1995). Whether high molecular

weight FGF-2 has this effect in adipocytes is not yet known but suggests the possibility of multiple functions.

The reduction in FGFR1 and FGF-2 expression as differentiation progresses may be due to the anti-adipogenic effects of FGF-2. A number of studies have shown FGF-2 to be anti-adipogenic in both human and murine models of adipogenesisis (Navre and Ringold 1989; Teichert-Kuliszewska *et al.* 1992; Hauner *et al.* 1995b; Krieger-Brauer and Kather 1995). Other studies have found FGF-2 to be stimulatory to differentiation in human adipocytes (Hauner et al 1995). This discrepancy may be due to the effect of different concentrations of FGF-2 because high concentrations of FGF-2 were shown to inhibit differentiation whereas low concentrations were stimulatory (Hauner *et al.* 1995b; Krieger-Brauer and Kather 1995). The antiadipogenic effects of FGF-2 may be reduced by decreasing expression of FGF-2 and its receptor FGFR1 as differentiation progresses and with the appearance of lipid droplets. However expression of both proteins remains detectable even when preadipocytes are fully differentiated.

FGF-2 secretion is higher in differentiated preadipocytes than preadipocytes. This may be due to the paracrine role of FGF-2 in adipocyte growth as well as its autocrine role. Adipose tissue is able to grow and regress throughout adulthood and vascular remodelling and angiogenesis are required for this to occur (Crandall *et al.* 1997). FGF has been found to be angiogenic (Folkman and Shing 1992) and it is possible that it may be secreted by the adipocyte to influence the surrounding vasculature and epithelium to undergo remodelling during adipocyte growth (Rupnick *et al.* 2002).

During differentiation of adipocytes, preadipocytes undergo a morphology change and accumulate lipid and FGF-2 secretion may accommodate this change by influencing the surrounding vasculature and epithelium.

FGF-10 has been found to be involved in the differentiation of adipocytes in 3T3-L1 cells and mouse preadipocytes. It is expressed during differentiation and blocking FGF-10 by an FGF-10 neutralising antibody inhibited their differentiation (Sakaue *et al.* 2002). We found FGF-10 mRNA expressed in human preadipocytes, Day 6 differentiated preadipocytes, and mature adipocytes confirming previous findings. The data do not preclude a role for FGF-10 in human preadipocyte differentiation as FGF-10 signals through FGFR1. Since blocking FGFR1 signalling inhibits differentiation, FGF-10 may be involved (Sakaue *et al.* 2002). Further studies need to be carried out to determine whether FGF-10 protein is expressed in human adipocytes and if so does it play a role in adipocyte differentiation.

Other FGFs may be important in adipocytes and adipogenesis. One study has shown the expression of mRNAs for FGF 1, 7, 9, 18 as well as FGF 2 and 10 in human adipose tissue (Gabrielsson *et al.* 2002). These other FGF's may have a role in adipocyte function with FGF-1 being found to stimulate differentiation at low concentrations, but inhibiting at higher concentrations (Krieger-Brauer and Kather 1995). Collectively all these FGFs may have effects on the adipocyte and their protein expression in the human adipocytes will have to be confirmed before their role in the adipocyte can be determined. Once expression is confirmed, further studies

will need to be carried out to determine which of the expressed FGFs is important in adipocyte growth and differentiation or whether collectively they all play a role.

FGF signalling activates many pathways involved in processes such as growth, differentiation and migration (Powers et al 2000). FGF-2 has been shown to activate MAPKs such as p38 MAPK, which has been shown to be important in adipocyte differentiation (Patel *et al.* 2003). Other FGFs, which signal through the FGFR's, could also facilitate adipocyte function and differentiation. This may explain why blocking FGFR signalling has such a devastating effect on growth and differentiation in the adipocyte. In conclusion, autocrine FGF is required for preadipocyte survival and the commitment to differentiate into mature adipocytes.

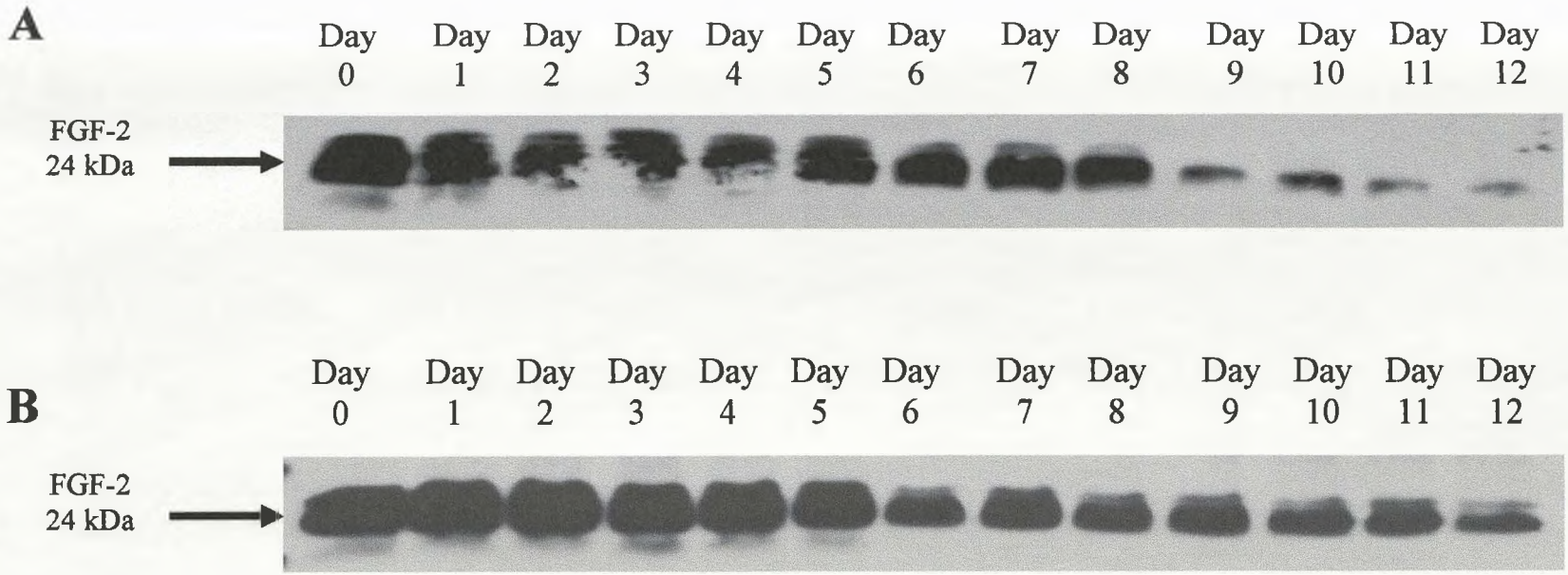


Figure 6.1 Western blot analysis of FGF-2 protein expression over the course of preadipocyte differentiation. 100μg of protein was loaded per lane. (A) Preadipocytes differentiated over the course of 12 days in differentiation medium. (B) Preadipocytes differentiated over the course of 12 days in differentiation medium containing rosiglitazone (10^{-6}M).

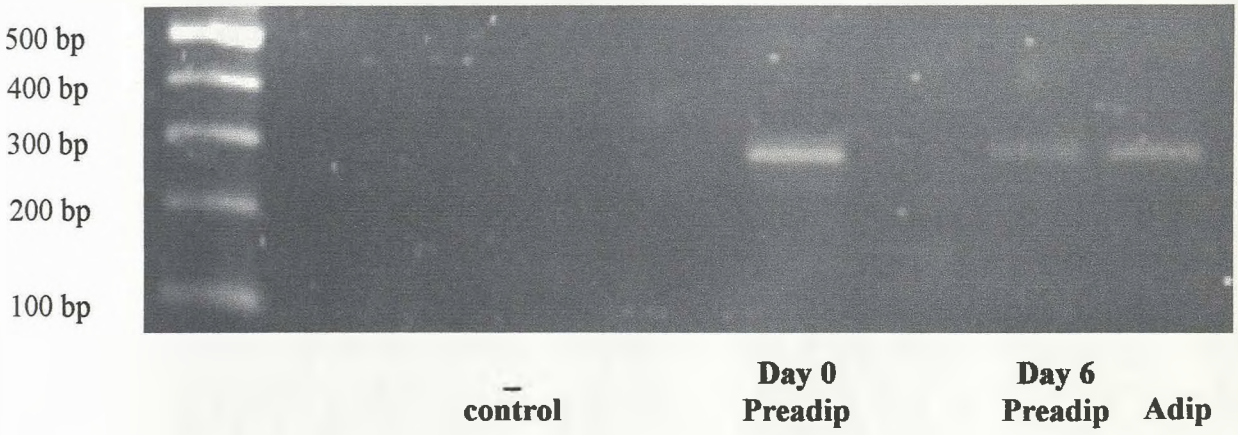


Fig 6.2 Expression of FGF-10 using RT PCR. – control = H_2O, Day 0 Preadip = Day 0 preadipocytes, Day 6 Preadip = Day 6 differentiated preadipocytes, Adip = adipocytes. Expected product size FGF-10 = 313 bp.

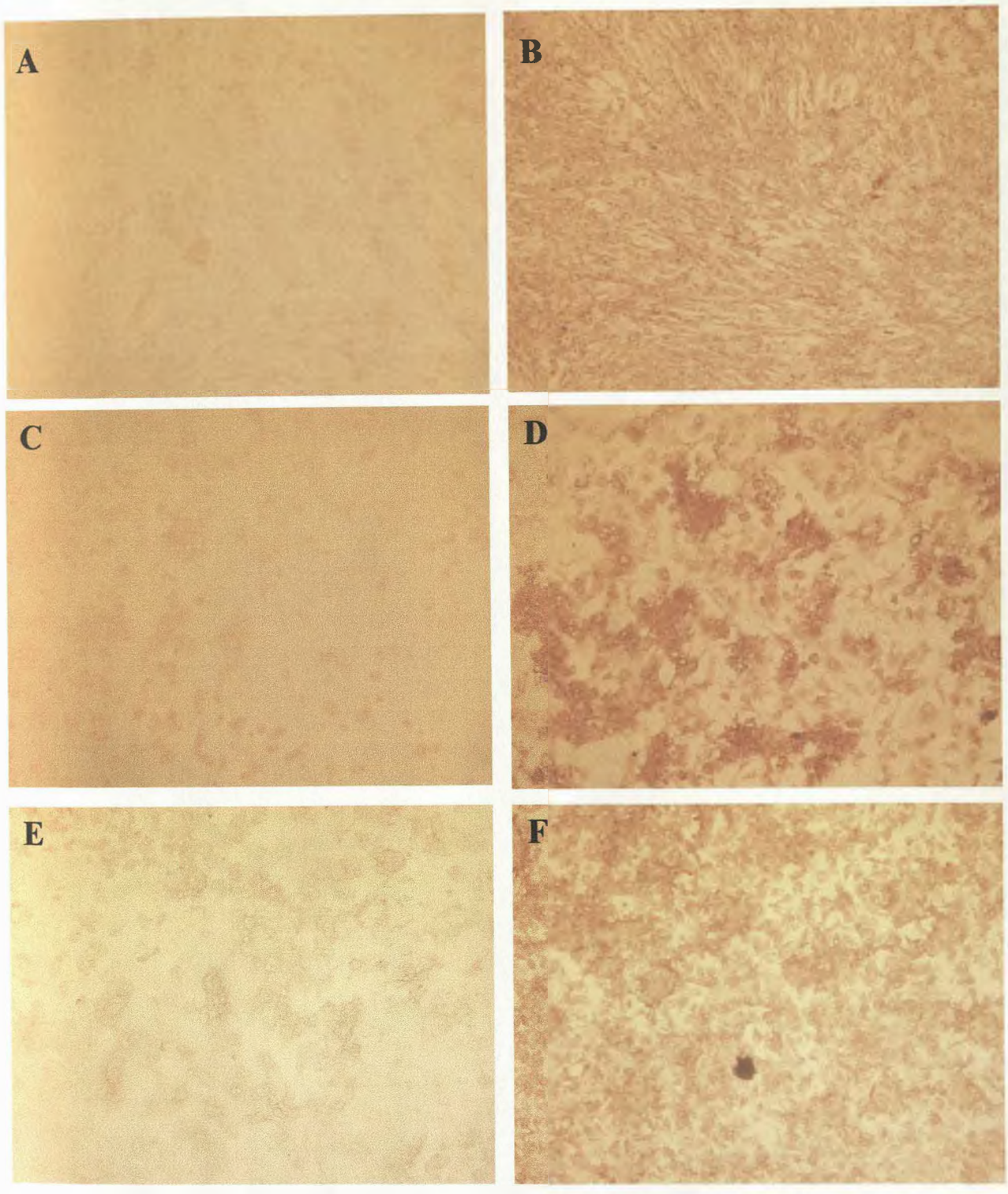


Fig 6.3 Immunostaining for FGFR1 Cells have been immunostained with an antibody specific for the C-terminus of FGFR1. (A) Control preadipocytes not incubated with FGFR1 antibody. (B) Preadipocytes incubated with FGFR1 antibody. (C) Control 12 day preadipocytes differentiated in normal differentiation medium not incubated with FGFR1 antibody. (D) 12 day preadipocytes differentiated in normal differentiation medium incubated with FGFR1 antibody. (E) Control 12 day preadipocytes differentiated in rosiglitazone containing differentiation medium not incubated with FGFR1 antibody. (F) 12 day preadipocytes differentiated in rosiglitazone containing differentiation medium incubated with FGFR1 antibody. Magnification x100.

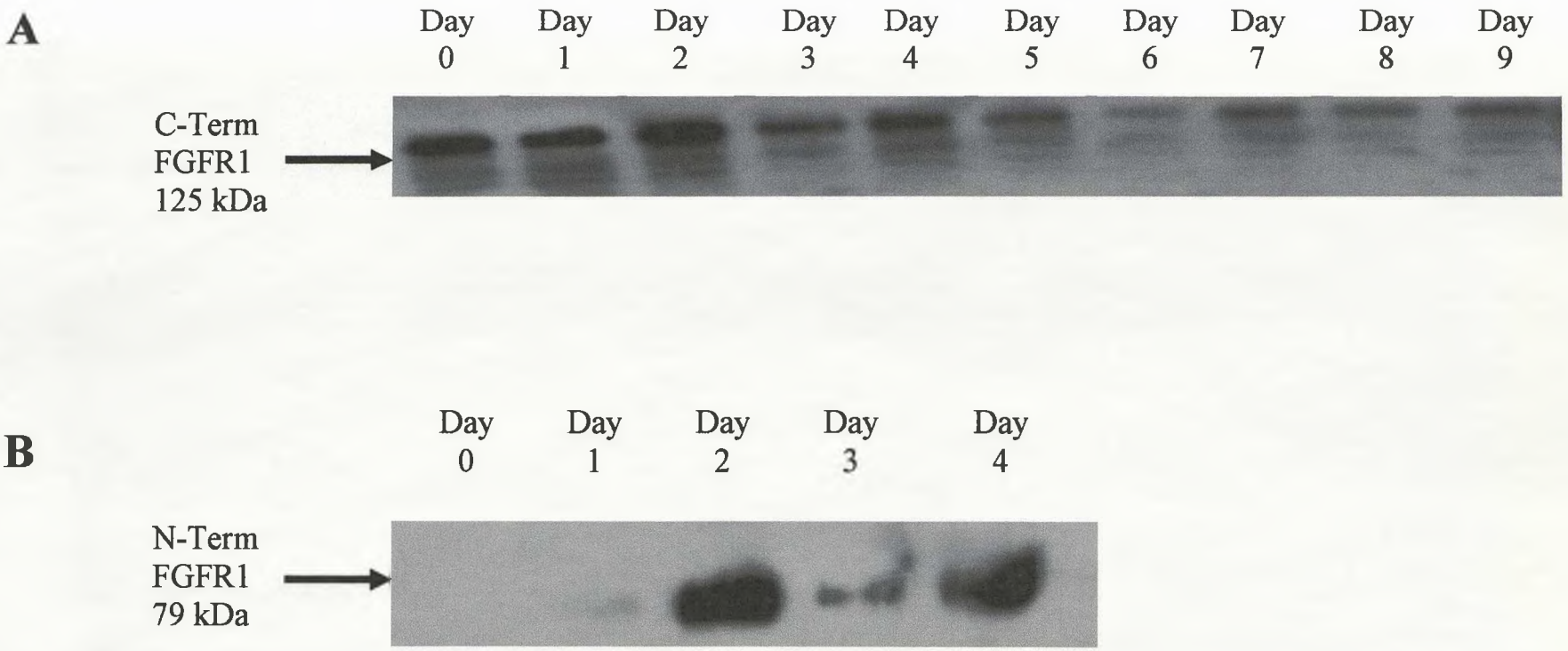


Figure 6.4 Western blot analysis of full length and truncated FGFR1 protein expression over the course of preadipocyte differentiation. 100μg of protein was loaded per lane. (A) Preadipocytes differentiated over the course of 9 days in differentiation medium infected with RAdDN-FGFR1. Cell layer protein was probed with an antibody to the C-terminus of FGFR1 to determine expression of full length FGRFR1. (B) Preadipocytes transduced with RAdDN-FGFR1 and differentiated over the course of 4 days in differentiation medium. Cell layer protein was probed with an antibody to the N-terminus of FGFR1 to determine expression of truncated (DN) FGFR1.

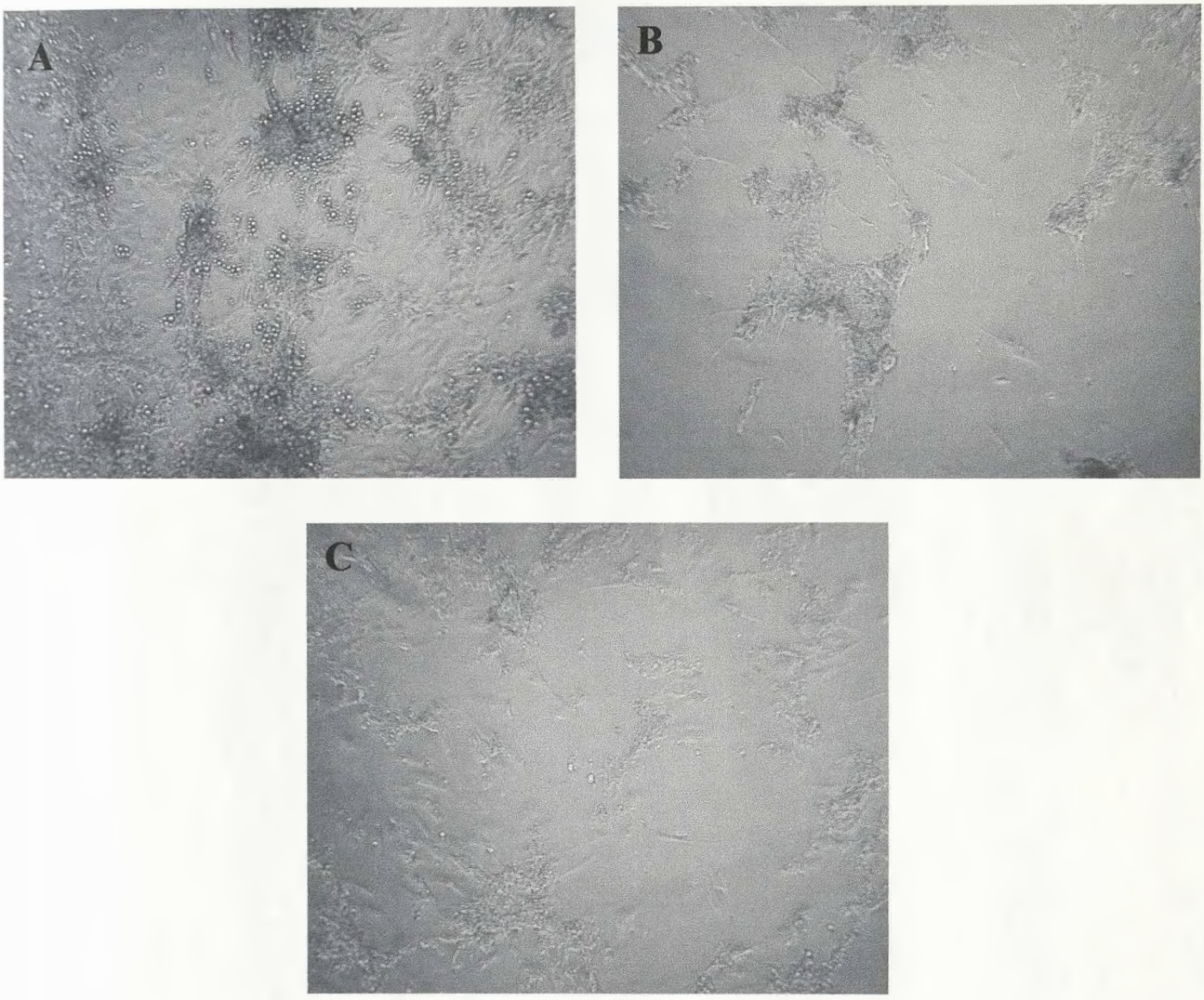


Fig 6.5 The effect of the RAdDN-FGFR1 and the PD166866 compound on adipocyte differentiation. (A) 12-day culture of preadipocytes transduced with the RAd-β-galactosidase virus in differentiation medium. (B) 12-day culture of preadipocytes transduced with the RAdDN-FGFR1 virus in differentiation medium. (C) 12-day culture of preadipocytes in differentiation medium treated with the PD166866 (10^{-7}M). Magnification x100.

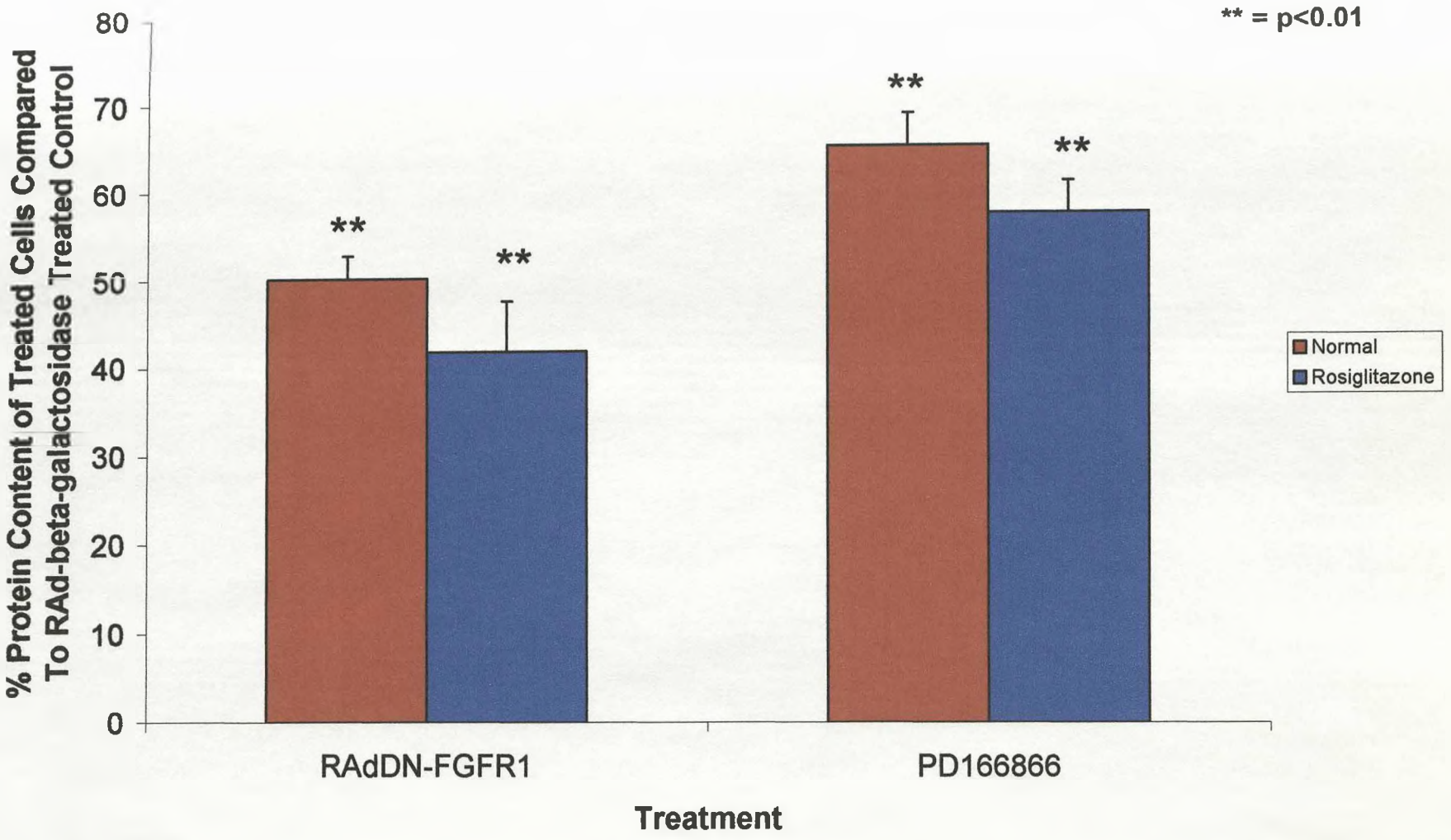


Fig 6.6 The effect of RAdDN-FGFR1 and PD166866 on the protein content of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 12 days, protein content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to RAd-β-galactosidase treated control differentiated preadipocytes ± SEM of 4 independent samples (n=4).

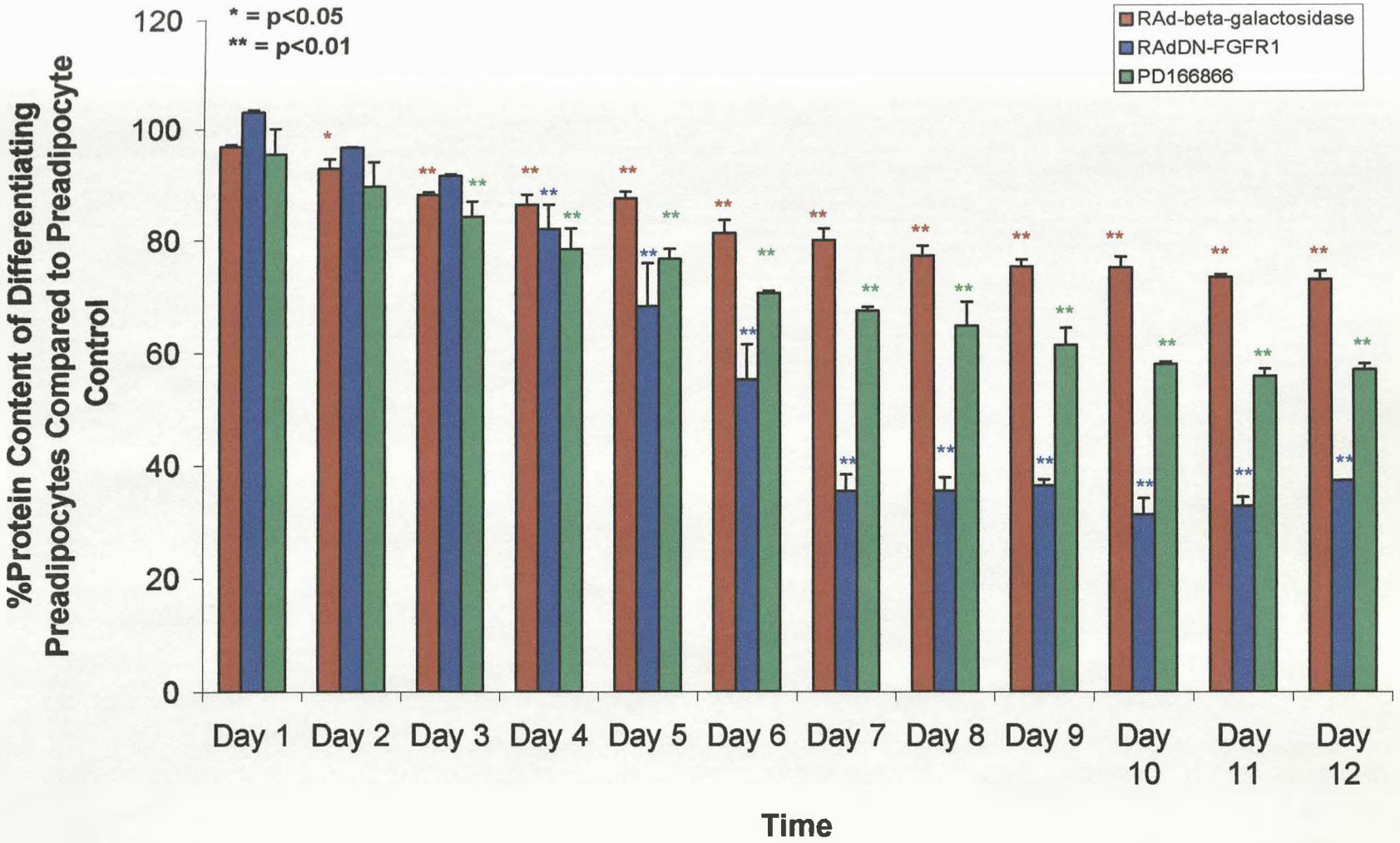


Fig 6.7 The effect on protein content in differentiated preadipocytes transduced with RAd-β-galactosidase, RAdDN-FGFR1, and PD166866 over 12 days. Protein content was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes ± SEM of 3 independent samples (n=3).

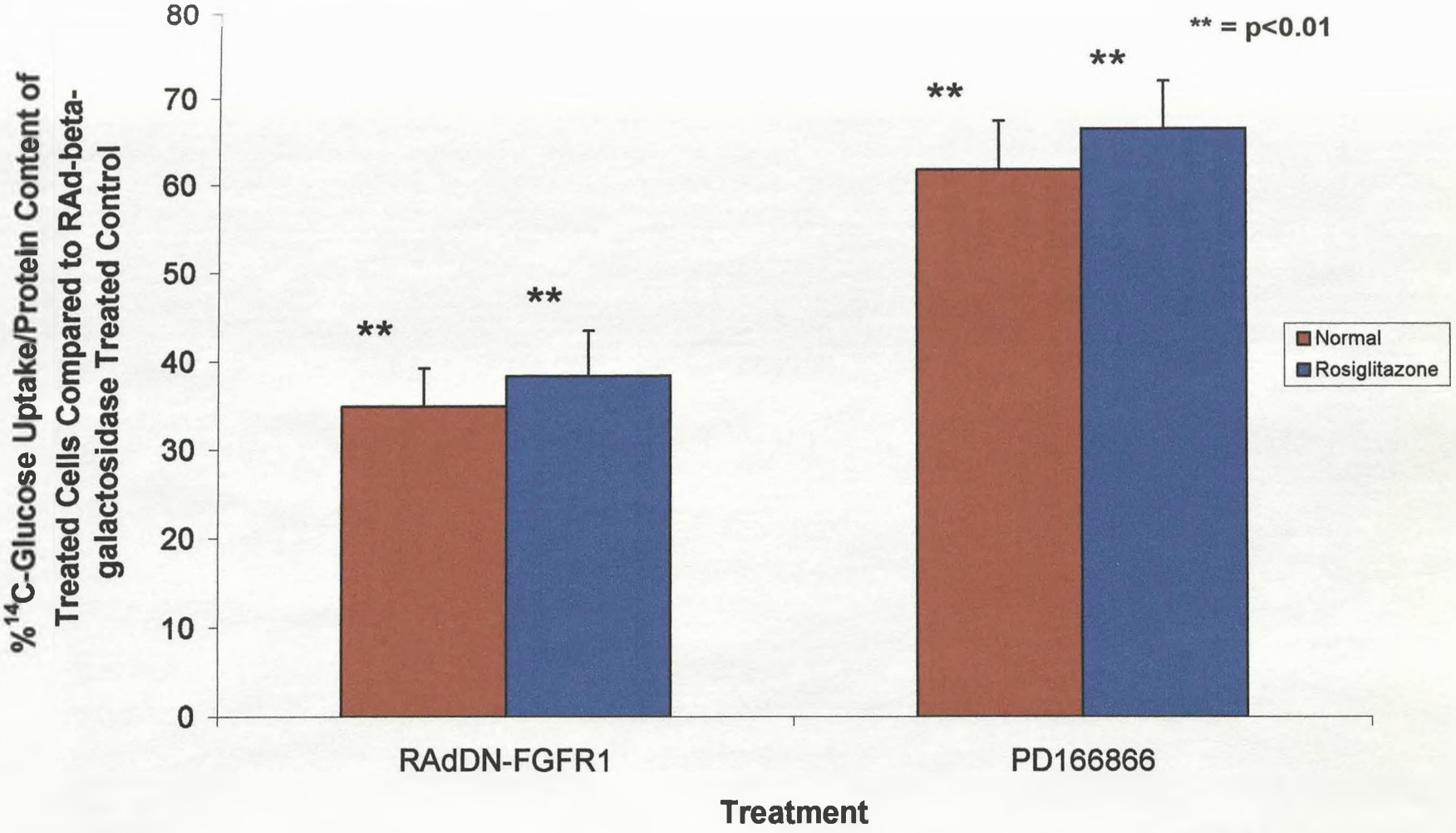


Fig 6.8 The effect of RAdDN-FGFR1 and PD166866 on ^{14}C-Glucose uptake in differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 12 days, ^{14}C-Glucose uptake was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to RAd-β-galactosidase treated control differentiated preadipocytes and corrected for protein content ± SEM of 4 independent samples (n=4).

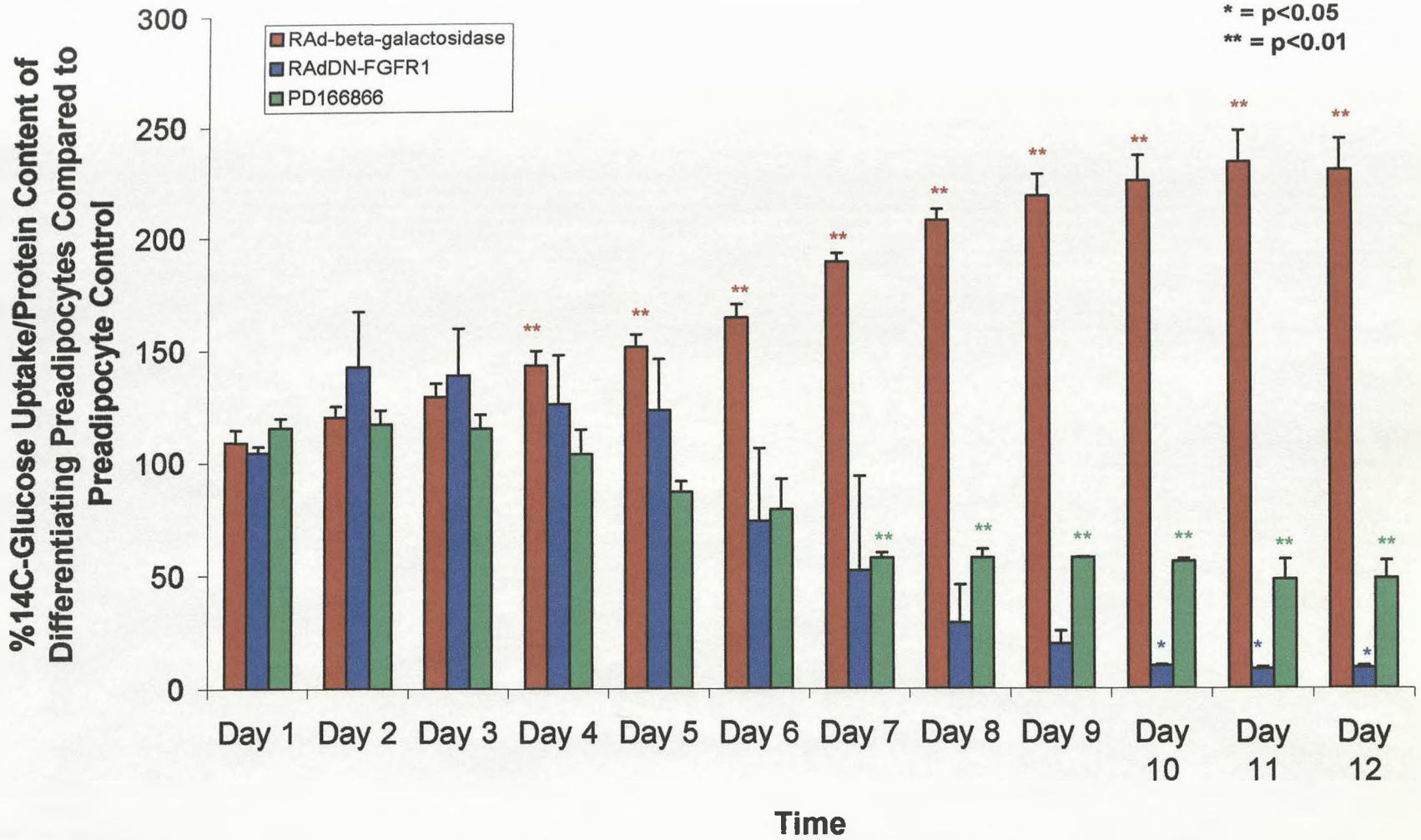


Fig 6.9 The effect on ^{14}C-Glucose uptake in differentiated preadipocytes transduced with RAd-β-galactosidase, RAdDN-FGFR1, and PD166866 over 12 days. ^{14}C-Glucose uptake was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes and corrected for protein content ± SEM of 3 independent samples (n=3).

CHAPTER 7

Role of the Angiogenic Proteins, VEGF and Angiopoietins in Adipocyte Growth and Differentiation

7.1 Introduction

In previous chapters the roles of insulin and FGF receptor signalling in adipocyte function and growth were examined. Both insulin and FGFs signal through the use of specific RTKs (reviewd in Patti and Kahn 1998; Powers *et al.* 2000). Binding of the ligand by its respective RTK causes tyrosine phosphorylation and activates downstream signalling pathways. FGF is known to be angiogenic (Folkman and Shing 1992; Powers *et al.* 2000), while insulin indirectly promotes angiogenesis by upregulating other angiogenic growth factors such as FGF-2 (Yamashita *et al.* 1995) and VEGF (Mick *et al.* 2002). Adipose is a highly vascularised, dynamic tissue which is able to grow and regress throughout adult life. Adipose tissue growth requires angiogenesis (Crandall *et al.* 1997) and adipocyte growth and differentiation are regulated through the vasculature (Rupnick *et al.* 2002). Known angiogenic factors include FGF-2, VEGFs, and the angiopoietins, which are secreted by many cell types. We have shown that FGF-2 is secreted by adipocytes and FGF signalling is important in adipocyte growth and function. There are several studies showing VEGF secretion by adipocytes and adipose tissue however these were performed in rodent WAT and BAT pads and 3T3-L1 cells (Claffey *et al.* 1992; Tonello *et al.* 1999; Asano *et al.* 2001; Mick *et al.* 2002). One study examined VEGF production from human omental adipocytes but there are no data from subcutaneous adipocytes (Zhang *et al.* 1997c). Angiopoietins include Ang 1 and Ang 2 whose receptor, Tie-2 is essential for angiogenesis (Partanen and Dumont 1999; Loughna and Sato 2001). Ang 1 and Ang 2 also function as cell adhesion molecules by interaction with integrins (Carlson *et al.* 2001). In 3T3-L1 adipocytes Ang 1 but not Ang 2 mRNA

was detected (Stacker *et al.* 2000). In contrast Cohen *et al* (2000) found Ang 2 mRNA in both mouse adipose tissue and 3T3-F442A cells and its mRNA levels were increased by leptin (Cohen 2003). There have been no studies to date examining Ang production from human adipose tissue.

The initial aim of this study was to determine whether VEGF, Ang 1, and Ang 2 are expressed in human subcutaneous preadipocytes. The roles of VEGF, Ang 1 and Ang 2 in the adipocyte were also determined. To examine this, adenoviruses expressing soluble forms of Tie-2, VEGFR1 and VEGFR2 were used. RAdExTek and RAdExTie2, which secrete the N-terminal, extracellular portion of Tie2 which binds Ang 1 and Ang 2, and RAd-sVEGFR1, and RAd-sVEGFR2 which secrete the N-terminal, extracellular portion of VEGFR1 and VEGFR2 which bind VEGFs. These soluble receptors prevent signalling through Tie-2 and VEGFRs enabling the role, if any of these molecules in adipocyte growth and differentiation to be determined.

Tie-2 has been found to be expressed in endothelial and haematopoietic cells (Jones *et al.* 2001) and was believed to be specific to those cells. However Tie-2 expression has been shown in thyroid follicular cells which are epithelial (Ramsden *et al.* 2001). VEGF receptors such as VEGFR1 and VEGFR2 were also initially believed to be limited to endothelial cells (Jakeman *et al.* 1992; Jakeman *et al.* 1993) but have also been found to be expressed on other cells such as uterine smooth muscle cells (Brown *et al.* 1997) and pancreatic cancer CAPAN-1 cells (Itakura *et al.* 2000) with VEGFR2 mediating the major growth and permeability actions of VEGF. There are no reports that Tie-2 or VEGFRs have been identified in adipocytes even though their ligands

have been identified. Once the expression of Ang 1, Ang 2 and VEGF has been established, the next aim of this study was to determine whether Tie-2 and VEGFR2 are expressed by human subcutaneous adipocytes.

7.2 Results

7.2.1 Expression of Angiopoietin 1, Angiopoietin 2, and Tie-2 mRNA in Human Preadipocytes and Differentiated Preadipocytes

To establish whether preadipocytes and adipocytes expressed Ang 1, Ang 2, and Tie-2, RT-PCR was performed on mRNA isolated from Day 0 preadipocytes, Day 6 preadipocytes differentiated in normal differentiation medium, and pure adipocytes (provided by Alison Harte). Human thyroid cells (provided by Margaret Eggo) were used as a positive control for Ang 1, Ang 2, and Tie-2. H_20 containing the primers for Ang 1, Ang 2, and Tie-2 in combination were used as a negative control. RT-PCR was carried out using primer pairs for Ang 1, Ang 2, and Tie-2 as described in the Chapter 2. Ang 1, Ang 2, and Tie-2 were expressed in Day 0 preadipocytes, Day 6 preadipocytes differentiated in normal differentiation medium, and pure adipocytes (**Fig 7.1**).

7.2.2 Expression of VEGF and VEGFR2 mRNA in Human Preadipocytes and Differentiated Preadipocytes

To establish whether mature adipocytes, preadipocytes and preadipocytes following differentiation expressed VEGF and VEGFR2, quantitative PCR was performed on mRNA isolated from Day 0 preadipocytes, Day 4 and Day 6 preadipocytes differentiated in normal differentiation medium, and pure adipocytes. Quantitative PCR was carried out using primer pairs for VEGF and VEGFR2 as described in the Chapter 2. VEGF and VEGFR2 expression are expressed relative to a value of 1.0 for Day 0 preadipocytes as shown in **Fig 7.2**. Mature adipocytes expressed very little VEGF mRNA compared with preadipocytes. VEGF mRNA in Day 4 differentiated preadipocytes and Day 6 differentiated preadipocytes significantly decreased in expression compared to Day 0 preadipocytes. The expression of VEGFR2 showed a similar pattern to that of VEGF as shown in **Fig 7.3**. Mature adipocytes by comparison with Day 0 preadipocytes expressed very little VEGFR2 mRNA. VEGFR2 mRNA reduced in expression throughout differentiation compared to Day 0 preadipocytes.

7.2.3 Expression of VEGFR2 in Human Differentiating Preadipocytes

Preadipocytes were cultured on glass coverslips and were induced to differentiate in differentiation medium with and without rosiglitazone. Immunohistochemistry was carried out on preadipocytes, Day 12 normally differentiated preadipocytes and Day 12 rosiglitazone differentiated preadipocytes. These were stained with an antibody

specific for VEGFR2. Control incubations had no primary antibody present. Human thyroid sections were used as a positive control for the VEGFR2 antibody (data not shown). There was no positive staining seen for VEGFR2 in either preadipocytes, Day 12 normally differentiated preadipocytes or Day 12 rosiglitazone differentiated preadipocytes compared to control incubations (**Fig 7.4**).

7.2.4 Expression of Tie-2 in Preadipocytes and Day 12 Differentiated Preadipocytes

Cell layer proteins were isolated from preadipocytes (Day 0) and differentiated preadipocytes (Day 12), and were analysed by Western blotting. Cell layer proteins from human thyroid cells were used as a positive control for Tie-2. 100μg protein was loaded in each lane of the gel, and an antibody to the C-terminus of Tie-2 was used. There was no detectable expression of Tie-2 in preadipocytes or Day 12 differentiated preadipocytes (**Fig 7.5**).

7.2.5 Expression of Tie-2 in Preadipocytes Transduced With RAdExTie2 Virus Differentiated Over 12 Days

To establish whether RAdExTie2 virus secretes soluble, truncated Tie-2 in preadipocytes and to determine the time course of expression of soluble Tie-2, preadipocytes transduced with RAdExTie2, were induced to differentiate over 12 days in differentiation medium. Conditioned media was isolated daily from preadipocytes

(Day 0) to differentiated preadipocytes (Day 12) and were analysed by Western blotting using an antibody to the N-terminus of Tie-2 (**Fig 7.6**). There was no detectable Tie-2 in Day 0 conditioned medium from cells not infected with RAdExTie2. After RAdExTie2 infection a faint band of 110 kDa corresponding to Tie 2 was detected at Day 1 which increased in intensity at Day 2. Following a medium change, Tie-2 levels dropped as existing soluble Tie-2 was removed but soluble Tie-2 was detected at Day 4 and this fell again as existing soluble Tie-2 was removed at Day 5 after a medium change. From Day 6-8 there was an increase in Tie-2 expression before a final medium change with no further Tie-2 expression in conditioned medium. Control uninfected preadipocytes differentiated for 12 days showed no expression of Tie-2 in the conditioned medium (data not shown).

7.2.6 Effects of RAdExTek, RAdExTie2, RAd-sVEGFR1, and RAd-sVEGFR2 Viruses on Protein Content in Differentiated Preadipocytes

Preadipocytes were transduced with RAdExTek, RAdExTie2, RAd-sVEGFR1, and RAd-sVEGFR2 viruses at a MOI of 20 pfu/cell. Control cultures were transduced with RAd-β-galactosidase virus at a MOI of 20 pfu/cell. Cells were induced to differentiate over 12 days in differentiation medium with and without 10^{-6} M rosiglitazone. **Fig 7.7** shows the effect of the RAdExTek, RAdExTie2, RAd-sVEGFR1, and RAd-sVEGFR2 viruses on adipocyte differentiation. **Fig 7.7A** shows 12 day old RAd-β-galactosidase-treated differentiated preadipocytes, **Fig 7.7B-C** show 4 day old differentiated preadipocytes treated with RAdExTek and RAdExTie2, while **Fig 7.7D-G** show 12 day old differentiated preadipocytes treated with

RAdExTek, RAdExTie2, RAd-sVEGFR2, and RAd-sVEGFR1 viruses respectively. After 4 days differentiation, cells infected with RAdExTek and RAdExTie2 began to elongate compared to day 4 RAd-β-galactosidase differentiated preadipocytes (**Fig 7.7B-C**), and by day 12 there were very few cells adherent with little or no lipid accumulation. 12 day old differentiated preadipocytes treated with RAd-sVEGFR1, and RAd-sVEGFR2 were very similar to 12 day old RAd-β-galactosidase treated differentiated preadipocytes. After the 12 day differentiation period, the cell layer was removed and the amount of protein present was determined. Results of treated cells are given as percentage mean values ± SEM compared to RAd-β-galactosidase treated control differentiated preadipocytes (**Fig 7.8**). RAdExTek virus and RAdExTie2 significantly reduced the level of protein content. RAd-sVEGFR1 virus produced a small but significant increase in the protein content, while the effects of RAd-sVEGFR2 were not significant. In preadipocytes differentiated in the presence of rosiglitazone, RAdExTek virus and RAdExTie2 again significantly reduced protein content, while RAd-sVEGFR2 virus significantly increased the level of protein content and RAd-sVEGFR1 had no effect (**Fig 7.8**).

To examine the protein content in the cell layer over 12 days of differentiation, preadipocytes were treated with RAd-β-galactosidase virus, RAdExTek virus, RAdExTie2 virus and induced to differentiate over 12 days in differentiation medium. Cell layer proteins were isolated daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12) and the daily protein content was measured. Results of treated cells are given as percentage mean values ± SEM compared to control Day 0 preadipocytes (**Fig 7.9**). RAd-β-galactosidase, RAdExTek, and RAdExTie2 virus

treated cells showed significant reduction in protein content over the course of preadipocyte differentiation compared to their respective Day 0 control preadipocytes. Both RAdExTek, and RAdExTie2 virus treated cells showed a greater decrease in protein content than RAd-β-galactosidase cells. This reduction in protein content was reflected in the cell density (**Fig 7.7**).

7.2.7 Effects of RAdExTek, RAdExTie2, RAd-sVEGFR2, and RAd-sVEGFR1 Viruses on Glucose Uptake in Differentiated Preadipocytes

The uptake of ^{14}C-glucose in 12 day old preadipocytes transduced with adenoviruses expressing soluble Tie-2, VEGFR1 and VEGFR2 and differentiated with and without rosiglitazone in the differentiation medium is shown in **Fig 7.10**. Data were corrected for protein content. RAdExTek virus and RAdExTie2 virus significantly reduced the level of ^{14}C-glucose uptake into cells, while RAd-sVEGFR1 virus and RAd-sVEGFR2 virus small but significantly increased the level of ^{14}C-glucose uptake into cells compared to RAd-β-galactosidase control cells (**Fig 7.10**). Similar effects were found in preadipocytes differentiated in the presence of rosiglitazone. RAdExTek virus and RAdExTie2 again significantly reduced the level of ^{14}C-glucose uptake in cells, while RAd-sVEGFR1 virus significantly increased the level of ^{14}C-glucose uptake into cells with RAd-sVEGFR2 having no effect (**Fig 7.10**).

To examine the time course of ^{14}C-glucose uptake into cells over 12 days of differentiation, preadipocytes were treated with RAd-β-galactosidase, RAdExTek, RAdExTie2 virus and induced to differentiate over 12 days in differentiation medium.

The uptake of ^{14}C-glucose was measured daily from preadipocytes (Day 0) to differentiated preadipocytes (Day 12) and corrected for protein content. Results of treated cells are given as percentage mean values ± SEM compared to respective control Day 0 preadipocytes (**Fig 7.11**). RAd-β-galactosidase treated cells showed significant increase in glucose uptake while both RAdExTek, and RAdExTie2 virus treated cells showed significant reduction in glucose uptake over the course of preadipocyte differentiation compared to their respective Day 0 control preadipocytes.

7.3 Discussion

In this study we show that mRNAs for Tie-2 and its ligands, Ang 1 and Ang 2 are present in primary cultures of human preadipocytes and differentiating preadipocytes. However Western blotting for Tie-2 protein did not substantiate the mRNA studies and we conclude that Tie-2 mRNA is likely from contaminating endothelial cells. For the angiopoietins further work demonstrating secretion of Ang 1 and Ang 2 is required to confirm our mRNA data in the primary human preadipocyte cultures. None of the studies examining angiopoietin production in adipocytes (Stacker *et al.* 2000; Cohen *et al.* 2001) have shown protein data, however many cell types secrete angiopoietins and it is probable that adipocytes also secrete these angiogenic proteins. Further evidence that angiopoietins are secreted by adipocytes comes from our work with the recombinant adenoviruses expressing soluble versions of Tie-2. Our data indicate that the angiopoietins may have an autocrine role because when sequestered by soluble ligand-binding forms of Tie-2, preadipocyte growth and function are decreased. Ang 1 and Ang 2 have been shown to mediate cell adhesion through

integrins (Carlson *et al.* 2001). Integrins are cell surface receptor proteins which mediate adhesion to the ECM. Angiopoietins can bind integrins as well as ECM proteins such as vitronectin and facilitate cell-cell and cell-ECM adhesion (Carlson *et al.* 2001). The changes observed in the morphology of preadipocytes may be due to the removal of Ang 1 and Ang 2 by the soluble Tie-2 receptor thus preventing cell adhesion. Loss of cell adhesion would compromise preadipocyte growth and function.

The vasculature regulates adipose tissue growth and differentiation and this will require angiogenesis (Crandall *et al.* 1997; Rupnick *et al.* 2002). Tie-2 and its ligands, Ang 1 and Ang 2 will be required for angiogenesis. Secreted Ang 1 and Ang 2 are likely acting in a paracrine manner interacting with the surrounding endothelial cells to facilitate the changes required for adipocyte differentiation and growth. A previous study has shown that Ang 1 expression increases as differentiation progresses in 3T3-L1 cells. Once preadipocytes have matured into adipocytes, levels of Ang 1 drop (Stacker *et al.* 2000). Although apparently contradictory, the authors speculate that Ang 1 is required as differentiation progresses for angiogenesis but once adipocytes are fully mature, angiogenesis is not required hence the fall in Ang 1. Another study by Cohen *et al* (2001) demonstrates that leptin induces Ang 2 in adipose and causes apoptosis of adipose endothelial cells (Cohen *et al.* 2001). They speculate that the reduction in fat mass by increased leptin is due to Ang 2 induction, which causes adipose tissue regression. Both these studies are consistent with our study which shows that that Ang 1 and Ang 2 are important in adipocyte growth and differentiation. We have additionally shown that Ang 1 and Ang 2 do play some role

in the adipocyte as the removal of Ang 1 and Ang 2 causes changes in the adipocyte morphology, function and growth.

In this study we show that VEGF mRNA is expressed by preadipocytes with levels decreasing as differentiation progresses. We find by adding soluble VEGFR2 and VEGFR1 receptors to differentiating preadipocytes, there is a slight increase in differentiation especially with the addition of soluble VEGFR2 receptor.

VEGF is a specific promoter of endothelial cell growth and migration, being a mitogen and angiogenic factor for endothelial cells (Yancopoulos *et al.* 2000). VEGF has been shown to be secreted by the human omentum (Zhang *et al.* 1997c) and in 3T3-L1 cells, VEGF mRNA and VEGF protein secretion increases as adipocyte differentiation progresses (Claffey *et al.* 1992). In contrast in our study in human preadipocytes we show that as differentiation progresses, there is a decrease in mRNA expression of VEGF. The differences seen may be due to species. Another explanantion is that initial isolates of preadipocytes are contaminated with endothelial cells expressing VEGF. During isolation, the mature adipocytes float and the endothelial cells will partition with the pellet of preadipocytes. As cells are cultured in serum-free conditions, endothelial cell contamination will fall as the endothelial cells die. This could explain the rapid fall in VEGF expression.

By inhibiting VEGF with soluble, truncated receptors of VEGFR2 and VEGFR1 we are removing VEGF from differentiating preadipocytes. This leads to an increase in differentiation which suggests that VEGF may have an inhibitory role in adipocyte

differentiation. However these changes are small. Adenoviral infection is known to trigger some signalling pathways e.g. cAMP (Suomalainen *et al.* 2001). Elevation of cAMP is known to increase adipocyte differentiation (Williams and Polakis 1977) which may explain this phenomenon.

VEGF signals through a family of closely related RTKs such as VEGFR2 but there have been no studies examining the presence of VEGFR2 in adipocytes. Quantitative PCR and immunohistochemistry were used to determine the expression of VEGFR2 in adipocytes. We found VEGFR2 mRNA in the initial isolates of preadipocytes but levels fell with culture for 6 days. mRNA levels were much lower in isolated adipocytes compared with preadipocytes. Using immunohistochemistry, we did not find expression of VEGFR2 in either preadipocytes or adipocytes. We conclude that primary cultures of preadipocytes are contaminated with endothelial cells. During isolation, the mature adipocytes float and the endothelial cells will partition with the pellet of preadipocytes. As culture in serum-free conditions continues, this contamination of the preadipocytes is reduced, as the endothelial cells die.

In conclusion the angiogenic growth factors Ang 1, Ang 2 and VEGF are expressed in adipocytes. Tie-2 expression though detectable by RT-PCR, was not detected by Western blotting. Expression of the VEGFR2, while detectable by quantitative PCR fell as differentiation progressed and may be derived from contaminating endothelial cells. Blocking angiopoietin function with either RAdExTie2 or RAdExTek had profound effects on adipocyte growth, function and morphology. We conclude that

this may be due to the prevention of cell-cell and cell-substrate adhesion which has been shown to be mediated through angiopoietin binding to integrins.

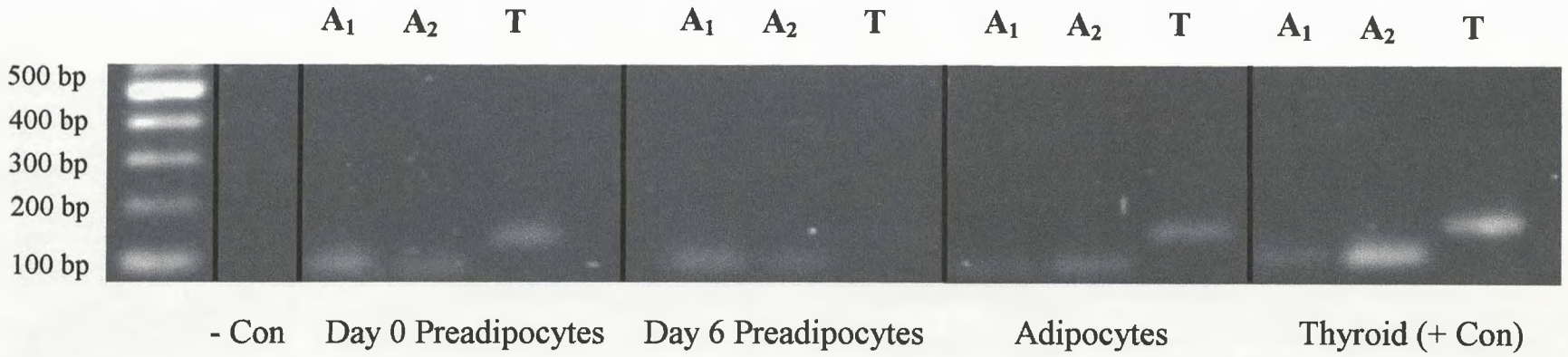


Fig 7.1 Expression of Ang 1 (A_1), Ang 2 (A_2), and Tie-2 (T) by RT PCR in day 0 preadipocytes, day 6 differentiated preadipocytes, and adipocytes. Thyroid (+ Con) = human thyroid cells were used as a positive control for expression of Ang 1, Ang 2, and Tie-2, – Con = H_2O+Ang 1,Ang 2, and Tie-2 primers. Expected product sizes Ang 1 = 84 bp, Ang 2 = 84 bp, Tie-2 = 131 bp.

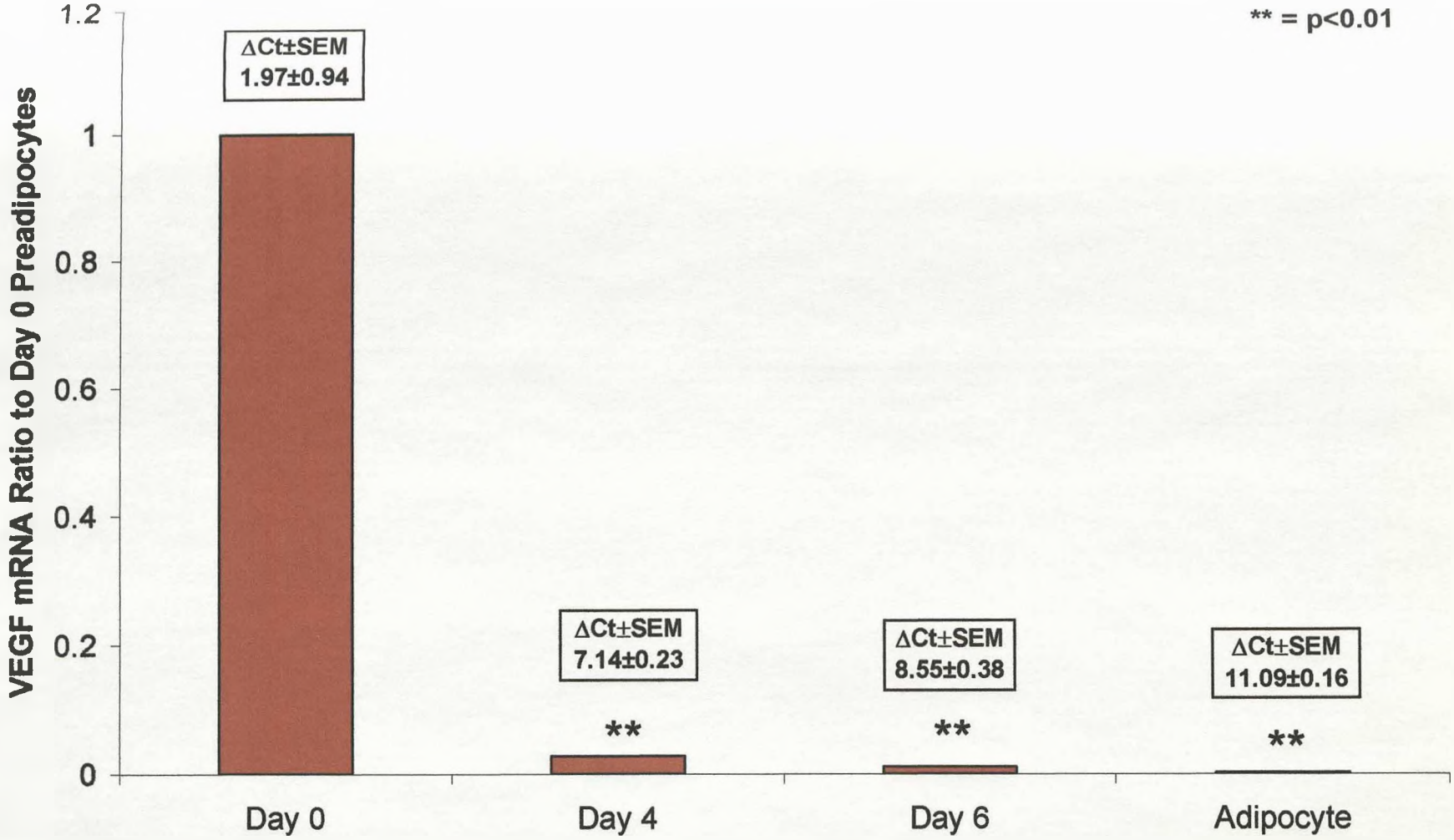


Fig 7.2 VEGF mRNA expression in Day 0 preadipocytes, Day 4 differentiated preadipocytes, Day 6 differentiated preadipocytes, and pure adipocytes. VEGF mRNA expression is expressed relative to a value of 1.0 for Day 0 preadipocytes which act as the control. ΔCt values (±SEM) of 3 independent samples (n=3) are shown above the histogram bars (low ΔCt values represent high gene expression). Statistics were carried out on ΔCt values

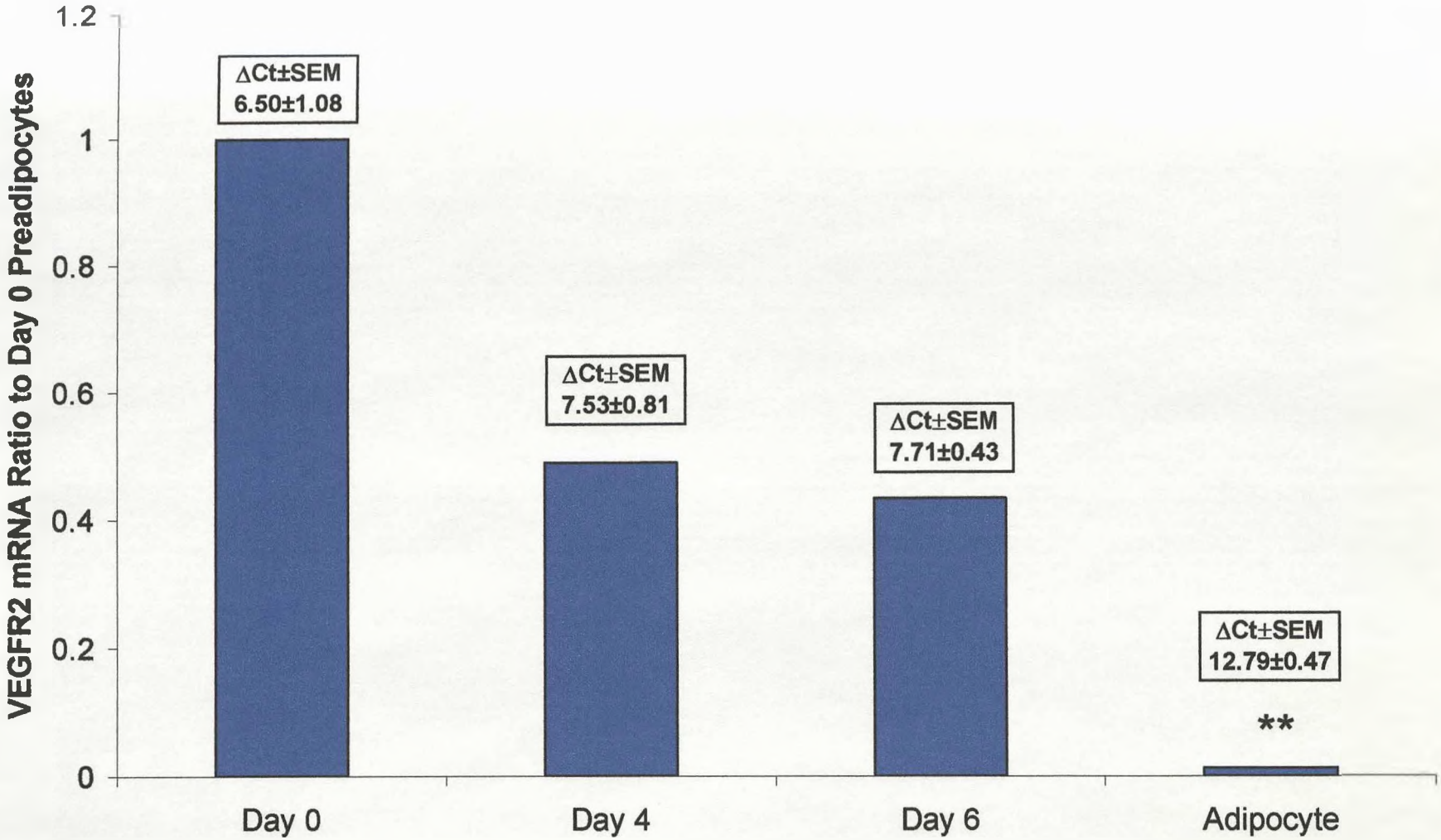


Fig 7.3 VEGFR2 mRNA expression in Day 0 preadipocytes, Day 4 differentiated preadipocytes, Day 6 differentiated preadipocytes, and pure adipocytes. VEGFR2 mRNA expression is expressed relative to a value of 1.0 for Day 0 preadipocytes which act as the control. ΔCt values (±SEM) of 3 independent samples (n=3) are shown above the histogram bars (low ΔCt values represent high gene expression). Statistics were carried out on ΔCt values

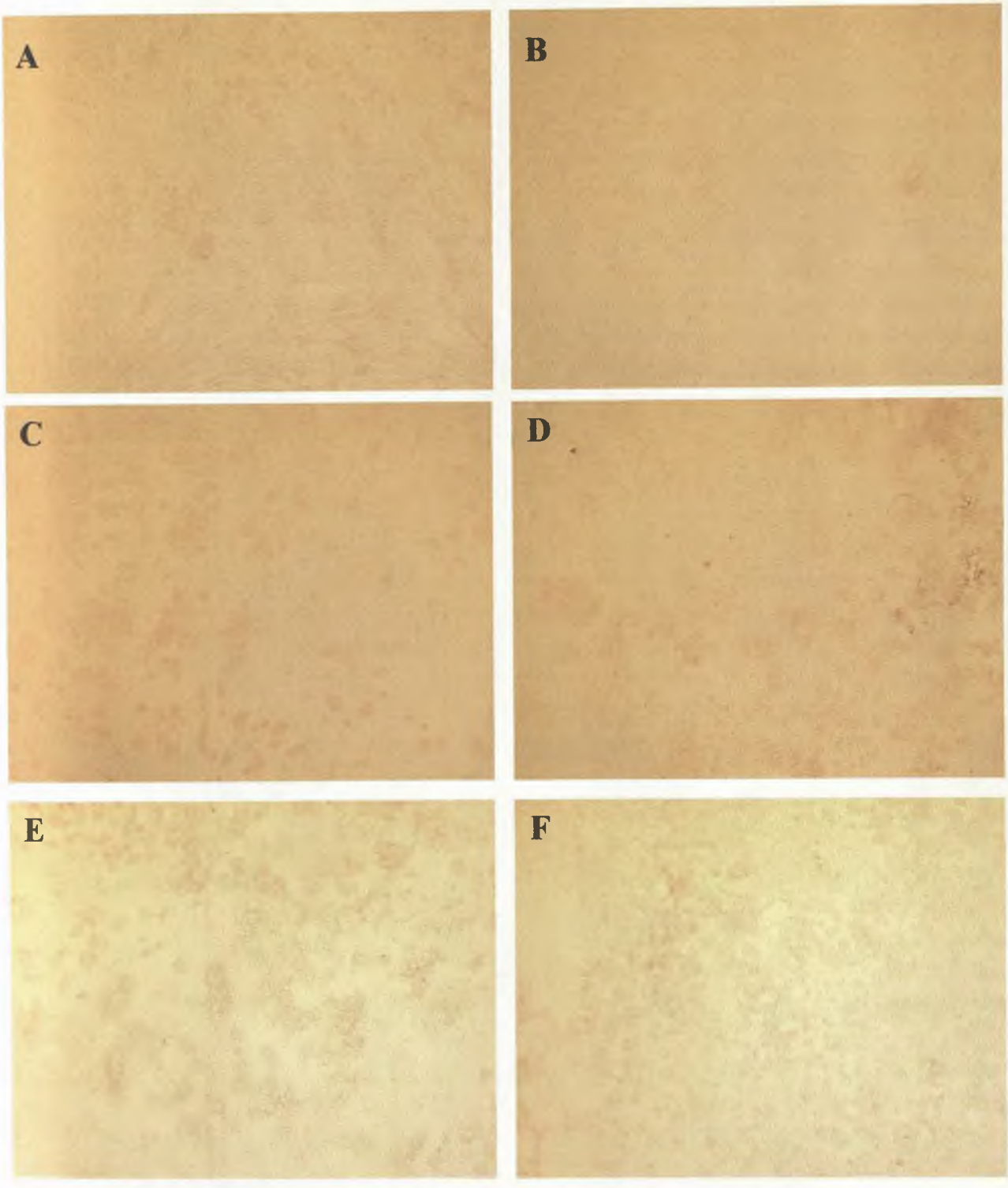


Fig 7.4 Immunostaining for VEGFR2 Cells have been immunostained with an antibody specific for VEGFR2. (A) Control preadipocytes not incubated with VEGFR2 antibody. (B) Preadipocytes incubated with VEGFR2 antibody. (C) Control 12 day preadipocytes differentiated in normal differentiation medium not incubated with VEGFR2 antibody. (D) 12 day preadipocytes differentiated in normal differentiation medium incubated with VEGFR2 antibody. (E) Control 12 day preadipocytes differentiated in rosiglitazone containing differentiation medium not incubated with VEGFR2 antibody. (F) 12 day preadipocytes differentiated in rosiglitazone containing differentiation medium incubated with VEGFR2 antibody. Magnification x100.

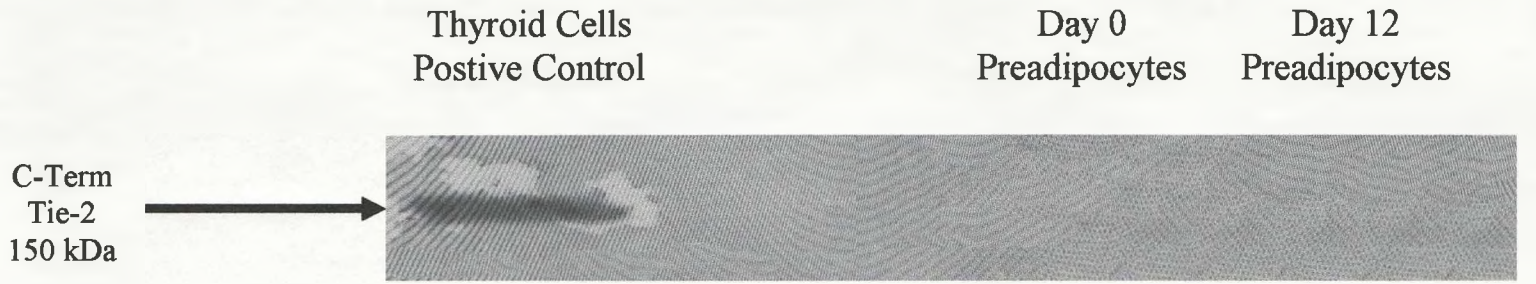


Figure 7.5 Western blot analysis of Tie-2 expression in Day 0 preadipocytes and Day 12 differentiated preadipocytes. 100μg of protein was loaded per lane. Cell layer proteins were probed with an antibody to the C-terminus of Tie-2 to determine expression. Cell layer proteins from human thyroid cells were used as a positive control for Tie-2 expression.

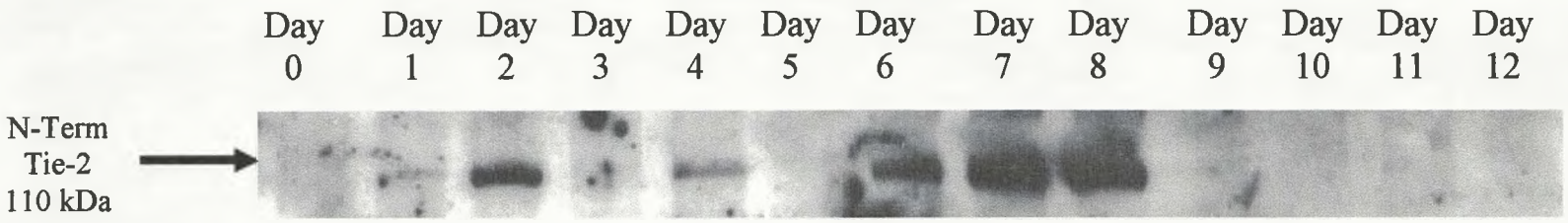


Figure 7.6 Western blot analysis of conditioned medium collected daily from preadipocytes differentiated over the course of 12 days in differentiation medium transduced with RAdExTie2 virus. Medium was changed on day 2, day 4, and day 8 of differentiation. 1ml of conditioned medium was ethanol precipitated and run on a western and probed with antibody to N-terminus of Tie-2.

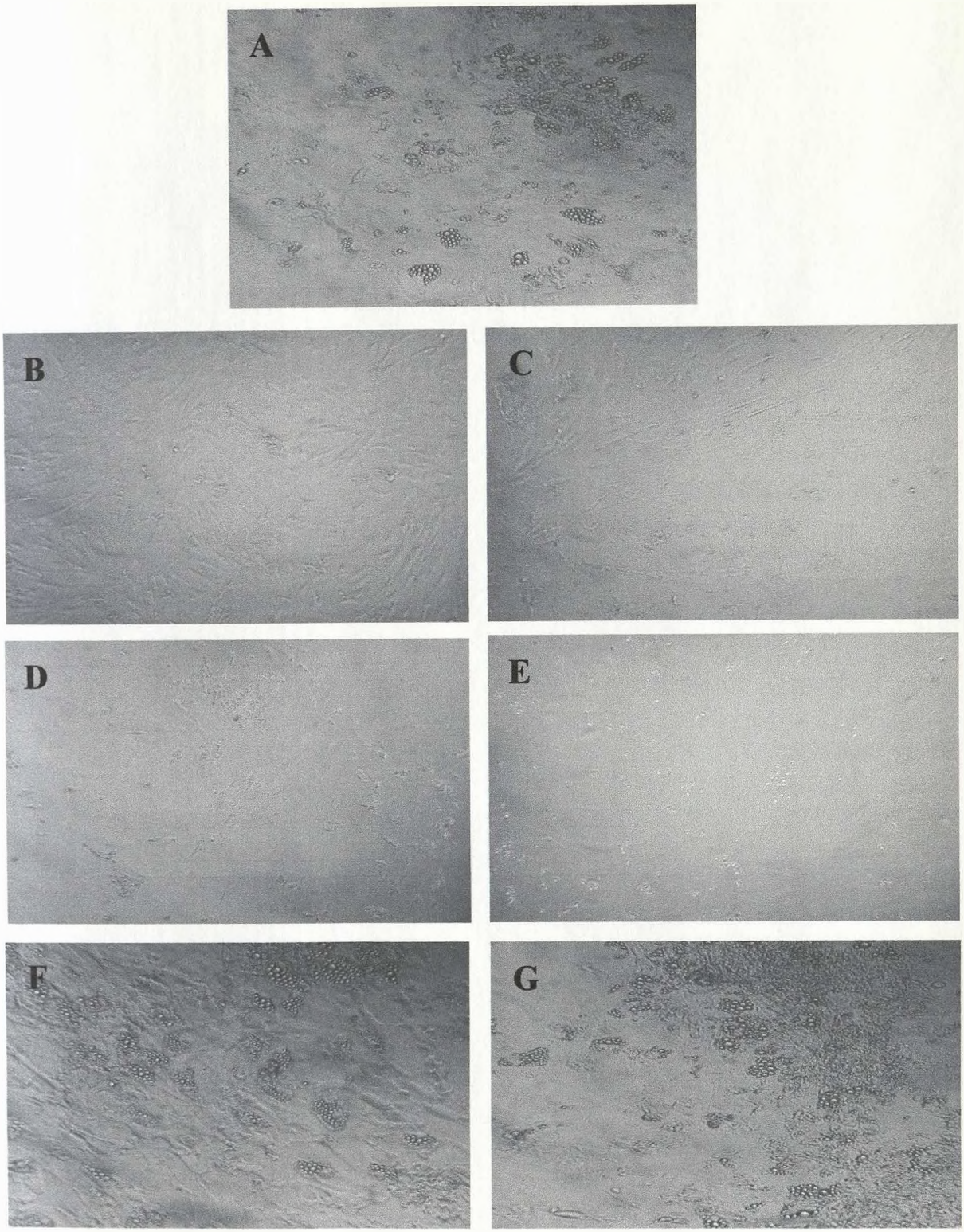


Fig 7.7 The effect of the RAdExTek, RAdExTie2, RAd-sVEGFR2, and RAd-sVEGFR1 viruses on adipocyte differentiation. (A) 12-day culture of preadipocytes transduced with the RAd-β-galactosidase virus in differentiation medium. (B) 4-day culture of preadipocytes transduced with the RAdExTek virus in differentiation medium. (C) 4-day culture of preadipocytes transduced with RAdExTie2 virus in differentiation medium. (D) 12-day culture of preadipocytes transduced with the RAdExTek virus in differentiation medium. (E) 12-day culture of preadipocytes transduced with the RExAdTie2 virus in differentiation medium. (F) 12-day culture of preadipocytes transduced with the RAd-sVEGFR2 virus in differentiation medium. (G) 12-day culture of preadipocytes transduced with the RAd-sVEGFR1 virus in differentiation medium. Magnification x100.

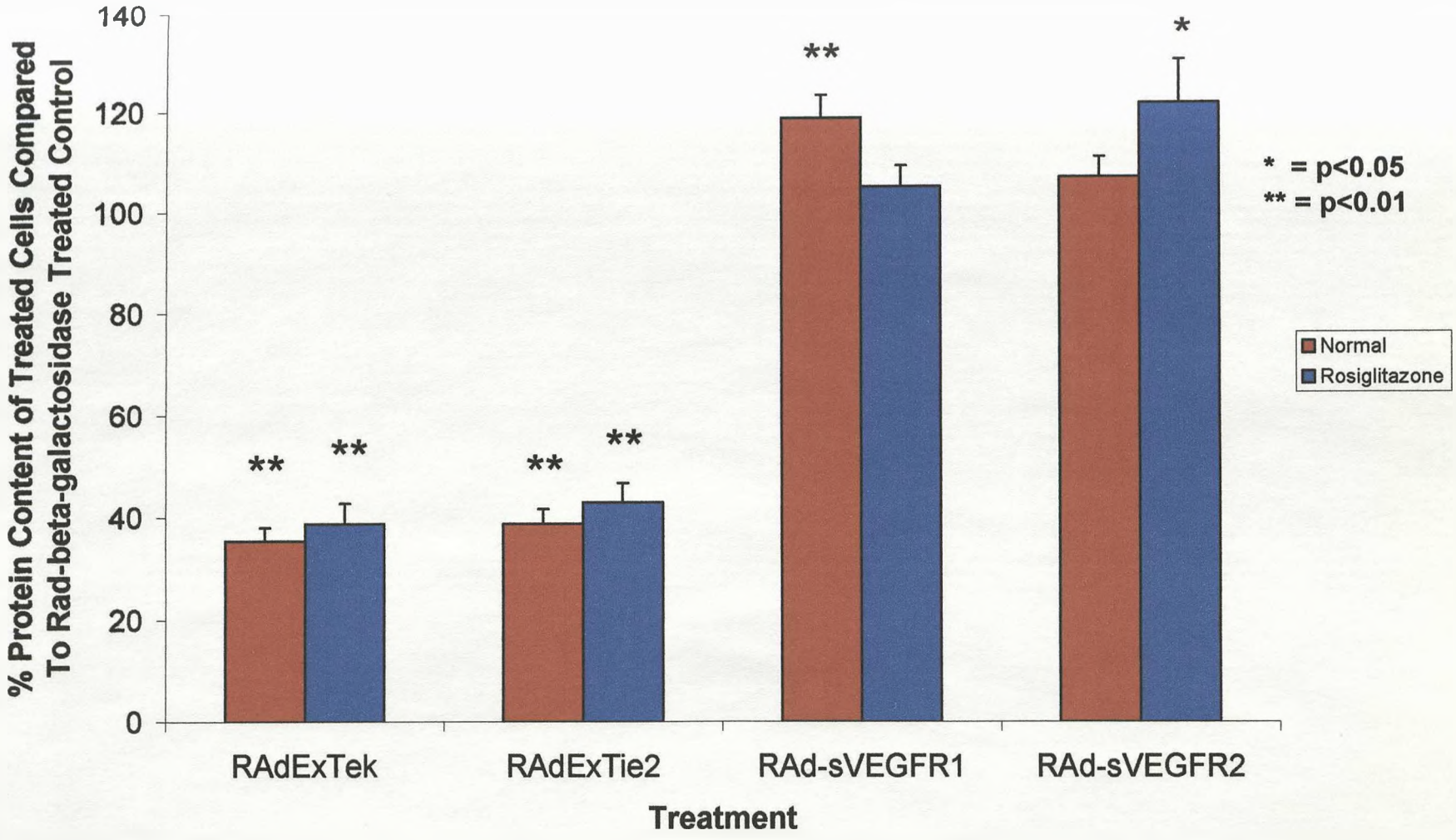


Fig 7.8 The effect of RAdExTek, RAdExTie2, RAd-sVEGFR2, and RAd-sVEGFR1 on the protein content of differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 12 days, protein content was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to RAd-β-galactosidase treated control differentiated preadipocytes ± SEM of 4 independent samples (n=4).

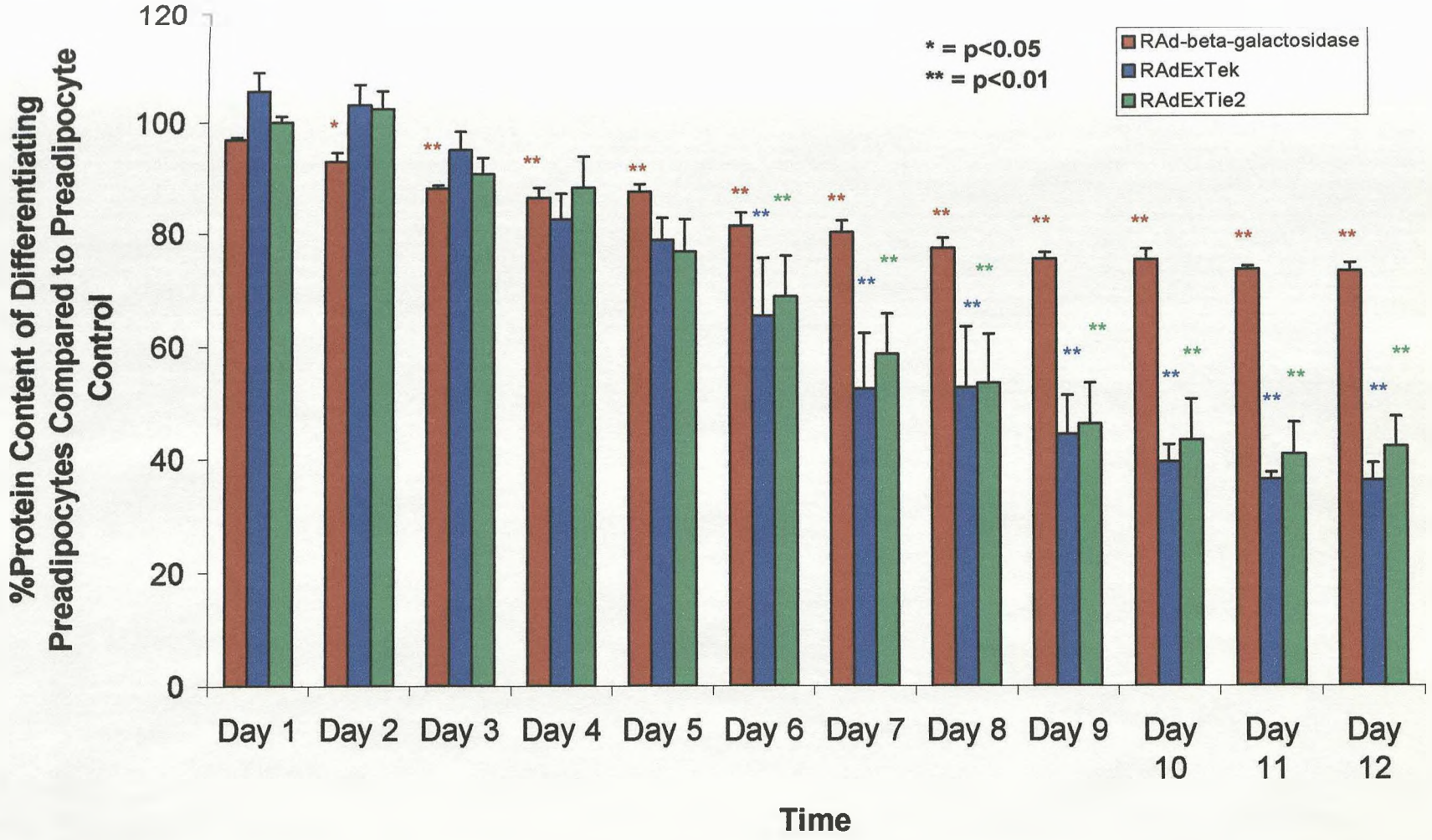


Fig 7.9 The effect on the protein content of differentiating preadipocytes transduced with RAd-β-galactosidase. RAdExTek and RAdExTie2 virus over 12 days. Protein content was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes ± SEM of 3 independent samples (n=3).

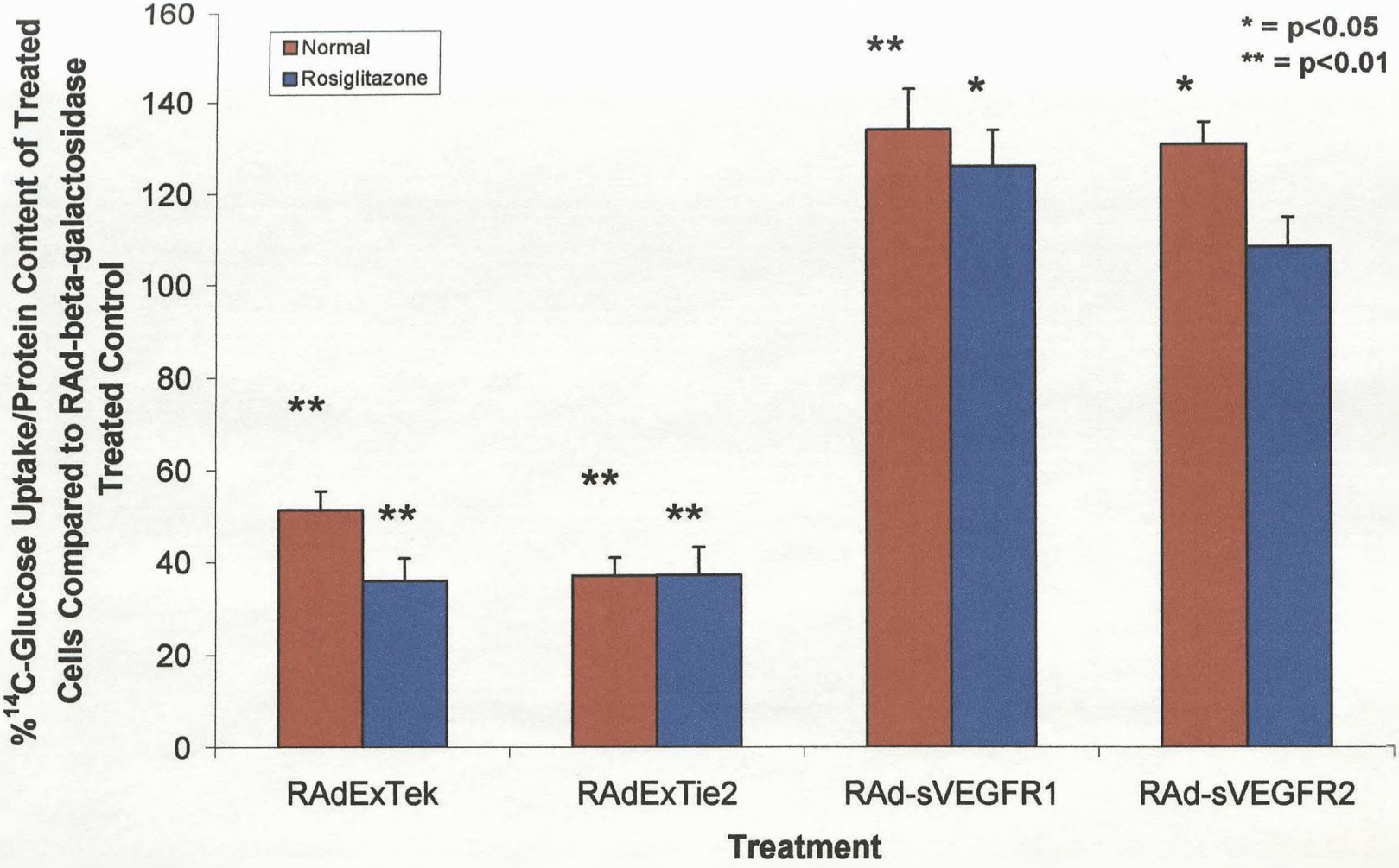


Fig 7.10 The effect of RAdExTek, RAdExTie2, RAd-sVEGFR1, and RAd-sVEGFR2 on ^{14}C-Glucose uptake in differentiated preadipocytes with and without rosiglitazone (10^{-6}M). After 12 days, ^{14}C-Glucose uptake was measured as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to RAd-β-galactosidase treated control differentiated preadipocytes and corrected for protein content ± SEM of 4 independent samples (n=4).

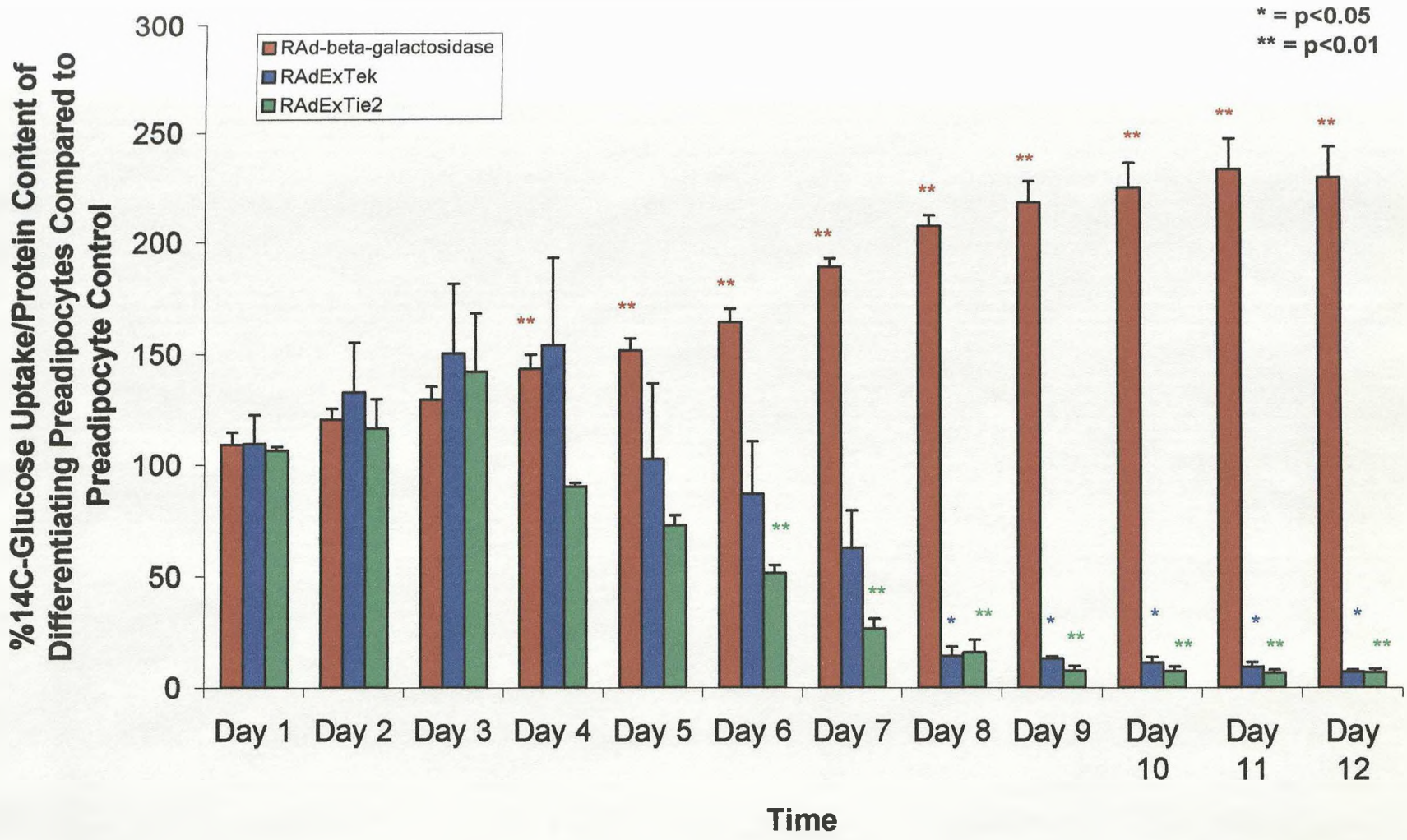


Fig 7.11 The effect on ^{14}C-Glucose uptake in differentiated preadipocytes transduced with RAd-β-galactosidase. RAdExTek and RAdExTie2 virus over 12 days. ^{14}C-Glucose uptake was measured daily as described in the Materials and Methods chapter. Results are given as the percentage mean value compared to Day 0 preadipocytes and corrected for protein content ± SEM of 3 independent samples (n=3).

CHAPTER 8

Final Discussion

Adipose tissue is able to grow and regress throughout the lifetime of an individual. This occurs by the replication and differentiation of preadipocytes and angiogenesis to support the increased mass. These processes require many factors. The aim of this thesis was to examine some of these factors and their signalling mechanisms to determine how they are important in the growth and differentiation of adipocytes.

Primary preadipocytes isolated from the subcutaneous abdominal region were used as the model of adipogenesis as they are more relevant to human disease than rodent models and cell lines. Initially we wished to optimise adipocyte differentiation and to determine the effects of the supplements most frequently used to induce adipocyte differentiation including insulin, dexamethasone, T3 and IBMX (Chapter 3). We examined their effects on different aspects of differentiation such as lipid accumulation, lipogenesis, leptin secretion, protein content and DNA content. We found that individually the supplements had no significant effect on adipocyte differentiation though T3 did stimulate lipogenesis, but when all 4 supplements were used in combination (normal differentiation medium) a high level of differentiation was observed (**Fig 8.1**). This suggests that these supplements (which individually activate different signalling pathways), when used in combination, synergise and cause adipocyte differentiation.

We found that the transcription factor PPAR-γ was expressed during differentiation with levels increasing during the early stages of differentiation suggesting its importance in adipogenesis. Using the PPAR-γ activator rosiglitazone did not have a significant effect on adipocyte differentiation when used alone. When used in

combination with normal differentiation medium, a high level of differentiation was observed reinforcing the view that the supplements when used in combination activate signalling pathways which synergise to induce adipocyte differentiation. Therefore the activation of PPAR-γ and the signalling pathways that are associated with it are important for adipocyte differentiation *in vitro*. Induction of PPAR-γ is mediated by C/EBP-β, C/EBP-γ, and ADD1/SREBP1 which are all induced *in vitro* by hormonal inducers such as insulin, dexamethasone, and IBMX. PPAR-γ can also be induced by the agonist rosiglitazone but the hormonal inducers *in vivo* have yet to be established. Further studies need to be carried out to determine what stimulates adipocyte differentiation *in vivo* and whether it is similar to the inducers of differentiation *in vitro*.

Once the effects of the most commonly used supplements to induce adipocyte differentiation and a model for adipocyte differentiation had been established, a study of the importance of several different signalling pathways in adipocyte differentiation was possible. We used inhibitors of well-documented specificity i.e. rapamycin, PD98059, wortmannin, and SB203580, which inhibit $p70^{S6K}$, p42/44 MAPK, PI3K, and p38 MAPK signalling pathways respectively. Their effects on different aspects of adipocyte differentiation were examined. These signalling pathways are known to be important in cell differentiation but the long term effects of sustained inhibition have not been documented. Inhibiting either the $p70^{S6K}$ or the p38 MAPK signalling pathways significantly reduced lipid accumulation and lipogenesis while the inhibition of the PI3K and p42/44 MAPK signalling pathways had no effect. However inhibition of any of the four signalling pathways inhibited leptin secretion, a

late marker of differentiation. This suggests that lipogenesis and leptin secretion, both markers of differentiation, are controlled by different signalling pathways. The requirement of multiple signalling pathways to induce adipocyte differentiation agrees with the findings of Chapter 3 were the combination of supplements activates signalling pathways that synergise to induce differentiation.

To further the work from Chapter 4, inhibition of signalling pathways upstream and downstream other than the ones which we have used in this study could help indicate more precisely which part of the pathway is essential for growth and differentiation. For example the inhibitor compounds H89 (Chijiwa *et al.* 1990) and UCN01 (Komander *et al.* 2003) which inhibit PKA and PDK1 respectively would allow the role of these pathways in adipocyte growth and differentiation to be established. Another possible way of doing this is by the use of small interfering RNA (siRNA) to silence genes such as p42/44 MAPK, PKB, and PI3K. siRNAs can be designed to silence specific genes of the signalling pathway allowing the role of individual proteins within a pathway and the overall pathway in adipocyte differentiation to be determined. Since the differentiating adipocyte is a non-dividing population, this is a good system in which to discover the role of signalling pathways in differentiation.

It would be interesting to analyse the effects of inhibitors and supplements used for adipocyte differentiation on other markers of growth and differentiation such as apoptosis, lipolysis, resistin, PPAR-γ, TNF-α and adiponectin expression. Gene arrays (Burton *et al.* 2002; Gerhold *et al.* 2002; Jessen and Stevens 2002) and proteomics (Halvorsen *et al.* 2000; Wilson-Fritch *et al.* 2003) would be an interesting

approach to determine the roles of the individual signalling pathways as these techniques allow the identification of mRNAs and proteins whose expression change during the course of adipocyte differentiation. As we have shown inhibiting specific signalling pathways inhibit certain parameters of growth and differentiation but not others, the use of gene arrays and proteomics would allow us to determine what parameters of growth and differentiation are controlled by these pathways and which are not.

Surprisingly we found that the p42/44 MAPK pathway is not important in some aspects of adipocyte differentiation such as lipogenesis, lipid accumulation and survival, but it was important in leptin secretion. Other studies have shown a role in lipolysis (Greenberg *et al.* 2001). We decided to explore whether this pathway was activated by compounds used to induce adipocyte differentiation and fat storage in preadipocytes and differentiated preadipocytes and compared their effects with those of insulin, a bona fide activator of p42/44 MAPK (Chapter 5). It is possible that these compounds use this pathway to mediate some of their effects in the adipocyte. The steroids, cortisol, dexamethasone, oestrogen, and the insulin sensitizer, rosiglitazone did not activate the p42/44 MAPK signalling pathway. T3 caused rapid and sustained activation of p42/44 MAPK in both preadipocytes and differentiated preadipocytes. Activation of p42/44 MAPK by T3 may be important in lipolysis which is known to be stimulated by T3 *in vivo*. Further studies will be required to examine whether the activation of p42/44 MAPK by T3 is important in lipolysis *in vitro*. This could be achieved by using the p42/44 MAPK inhibitor PD98059 to see whether T3-induced lipolysis is reduced or whether p42/44 MAPK activation is important for other aspects

of adipocyte function. It is possible that p42/44 MAPK activation by T3 may potentiate TRβ transcriptional activity (Davis *et al.* 2000; Chen *et al.* 2003). Davis *et al* (2000) found that activation of p42/44 MAPK by thyroid hormone but especially T4 was rapid (10 minutes post-treatment), so it is unlikely to be acting through its classical genomic pathway but rather through a nongenomic one (Davis *et al.* 2000). The identification of how thyroid hormone couples to p42/44 MAPK signalling would be useful to determine. Nongenomic activation by the steroid hormones involves membrane receptors (Losel and Wehling 2003). Membrane receptors for thyroid hormone have been identified in other cells such as VSMC (Ojamaa *et al.* 1996) so there may be a membrane receptor for T3 present in adipocytes which causes the nongenomic activation of p42/44 MAPK. The effects of these compounds on other signalling proteins such as PI3K or PKB would help to further identify which signalling pathways are activated and this could be determined by gene arrays and proteomic approaches.

In the early chapters, we have shown that adipogenesis requires the cooperative effects of several signalling pathways. Although differentiation is dependent on these exogenously added factors, factors secreted by the adipocyte are known to influence adipocyte differentiation and growth. We examined the role of FGF signalling in the adipocyte (Chapter 6). We found that both FGF-2 and FGF-10 were expressed by the adipocyte with levels of FGF-2 decreasing as differentiation progressed. FGF-2 was secreted by the adipocyte with higher levels of secretion in the differentiated preadipocytes compared to preadipocytes. We established that FGFR1 was expressed in preadipocytes and this reduced in expression as differentiation progressed and by

blocking FGFR1 by PD166866, there were large reductions in both growth and adipogenesis. By blocking all FGFRs with RAdDN-FGFR1, the reduction in growth and adipogenesis were further enhanced suggesting that more than one receptor is involved in adipose growth and differentiation. This demonstrates that FGFs acts as autocrine factors and that FGF signalling is important in growth and adipogenesis (**Fig 8.2**).

Our work on FGFs and their signalling demonstrates that multiple FGFs signalling through different FGFR receptors are required for adipocyte growth and differentiation. This suggests that FGFs have multiple roles within the adipocyte. To further the work on FGF signalling, the identity of each FGF expressed and its cognate receptor must be revealed. This will allow the role of each individual FGF and the FGFR through which it signals in adipocyte growth and differentiation to be determined. Conclusions can then be made on whether they act in combination to elicit their effects or whether each individual FGF plays a significant role in adipocyte growth and function.

Due to the important role that the angiogenic molecule, FGF plays in growth and adipogenesis, the possibility that other angiogenic molecules are important in growth and adipogenesis needed to be explored. Chapter 7 initially sought to establish whether the angiopoietins and VEGF were expressed in adipocytes. We showed that VEGF, Ang 1 and Ang 2 are expressed in adipocytes. However protein studies need to be carried out to confirm their presence and expression levels over the course of differentiation as well as identify how they signal. The role of these molecules in

growth and adipogenesis was examined using recombinant adenoviruses expressing soluble receptors for angiopoietins and VEGF, which bind these molecules and prevent them from signalling. We found that angiopoietins are important in growth and adipogenesis and this effect may be due to their effects on cell adhesion (**Fig 8.2**) (Carlson et al 2001). Further investigations are required to determine whether the effects of angiopoietins are due to this or whether they act through an alternative pathway. The role of VEGF was unclear as the removal of VEGF causes a small increase in adipocyte differentiation. This suggests that VEGF is acting in a paracrine manner (**Fig 8.2**). However the effect on differentiation could be due to elevations in cAMP caused by adenoviruses (Suomalainen *et al.* 2001) which could stimulate differentiation (Williams and Polakis 1977).

The inhibition of signalling by both FGF and angiopoietins severely inhibited adipocyte growth and differentiation. Whether the effect of inhibiting these pathways is the same *in vivo* remains to be determined. Although others in the lab have used these recombinant adenoviruses *in vivo*, the animals were not observed for long enough (14 days) to determine whether there was any effect on fat mass. A previous study by Rupnick *et al* (2002) showed that adipose mass in mice can be regulated through the vasculature by the use of angiogenic inhibitors (Rupnick *et al.* 2002). By using the adenoviruses blocking FGF and angiopoietin signalling, we could see whether adipose mass is regulated *in vivo* by FGFs and angiopoietins and whether they act in a paracrine manner on the surrounding endothelium as well as autocrine effects. However targeting adenoviruses to adipose tissue *in vivo* is difficult and their use may result in the inhibition of signalling pathways in other tissues. As FGFs,

angiopoietins, and VEGF are not specific to adipose, their inhibition may result in deleterious effects. It is vital that the downstream signalling pathways from these angiogenic molecules which are important in adipose tissue growth and differentiation be determined. This may be achieved by use of inhibitor compounds or siRNA as discussed previously which will help identify the exact component of the signalling pathway downstream from FGFs and angiopoietins which are important in adipose tissue growth and differentiation. This will potentially enable more specific targeting for therapies to regulate adipose mass.

In conclusion paracrine and autocrine factors such as angiopoietins, FGF and VEGF produced by the adipocyte can affect aspects of adipocyte function, growth and differentiation. Exogenous factors such as insulin, T3, glucocorticoids and IBMX all can influence adipocyte function, growth and differentiation. These activate signalling pathways which work in conjunction with each other to help elicit their effects on the adipocyte. Overall this thesis illustrates the complexity of the adipocyte with many factors activating signalling pathways. These signalling pathways control all aspects of the adipocyte and therefore illustrate their importance. The identification and the role of these factors and signalling pathways will help gain a better understanding of the adipocyte and possibly potential ways to combat obesity, Type 2 diabetes and other conditions associated with the adipocyte.

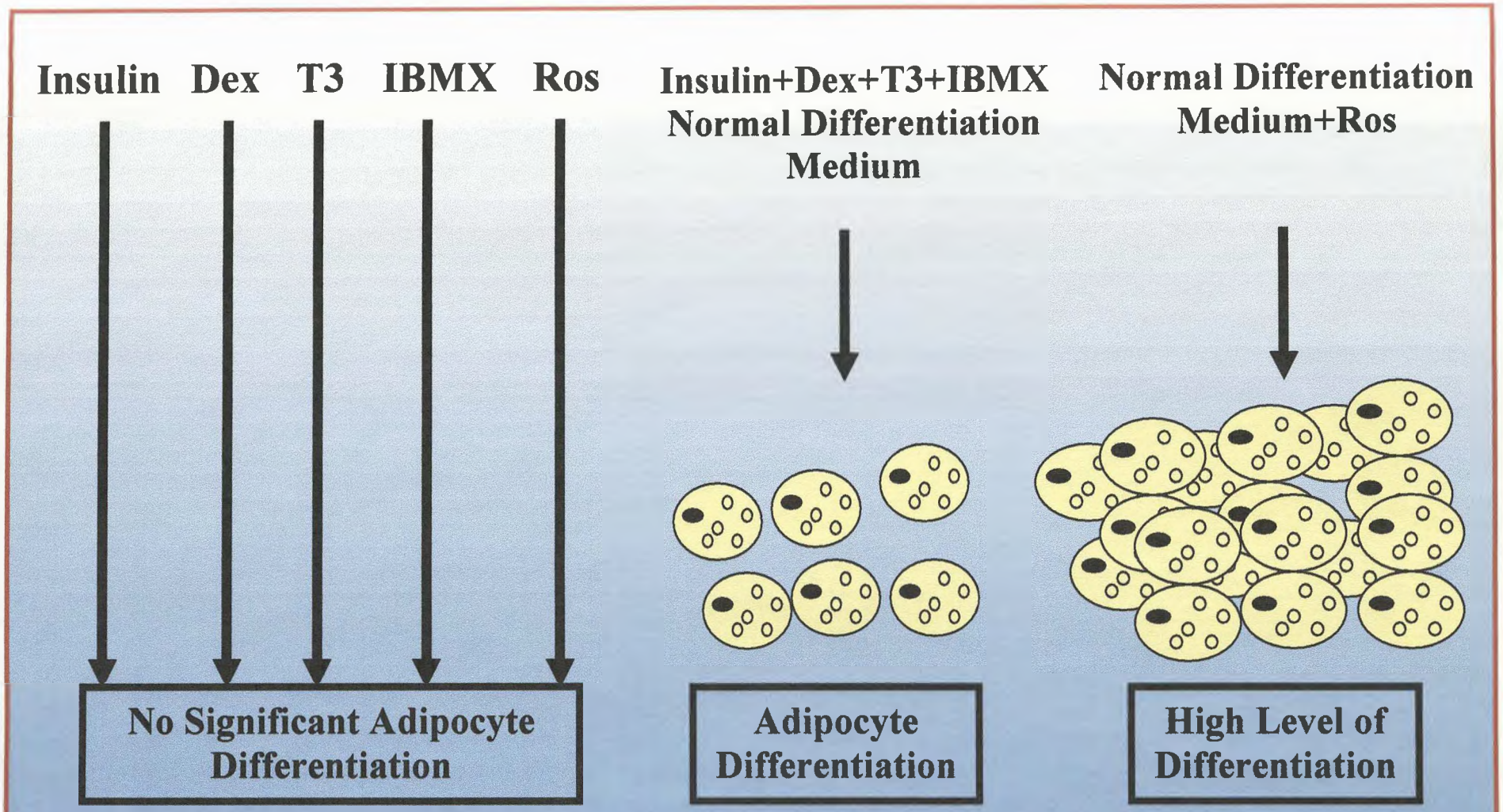


Fig 8.1 Effect of the medium supplements insulin, dexamethasone, T3, IBMX, and rosiglitazone on adipocyte differentiation individually and in combination. Individually each supplement caused no significant adipocyte differentiation but when used in combination, a high level of differentiation was observed with the greatest levels seen when all 5 supplements were used in combination.

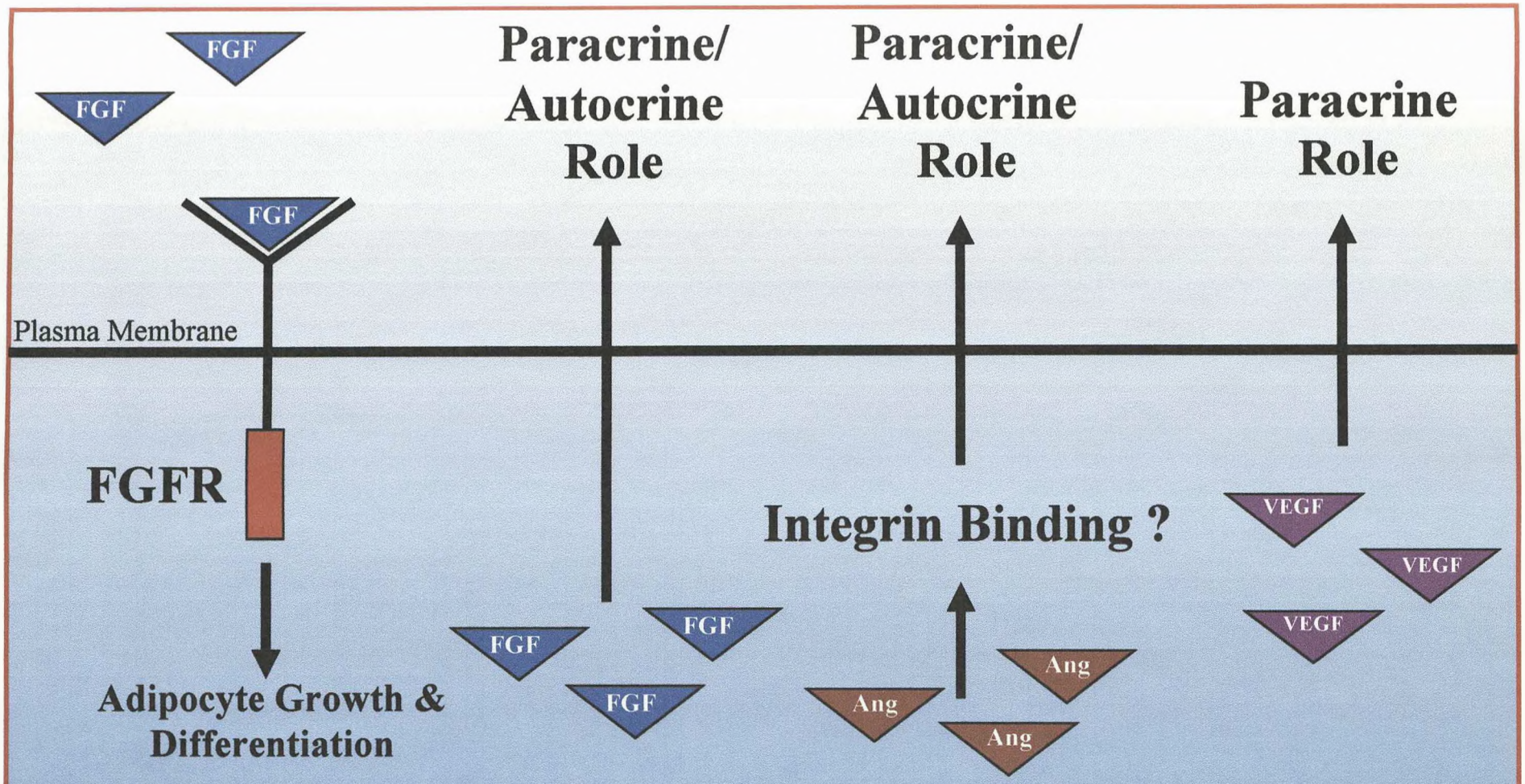


Fig 8.2 Model of action of angiogenic molecules in the adipocyte. FGF has an autocrine role in the adipocyte as FGF-2 and FGF-10 are expressed while inhibiting FGF signalling prevents adipocyte growth and differentiation. Angiopoietins are expressed by adipocytes and play a role in adipocyte growth and differentiation possibly to due integrin binding and cell adhesion. VEGF is expressed by the adipocyte and may play a paracrine role.

Appendix

Treatment	Protein Content (mg)	^{14}C-Lipid (DPM) per mg protein	Lipid Content (Abs 520nm) per mg protein	Leptin Content (ng/ml)	DNA content (485:535nm) arbitrary units
	Data Range	*Data Range*	*Data Range*	*Data Range*	*Data Range*
Day 20 preadipocytes cultured in DMEM/Ham's F-12 (n=6)	0.037-0.239	36.00-318.96	0.51-1.89	0.13-0.38	175-291
Day 20 insulin treated preadipocytes (n=6)	0.047-0.205	42.80-346.98	0.58-1.66	0.22-0.70	124-376
Day 20 dexamethasone treated preadipocytes (n=6)	0.032-0.127	45.43-160.41	0.87-2.18	0.06-0.78	213-388
Day 20 T3 treated preadipocytes (n=6)	0.024-0.192	42.29-816.89	0.62-3.59	0.01-0.72	135-372
Day 20 IBMX treated preadipocytes (n=6)	0.032-0.192	32.15-394.86	0.53-1.74	0.02-0.35	218-286
Day 20 rosiglitazone treated preadipocytes (n=6)	0.045-0.156	46.16-161.36	0.61-1.61	0.10-0.42	135-304
Day 20 differentiated preadipocytes in normal differentiation medium minus insulin (n=5)	0.047-0.086	153.61-1463.74	0.59-2.93		
Day 20 preadipocytes differentiated in normal differentiation medium containing rosiglitazone but minus insulin (n=5)	0.029-0.120	181.34-4456.11	0.61-3.37		
Day 20 differentiated preadipocytes in normal differentiation medium (n=30)	0.033-0.299	9.80-70155.60	0.01-2.86	6.46-16.59	69-290
Day 20 preadipocytes differentiated in normal differentiation medium containing rosiglitazone (n=30)	0.038-0.372	7.60-49041.60	0.02-4.90	6.96-9.51	89-363

Day 20 preadipocytes differentiated in normal differentiation medium containing 10nM rosiglitazone (n=5)	0.021-0.298	694.35-2986.60	0.17-0.43		
Day 20 preadipocytes differentiated in normal differentiation medium containing 100nM rosiglitazone (n=5)	0.010-0.319	859.75-3456.30	0.18-0.43		
Day 20 differentiated preadipocytes in normal differentiation medium containing 10nM insulin (n=5)	0.055-0.099	281.69-2448.08	0.54-2.69		
Day 20 preadipocytes differentiated in normal differentiation medium containing rosiglitazone and 10nM insulin (n=5)	0.068-0.122	146.89-8671.55	1.00-2.90		
Day 20 differentiated preadipocytes in normal differentiation medium containing 1000nM insulin (n=5)	0.068-0.115	587.51-4404.80	0.50-2.76		
Day 20 preadipocytes differentiated in normal differentiation medium containing rosiglitazone and 1000nM insulin (n=5)	0.091-0.169	135.19-11580.99	1.01-3.29		
Rapamycin treated 20 day differentiated preadipocytes (n=15)	0.076-0.670	10.80-90.00	0.02-0.55	1.74-4.67	
Rapamycin treated 20 day rosiglitazone differentiated preadipocytes (n=15)	0.079-0.524	9.50-103.60	0.02-0.68		
PD98059 treated 20 day differentiated preadipocytes (n=15)	0.043-0.345	9.80-1760.46	0.02-3.47	1.55-4.94	
PD98059 treated 20 day rosiglitazone differentiated preadipocytes (n=15)	0.042-0.312	9.60-7541.09	0.02-3.70		
Wortmannin treated 20 day differentiated preadipocytes (n=15)	0.032-0.350	7.50-1725.80	0.02-2.48	1.74-3.10	

Wortmannin treated 20 day rosiglitazone differentiated preadipocytes (n=15)	0.042-0.420	7.40-8954.90	0.02-3.70		
SB203580 treated 20 day differentiated preadipocytes (n=15)	0.037-0.088	24.61-841.20	0.27-1.67	0.03-1.74	
SB203580 treated 20 day rosiglitazone differentiated preadipocytes (n=15)	0.041-0.099	11.40-529.89	0.27-2.37		
RAd-β-gal treated 20 day differentiated preadipocytes (n=12)	0.019-0.228	417.16-22437.10			
RAd-β-gal treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.016-0.151	193.73-20627.49			
RAdDN-FGFR1 treated 20 day differentiated preadipocytes (n=12)	0.021-0.228	148.77-16880.18			
RAdDN-FGFR1 treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.104-0.319	1785.29-26598.27			
PD166866 treated 20 day differentiated preadipocytes (n=12)	0.032-0.148	230.96-8018.90			
PD166866 treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.060-0.174	984.16-14565.68			
RAdExTek treated 20 day differentiated preadipocytes (n=12)	0.013-0.156	144.75-3550.87			
RAdExTek treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.013-0.169	62.52-2683.23			

RAdExTie2 treated 20 day differentiated preadipocytes (n=12)	0.026-0.174	2846.12-11367.94			
RAdExTie2 treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.026-0.197	2019.03-14739.14			
RAd-sVEGFR1 treated 20 day differentiated preadipocytes (n=12)	0.045-0.252	478.98-6327.10			
RAd-sVEGFR1 treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.047-0.151	1232.73-3108.78			
RAd-sVEGFR2 treated 20 day differentiated preadipocytes (n=12)	0.038-0.213	12365.32-49877.32			
RAd-sVEGFR2 treated 20 day rosiglitazone differentiated preadipocytes (n=12)	0.021-0.159	11135.08-49935.57			

Range shows lower and upper values from which percentage values in Chapters 3-7 are derived.

n = number of patient samples

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Publications

List of Publications

Papers

1. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2003). Differential Regulation of Lipogenesis and Leptin Production by Independent Signaling Pathways and Rosiglitazone During Human Adipocyte Differentiation. *Diabetes*, 52(1): 43-50.

Abstracts

1. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2001). Insulin Signalling Pathways Used in Adipocyte Differentiation. *Diabetic Medicine*, 18 (Suppl 2) P23

2. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2001). Insulin Signalling Pathways Used in Adipocyte Differentiation. *Diabetes*, 50 (Suppl 2) A415, 1725-PO

3. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2002). Essential Role of p38 Mitogen-Activated Protein Kinase in Adipogenesis. *Journal of Endocrinology*, 3 (Suppl) P242

4. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2002). Importance of Autocrine FGF-2 in Human Preadipocyte Differentiation. *84th American Endocrine Society Annual Meeting, San Francisco*, P2-315

5. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2002). TNF-α Does Not Exert Its Dedifferentiating Effects on Adipogenesis Via The p38 Mitogen-Activated Protein Kinase Signalling Pathway. *Diabetes*, 51 (Suppl 2) 1850-P

6. **Patel NG**, Kumar S, Eggo MC (2003). Autocrine FGF-2 is Essential For Human Preadipocyte Differentiation. *Journal of Endocrinology*, 5 (Suppl) OC17, **Oral Presentation**

7. **Patel NG**, Holder JC, Smith SA, Kumar S, Eggo MC (2003). T3 Activates p42/44 MAPK in Human Adipocytes and Preadipocytes. *85th American Endocrine Society Annual Meeting, Philadelphia*, P3-242

8. **Patel NG**, Kumar S, Eggo MC (2003). Thyroid Hormones But Not Steroid Hormones Increase p42/44 MAPK Phosphorylation in Human Adipocytes. *European Thyroid Association Annual Meeting, Edinburgh,* O-43, **Oral Presentation**

Differential Regulation of Lipogenesis and Leptin Production by Independent Signaling Pathways and Rosiglitazone During Human Adipocyte Differentiation

Nayan G. Patel,[1] Julie C. Holder,[2] Stephen A. Smith,[2] Sudesh Kumar,[1] and Margaret C. Eggo[1]

Since leptin levels are independently correlated with risk of coronary heart disease, we have identified signaling pathways important in mediating leptin production and lipogenesis in human preadipocytes. We used inhibitors of $p70^{S6}$ kinase, p42/44 mitogen-activated protein kinase (MAPK), p38 MAPK, and phosphatidylinositol 3-kinase (PI3K). Human preadipocytes were induced to differentiate in insulin, dexamethasone, triiodothyronine, and 3-isobutyl-1-methylxanthine in the presence or absence of inhibitors and the peroxisome proliferator–activated receptor (PPAR)-γ activator rosiglitazone. Differentiation was assessed by measuring leptin secretion, lipid content, and lipogenic activity. Rosiglitazone increased cell protein by 15%, the lipid content of the cell layer was doubled, and the lipogenic activity increased sevenfold but did not stimulate leptin secretion. None of the inhibitors significantly inhibited protein content over 20 days, but lipid content and lipogenic activity were inhibited by $p70^{S6}$ kinase and p38 MAPK inhibition but not by p42/44 MAPK or PI3K inhibition. All of the inhibitors significantly decreased leptin secretion, and these inhibitory effects were increased by coincubation with rosiglitazone. We conclude that PI3K and p42/44 MAPK pathways are not critical to the differentiation program leading to lipid accumulation, but stimulation of leptin secretion is dependent on these as well as the $p70^{S6}$ kinase and p38 MAPK signaling pathways. *Diabetes* 52:43–50, 2003

Human obesity is a significant risk factor in a number of diseases, such as non–insulin-dependent diabetes (type 2 diabetes) and hypertension (1). Obesity is characterized by an increase in the size of lipid stores in adipocytes and an increase in the number of adipocytes. These are derived from a pool of existing preadipocytes, which are ready to differentiate when an appropriate signal is given, a process referred to as adipogenesis (2). Changes in the expression levels of roughly 300 proteins are estimated to occur in the structural and functional morphogenesis associated with adipocyte differentiation (3).

Adipocyte differentiation is regulated by a number of hormones, growth factors, and cytokines. Essential components include cAMP, insulin, and glucocorticoids, while tumor necrosis factor (TNF)-α, a product of adipocytes, is known to inhibit differentiation (2,4–6). Differentiation can be characterized by chronological changes in the expression of numerous genes. These include lipoprotein lipase (LPL), peroxisome proliferator–activated receptor (PPAR)-γ, GLUT4, glycerol-3-phosphate dehydrogenase (G3PDH), and leptin, which appear during different stages of differentiation, leading to the characteristic changes in morphology and the accumulation of triglyceride in the cytoplasm (2,5).

Leptin, the 167–amino acid product of the *ob* gene, is synthesized primarily in adipocytes and is a major regulator of fat and energy storage due to its effects on the hypothalamus and neuropeptide Y secretion. The leptin receptor is, however, widespread and is expressed on inflammatory blood cells, lung, liver, and intestine, and leptin has inhibitory effects on glucose metabolism and insulin secretion due to its effects on pancreatic β-cells (7). In obesity, there is leptin resistance to the elevated circulating leptin levels (7,8). Leptin has been shown in several studies to be an independent risk factor for coronary heart disease (9,10), but in obesity, sensitivity to effects on the sympathetic nervous system may not be decreased compared with metabolic effects. This may explain the hypertension observed in obesity (8).

Transcription factors play an important role in adipocyte differentiation. One important transcription factor is PPAR-γ, which, in gain-of-function studies where nonadipogenic fibroblastic cells were made to express PPAR-γ, a strong differentiation response was obtained (11,12). The use of synthetic ligands for PPAR-γ such as the thiazolidinediones has shown promotion of adipogenesis in vitro (13), although the requirement for insulin, dexamethasone, and cAMP in vitro remains (14). However, PPAR-γ activation inhibits leptin gene transcription in 3T3-L1 cells, and human studies show a reduction in circulating leptin with thiazolidinedione treatment (15,16). Autocrine TNF-α also inhibits leptin transcription in human adipose cells and inhibits other aspects of differentiation, such as

From the [1]Division of Medical Sciences, University of Birmingham, Birmingham, U.K.; and [2]GlaxoSmithKline, Harlow, Essex, U.K.

Address correspondence and reprint requests to Dr. Margaret C. Eggo, The Medical School, Birmingham, B15 2TT, U.K. E-mail: m.c.eggo@bham.ac.uk.

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C/EBP, CCAAT/enhancer-binding protein; Dex, dexamethasone; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; G3PDH, glycerol-3-phosphate dehydrogenase; FKBP12, FK-506–binding protein-12; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; IBMX, 3-isobutyl-1-methylxanthine; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator–activated receptor; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; T3, triiodothyronine; TBS-T, Tris-buffered saline–Tween 20; TNF, tumor necrosis factor.

G3PDH, GLUT4, and PPAR-γ expression. TNF-α also causes the dedifferentiation of human mature adipocytes (17,18).

This study aims to determine whether the differentiation program in adipocytes is differentially susceptible to inhibition by specific inhibitors of signaling pathways and PPAR-γ activation. To accomplish this, we have treated differentiating human preadipocytes with four different inhibitor compounds: rapamycin, PD98059, wortmannin, and SB203580, which inhibit $p70^{S6}$ kinase, p42/44 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and p38 MAPK, respectively. Rapamycin inhibits the insulin-responsive kinase, $p70^{S6}$ kinase, by binding with FK-506–binding protein-12 (FKBP12), and this complex interacts with mTOR, causing the inhibition of $p70^{S6}$ kinase (19). PD98059 has been shown to inhibit MEK1, an enzyme that activates p42/44 MAPK (20). p42/44 MAPKs play a major role in the regulation of cell growth and differentiation in adipocytes and elsewhere (21). SB203580 is a pyridinyl imidazole that inhibits p38 MAPK (22), while wortmannin inhibits PI3K, an enzyme that selectively phosphorylates the 3-position of the inositol ring and is acutely activated by insulin and other growth factors (23). These compounds inhibit pathways known to be involved in cell differentiation. We have also examined the effect of PPAR-γ activation on differentiation and on the effects of the inhibitors.

RESEARCH DESIGN AND METHODS

Subjects. Subcutaneous adipose tissue was obtained from patients undergoing elective surgery in accordance with the guidelines of the South Birmingham ethics committee. None of the patients had diabetes or severe systemic illness, and none was taking medications known to influence adipose tissue mass, distribution, or metabolism.

Isolation and culture of human preadipocytes. Adipocytes were isolated by a variation of the method of Rodbell (24). Adipose tissue was digested with 1 mg/ml type I collagenase (Worthington Biochemical, Freehold, NJ) in Hank's balanced salt solution (HBSS), for 1 h at 37°C, and shaken at 100 cycles/min. The disrupted tissue was filtered through a double-layered cotton mesh, and isolated cells were washed with HBSS and centrifuged at 250*g* for 5 min to give a pellet containing preadipocytes. The cell pellet was resuspended in erythrocyte lysis buffer (154 mmol/l NH_4Cl, 5.7 mmol/l K_2HPO_4, and 0.1 mmol/l EDTA, pH 7.0) for 10 min and centrifuged at 250*g* for 5 min to remove erythrocyte contamination. The resulting pellet was washed in HBSS, centrifuged at 250*g* for 5 min, and resuspended in DMEM/Ham's F-12 medium (Invitrogen, Paisley, U.K.) supplemented with 15% bovine FCS (First Link, Brierley Hill, U.K.). The cells were plated in tissue culture dishes and grown until confluent. All media used was supplemented with 100 units/ml penicillin G (Sigma) and 0.1 mg/ml streptomycin sulfate (Sigma).

Differentiation of human preadipocytes. Confluent preadipocytes were washed twice with HBSS and cultured in differentiation medium containing 100 nmol/l insulin (Sigma), 100 nmol/l dexamethasone (Dex) (Sigma), 0.2 nmol/l triiodothyronine (T3) (Sigma), and, for the first 4 days of culture, 25 μmol/l 3-isobutyl-1-methylxanthine (IBMX) (Sigma). Cells were treated with four different inhibitor compounds, 10^{-7} mol/l rapamycin (Calbiochem, Nottingham, U.K.), 10^{-5} mol/l PD98059 (Calbiochem), 10^{-7} mol/l wortmannin (Alexis, San Diego, CA), 10^{-6} mol/l SB203580 (supplied by GlaxoSmithKline, U.K.) as well as the insulin-sensitizing agent 10^{-6} mol/l rosiglitazone (supplied by GlaxoSmithKline, U.K.). Stock solutions were made up in DMSO, and control cultures were treated with differentiation medium and the vehicle DMSO. Cells were incubated in 5% CO_2:95% air at 37°C, and differentiation medium and treatments were changed every 2–3 days for 20 days until cells had accumulated visible lipid droplets.

Lipogenesis assay. Lipogenesis was measured in 20-day-old differentiated preadipocytes using a variation of the method described by Moody et al. (25). Briefly cells were washed in HBSS and incubated overnight at 37°C, 5% CO_2:95% air, in DMEM (GibcoBRL Life Technology) containing 5 mmol/l glucose (Sigma), 100 nmol/l insulin, 100 nmol/l Dex, 0.2 nmol/l T3, and 2 μCi/ml D-[U-^{14}C]glucose (specific activity 291 mCi/mmol) (Amersham International, Slough, U.K.) as well as treatments with 10^{-7} mol/l rapamycin, 10^{-5} mol/l PD98059, 10^{-7} mol/l wortmannin, 10^{-6} mol/l SB203580, and 10^{-6} mol/l rosiglitazone. Cells were washed twice with HBSS, lipid was extracted using ethanol, and D-[U-^{14}C]glucose radioactivity was determined by liquid scintillation counting.

Lipid staining of differentiated preadipocytes. Lipid staining was carried out using a variation of the method described by Culling (26). Briefly cells were washed with HBSS and stained with 2.5% Oil Red O dissolved in isopropanol (Sigma) for 15 min at room temperature. Cells were briefly washed with 60% isopropanol (Fisher Scientific U.K., Loughborough, U.K.) at room temperature and then washed twice with distilled water. Cells were viewed under a light microscope. Lipid and Oil Red O were extracted using ethanol, and the absorbance was measured on a spectrophotometer at 520 nm wavelength.

Protein assay. The cell layer was dissolved in sample buffer containing 2% sodium dodecyl sulfate (SDS) solution (2% SDS, 62.5 mmol/l Tris-HCl, pH 6.8), and protein content was determined using a modified Lowry assay (BioRad, Preston, U.K.).

Leptin enzyme-linked immunosorbent assay. Conditioned medium was collected from differentiated preadipocytes daily for a period of 12 days. The total amount of secreted leptin during differentiation was measured using an enzyme-linked immunosorbent assay (ELISA) kit (DRG Diagnostics, Marburg, Germany). The procedure was modified by the addition of 0.1% Triton added to samples and standards. Conditioned medium was not diluted, 50 μl of sample was assayed directly, and standards were diluted in differentiation medium. These samples remained on the linear scale of the standard curve throughout the collection period.

Determination of DNA content. Nuclei of cells fixed in 95% ethanol were stained with acridine orange (10 μg/ml in HBSS) (Sigma) for 1 min in the dark at room temperature. Cells were washed twice with HBSS, and the cell layer was taken up in 1 mol/l NaOH. DNA content was determined by measuring the excitation and emission wavelength at 485 nm and 535 nm in a fluorescence microplate reader.

Western blot analysis for phosphorylated p38 MAPK. Cell-layer proteins isolated from preadipocytes and differentiated preadipocytes treated with TNF-α (100 ng/ml) (PeproTechEC, London, U.K.) and dissolved in SDS solution were separated by SDS-PAGE using a 12.5% polyacrylamide gel and a 7.5% stack. The cell layer was dissolved in sample buffer, reduced with 10% β-mercaptoethanol, heated for 5 min at 95°C, and run on a gel. Prestained molecular weight markers (Sigma) were used as standards. The separated proteins were transferred to a polyvinylidene difluoride (PVDF) hybond membrane (Amersham International) by electroblotting at 425 mA for 3 h in a vertical transfer apparatus. The membrane was blocked by incubating in 10% nonfat milk in Tris-buffered saline–Tween 20 (TBS-T) (10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.1% Tween 20) for 1 h at room temperature to prevent nonspecific binding. The membrane was incubated in anti-phospho-p38 MAPK primary antibody (New England Biolabs, Hertfordshire, U.K.) at a dilution of 1:1,000 in TBS-T with 10% nonfat milk for 3 h at room temperature. The membrane was washed three times in TBS-T and incubated with anti-rabbit IgG horseradish peroxidase (HRP) secondary antibody (Binding Site, Birmingham, U.K.) at a dilution of 1:10,000 in TBS-T for 1 h at room temperature. The antigens were detected by the enhanced chemiluminescence (ECL) system (Insight Biotechnology, Middlesex, U.K.) after exposure to X-ray film for 15 min.

Statistics. All experiments in the study were performed using adipose tissue from at least three patients ($n \geq 3$). One-way ANOVA and unpaired Student's *t* test were used for data analysis in this study. Data are shown as means ± SE. *P* values <0.05 were considered significant.

RESULTS

Differences in preadipocyte differentiation in the presence of rosiglitazone measured by cell morphology. Preadipocytes were induced to differentiate for 20 days in medium containing insulin, Dex, T3, and, for the first 4 days of culture, IBMX. Figure 1*A* shows preadipocytes before differentiation is induced. The preadipocytes have a characteristic fibroblastic appearance. When differentiation is induced, morphological changes occur with cells becoming spherical with cytoplasmic lipid droplets accumulating as shown in Fig. 1*B*. The addition of rosiglitazone to the differentiation medium markedly increased the number of visible lipid droplets as shown in Fig. 1*C* compared with preadipocytes differentiated in differentiation medium alone shown in Fig. 1*B*.

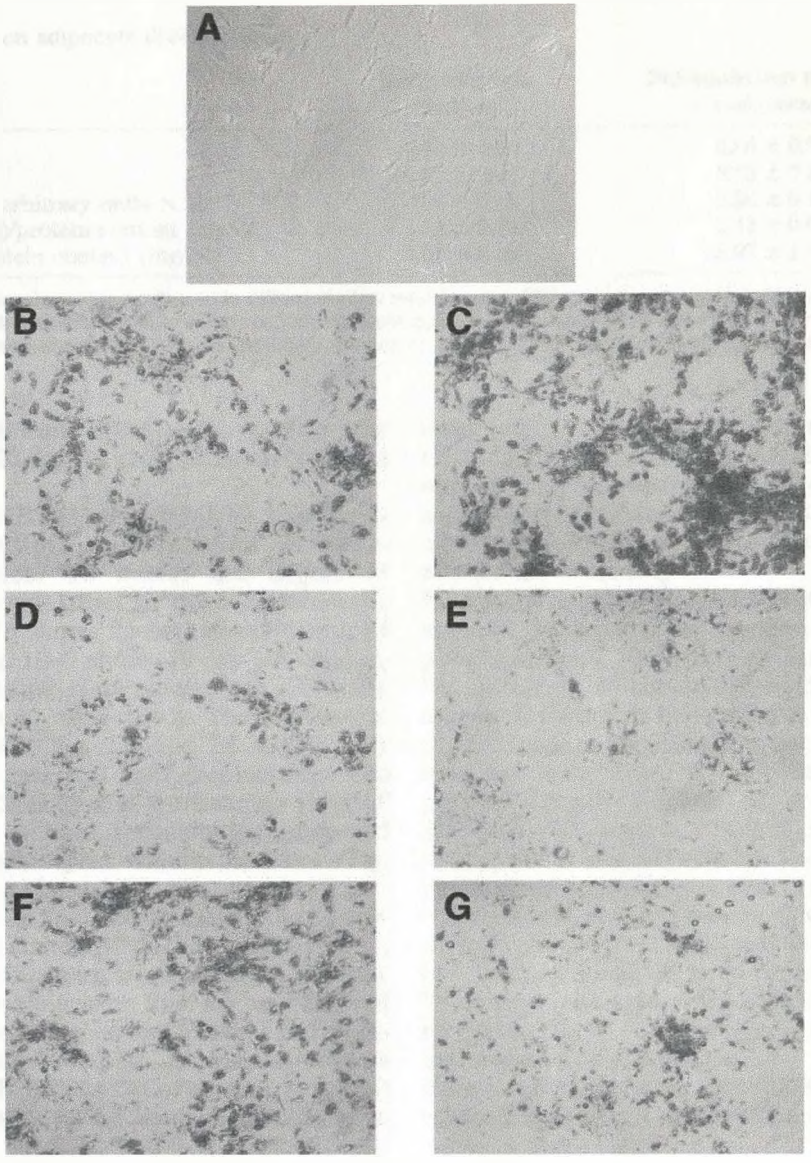

FIG. 1. The effect of rosiglitazone and the inhibitors on adipocyte differentiation. *A*: Human preadipocytes. *B*: Twenty-day culture of preadipocytes in differentiation medium. *C*: Twenty-day culture of preadipocytes in differentiation medium with rosiglitazone (10^{-6} mol/l). *D*: Twenty-day culture of preadipocytes in differentiation medium and rapamycin (10^{-7} mol/l). *E*: Twenty-day culture of preadipocytes in differentiation medium and SB203580 (10^{-6} mol/l). *F*: Twenty-day culture of preadipocytes in differentiation medium and PD98059 (10^{-5} mol/l). *G*: Twenty-day culture of preadipocytes in differentiation medium and wortmannin (10^{-7} mol/l). Magnification ×100.

Effects of rosiglitazone on cell survival, leptin secretion, lipid accumulation, and lipogenesis. To assess the effect of rosiglitazone on the differentiation, five different parameters were examined. These were protein content, total leptin secretion over 12 days of differentiation, total DNA content, the amount of accumulated lipid, and lipogenesis. The final two parameters have been corrected for protein content. Table 1 shows the results from a representative experiment. This experiment was repeated 15 times with comparable data. Relative effects were maintained, although individual preparations show variation in their extent of differentiation (27). The addition of rosiglitazone to the differentiation medium caused a significant increase of 15% in protein content of the cells ($P < 0.01$). DNA content was increased by a similar amount, although the effect was not statistically significant. Rosiglitazone increased the amount of lipid accumulated twofold ($P < 0.0001$), and the level of de novo lipogenesis was increased sevenfold ($P < 0.0001$) when compared with cells cultured for 20 days in differentiation medium alone. Rosiglitazone had no effect on the total amount of leptin secreted over 12 days of preadipocyte differentiation compared with cells cultured for 12 days in differentiation medium alone.

Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on protein synthesis of differentiated preadipocytes. Preadipocytes were induced to differentiate over 20 days in differentiation medium with and without rosiglitazone. Four inhibitor compounds, rapamycin, SB203580, PD98059, and wortmannin, were added to cells when differentiation was initiated and were maintained in the medium throughout the 20-day period. After this 20-day period, the cell layer was removed and the amount of protein present was determined. None of

TABLE 1
The effect of rosiglitazone on adipocyte differentiation

Assay	Differentiation medium	Differentiation medium + rosiglitazone	*P*
Protein content (mg/ml)	0.70 ± 0.00	0.85 ± 0.02	<0.01
Leptin content (ng/ml)	10.52 ± 3.09	8.22 ± 0.74	>0.05
DNA content (485:535nm) arbitrary units × 10^{-6}	2.04 ± 0.10	2.36 ± 0.17	>0.05
Lipid content (Abs 520 nm)/protein content (mg/ml)	1.21 ± 0.10	2.43 ± 0.00	<0.0001
^{14}C-lipid (dpm × 10^{-3})/protein content (mg/ml)	4.01 ± 0.35	28.97 ± 1.43	<0.0001

Data are means ± SE. Preadipocytes were cultured in differentiation medium and differentiation medium containing rosiglitazone for 20 days. After this period, the protein content, leptin content, cell count, lipid content, and the level of lipogenesis were assessed to determine the level of differentiation. The data shown are a representative experiment, and the lipid content and lipogenesis data have been corrected for protein content.

the inhibitors had a significant effect on the total amount of protein present after 20 days of differentiation (data not shown).

Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on lipid accumulation of differentiated preadipocytes. To assess the degree of differentiation, lipid accumulation in differentiated cells was measured. Figure 1*B* shows 20-day-old differentiated preadipocytes, while Fig. 1*D-G* shows 20-day-old differentiated preadipocytes treated with rapamycin, SB203580, PD98059, and wortmannin, respectively. The addition of rapamycin and SB203580 (Fig. 1*D* and *E*) significantly reduced the amount of spherical lipid droplets observed while the addition of PD98059 and wortmannin (Fig. 1*F* and *G*) had little effect on lipid accumulation compared with differentiated preadipocytes without inhibitor (Fig. 1*B*).

To quantify this effect on lipid accumulation, the 20-day-old preadipocytes were stained with the dye Oil Red O, and the amount of stain taken up was quantified. Figure 2 shows the effect of the inhibitors on lipid accumulation of differentiated cells in the presence and absence of rosiglitazone in the differentiation medium. Rapamycin (74% of control, $P < 0.05$) and SB203580 (77% of control, $P < 0.05$) significantly reduced the level of lipid accumulation in cells, while PD98059 and wortmannin had no significant effect (Fig. 2). In preadipocytes differentiated in the presence of rosiglitazone, rapamycin (63% of control, $P < 0.01$) and SB203580 (52% of control, $P < 0.01$) again significantly inhibited lipid accumulation, while PD98059 and wortmannin had no effect (Fig. 2).

Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on lipogenesis of differentiated preadipocytes. To determine lipogenesis, the uptake of ^{14}C-glucose in 20-day-old differentiated preadipocytes was assessed. Cells had been differentiated with and without rosiglitazone in the differentiation medium, and the inhibitors had been added to the cells throughout the 20-day period. Rapamycin (75% of control, $P < 0.05$) and SB203580 (53% control, $P < 0.01$) significantly reduced the uptake of ^{14}C-glucose into lipid, while PD98059 and wortmannin had no significant effect (Fig. 3). In preadipocytes differentiated in the presence of rosiglitazone, rapamycin (67% of control, $P < 0.01$) and SB203580 (36% of control, $P < 0.01$) again significantly reduced the incorporation of ^{14}C-glucose into lipid, while PD98059 and wortmannin had no effect (Fig. 3). The inhibitory effects were enhanced in the presence of rosiglitazone.

Effects of rapamycin, SB203580, PD98059, and wortmannin inhibitors on leptin secretion from differen-

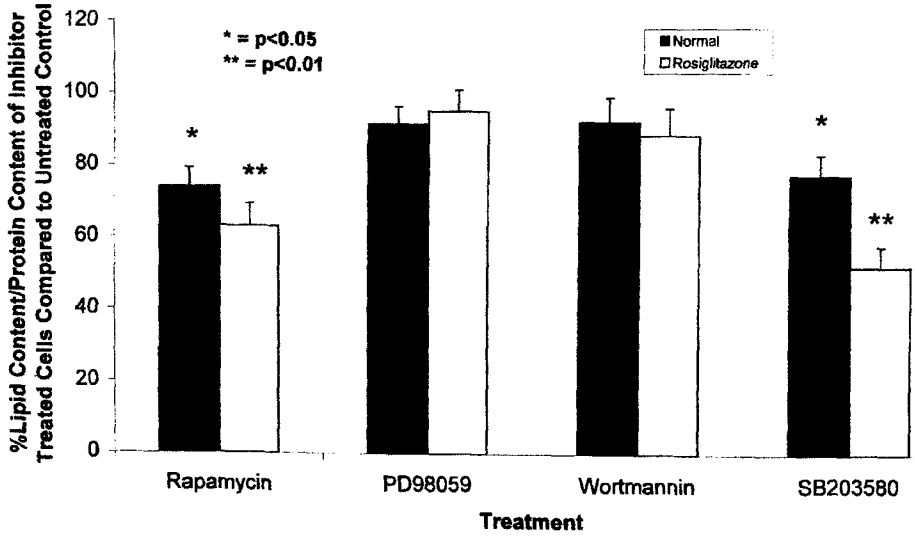

FIG. 2. The effect of the four inhibitors on the lipid content of differentiated preadipocytes with and without rosiglitazone (10^{-6} mol/l). After 20 days, lipid content was measured as described in RESEARCH DESIGN AND METHODS. Results are given as the percentage mean value compared with untreated control differentiated preadipocytes and are corrected for protein content ± SE of four independent samples ($n = 4$).

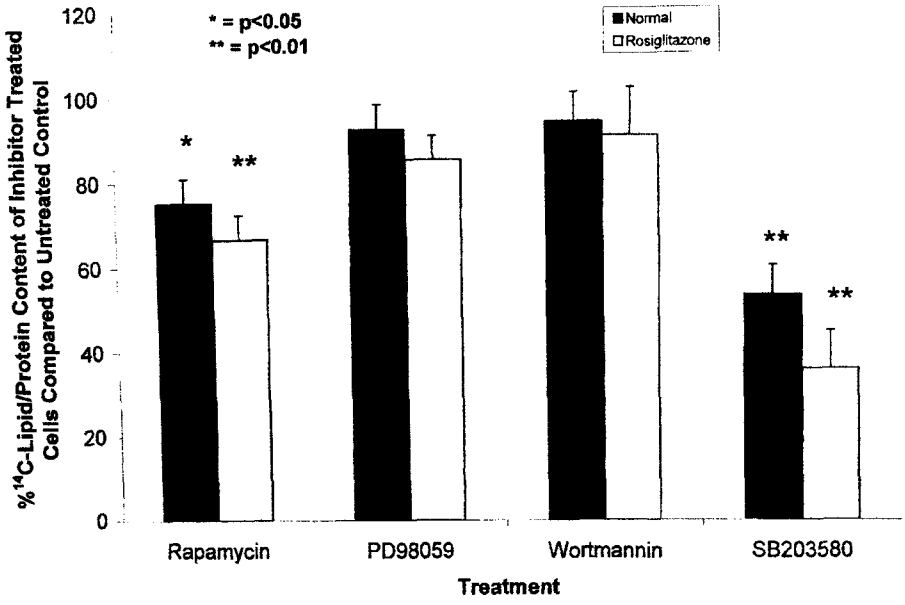

FIG. 3. The effect of the four inhibitors on lipogenesis of differentiated preadipocytes with and without rosiglitazone (10^{-6} mol/l). After 20 days, lipogenesis was measured as described in RESEARCH DESIGN AND METHODS. Results are given as the percentage mean value compared with untreated control differentiated preadipocytes and are corrected for protein content ± SE of four independent samples (n = 4).

tiated preadipocytes. Preadipocytes were differentiated in the presence of the four inhibitors rapamycin, SB203580, PD98059, and wortmannin over 12 days, and the conditioned medium was collected daily. To determine the total amount of leptin secreted, an ELISA was carried out on the conditioned medium collected over 12 days, allowing the determination of total leptin secreted after 12 days of differentiation.

Figure 4 shows the effect of the four inhibitors on leptin secretion during differentiation over a 12-day period. The addition of SB203580 (6% of control, $P < 0.01$) markedly inhibited leptin secretion, while the addition of wortmannin (22% of control, $P < 0.05$), rapamycin (34% of control, $P < 0.05$), and PD98059 (34% of control, $P < 0.05$) also significantly inhibited leptin secretion compared with control.

Effect of TNF-α treatment on phospho-p38 MAPK. Preadipocytes and differentiated preadipocytes were treated with TNF-α (100 ng/ml) for different time periods. Figure 5*A* shows the time course of phospho-p38 MAPK activation in preadipocytes, while Fig. 5*B* shows the time course of phospho-p38 MAPK activation in differentiated

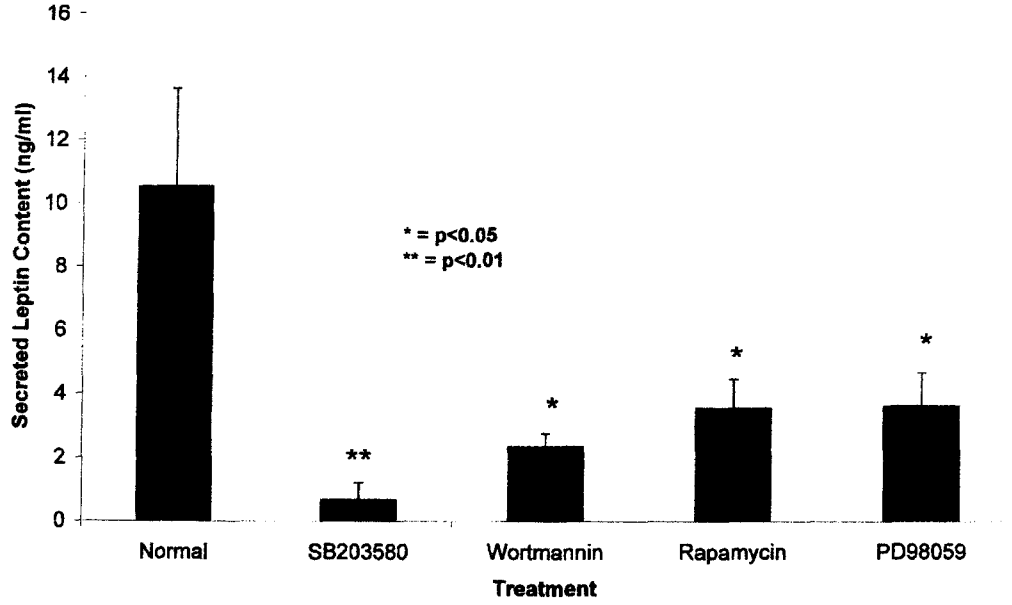

FIG. 4. The effect of the four inhibitors on the secreted leptin content of differentiated preadipocytes. Preadipocytes were differentiated over a 12-day period with the inclusion of the inhibitors. Conditioned medium was removed daily and the secreted leptin content was measured using an ELISA kit, allowing the total secreted leptin content of cells differentiated over 12 days to be determined. Results are given as the mean ± SE of three independent samples (n = 3).

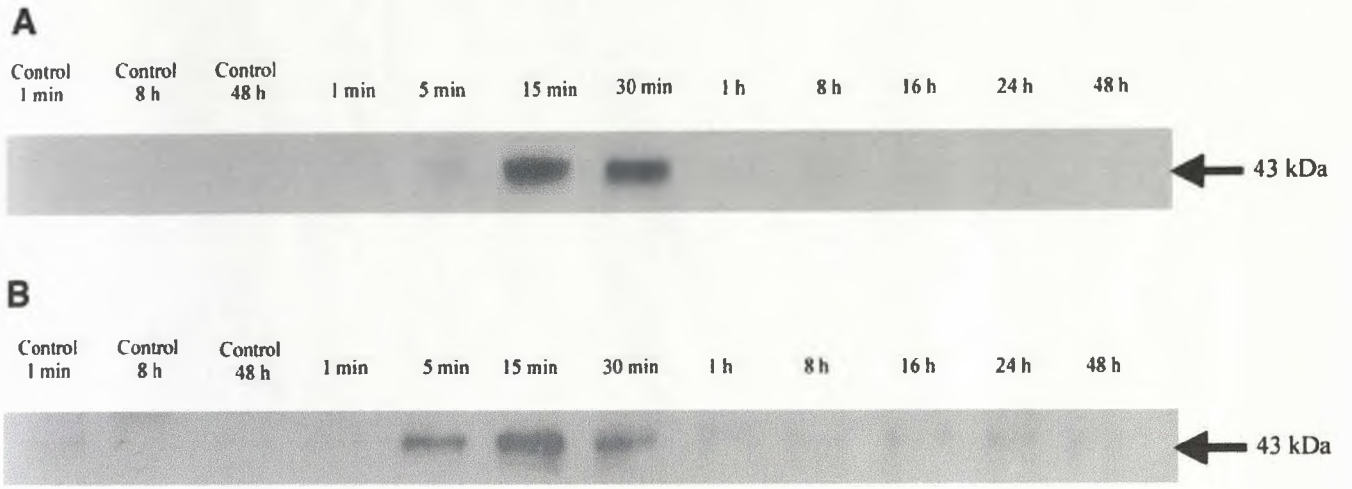

FIG. 5. The effects of TNF-α treatment on phospho-p38 MAPK activation. *A*: Preadipocytes were cultured in DMEM:F12 with TNF-α (100 ng/ml) for indicated incubation periods. Control lanes contain no TNF-α treatment. Phospho-p38 MAPK activation was detected in the cell layer by Western blotting. *B*: Twenty-day-old differentiated preadipocytes were cultured in DMEM:F12 with TNF-α (100 ng/ml) for indicated incubation periods. Control lanes contain no TNF-α treatment. Phospho-p38 MAPK activation was detected in the cell layer by Western blotting.

preadipocytes. A single band of 43 kDa was detectable after 5 min incubation with TNF-α in preadipocytes (Fig. 5*A*) and 15 min TNF-α incubation in differentiated preadipocytes (Fig. 5*B*). After 1 h, phospho-p38 MAPK levels reduced to undetectable levels.

DISCUSSION

In human preadipocyte differentiation, we found that rosiglitazone increased the protein and DNA content of the cell layer modestly. Lipid content of cells was increased twofold but lipogenesis was increased sevenfold compared with cells differentiated in differentiation medium without rosiglitazone. In contrast, leptin secretion was not increased with rosiglitazone treatment, consistent with a preferential inhibition of this marker of differentiation compared with lipogenesis. Studies with 3T3 cells (15) and short-term in vitro studies in mature human adipocytes showed that PPAR-γ activation reduces leptin mRNA. Our data show that throughout differentiation PPAR-γ activation increases the extent and rate of differentiation into mature fat cells while having no such stimulatory effect on leptin synthesis. Consistent with its role as an insulin sensitizer, PPAR-γ activation in human adipocytes was found to increase the transcription and insulin-induced activation of the p85α subunit of PI3K (28). Since insulin is reported to be a repressor of glucocorticoid-induced leptin synthesis (29), as well as having antilipolytic functions (30), our studies showing no increase in leptin secretion compared with elevated lipid stores following PPAR-γ activation are compatible with these data.

We used specific inhibitors of different signaling pathways and assessed the effect on markers of adipocyte differentiation. Inhibition of $p70^{S6}$ kinase and p38 MAPK inhibited adipocyte differentiation, assessed by the lipogenic activity of differentiated cells and the level of lipid accumulation within the cells. The inhibitory effects were greater for p38 MAPK inhibition. PI3K and p42/44 MAPK inhibition, however, had no inhibitory effect on lipid metabolism, but all four inhibitors markedly inhibited leptin secretion, a late marker for adipocyte differentiation.

$p70^{S6}$ kinase has been implicated in protein synthesis, especially in the transcriptional and translational regulation of insulin effects (31). We did not find inhibitory effects on protein synthesis over 20 days in culture, suggesting that rapamycin is not cytotoxic. However, new protein synthesis, which would be necessary to initiate a program of differentiation, may be limited.

In agreement with studies in 3T3-L1 cells (32), we found that p38 MAPK inhibition inhibits human adipocyte differentiation. Its essential roles in inflammation, growth, differentiation, the cell cycle, and cell death have been shown in other cell types (22). In 3T3-L1 cells, the active phosphorylated form of the transcription factor CCAAT/enhancer-binding protein (C/EBP) β was reduced by SB203580 (32). C/EBP β is an activator of C/EBP α and PPAR-γ, two potent adipogenic transcription factors (33). Inhibition of p38 MAPK may thus inhibit C/EBP α and PPAR-γ activation and block adipocyte differentiation (32) in this way.

We show here that TNF-α activates the p38 MAPK pathway in human preadipocytes and adipocytes consistent with data from 3T3-L1 cells (32) and fetal brown adipocytes (34). Because our data show that the p38 MAPK pathway is essential for differentiation, we conclude that the inhibitory effects of TNF-α on differentiation are not mediated by the p38 MAPK signaling pathway.

The noninvolvement of the p42/44 MAPK signaling pathway in adipocyte lipogenesis has been shown in some, but not all, studies with 3T3-L1 cells (4). In human cells, PPAR-γ is a substrate for p42/44 MAPK, and its phosphorylation leads to decreased adipocyte differentiation (6,35,36). A genetic mutation in PPAR-γ at this phosphorylation site has been found in obese individuals (37). Blocking p42/44 MAPK should therefore increase differentiation, but our studies in human adipocytes induced to differentiate in long-term culture show that other pathways downstream of p42/44 MAPK must mitigate this stimulation.

PI3K plays an essential role in the regulation of various cellular activities, including proliferation, differentiation,

and the prevention of apoptosis (38). In this study, we show that the inhibition of PI3K by wortmannin had no effect on human adipocyte lipogenesis. This contradicts the results of other studies, which have shown that wortmannin inhibits adipocyte differentiation (38,39). However, those studies have been carried out in cell lines such as 3T3-L1 cells where postconfluent mitoses are thought to be required early on for successful differentiation of this cell line (clonal expansion phase) (40). PI3K inhibition is likely to interfere with this phase, whereas this staging is not required in human adipocytes. Studies looking at insulin treatment on GLUT4 levels have shown differences between 3T3-F442A and primary rat adipocytes, thus reinforcing the apparent differences between cell lines and primary culture (41), and Ryden et al. (42) found constitutively active p42/44 MAPK in these cells. It is surprising that wortmannin is less potent than rapamycin in our assays, because PI3K is upstream of the rapamycin target $p70^{S6}$ kinase. Our data suggest that the pathways are not directly linked.

Insulin has a known antilipolytic action, and it was possible that the antilipolytic effect would be separable from stimulatory effects on lipogenesis. However, with all of our inhibitors, the effects on lipogenesis are directly comparable to the effects on lipid content in the cells. This suggests that the antilipolytic effects of insulin are not mediated through these pathways. For rosiglitazone, stimulatory effects were more marked on lipogenesis than lipid accumulation, suggesting preferential effects.

In contrast to the disparate effects on lipogenesis, all four inhibitors profoundly inhibited leptin secretion. PD98059 and wortmannin, which inhibit pathways involved in insulin signaling, inhibited leptin secretion without inhibition of lipogenesis and lipid storage. Since insulin stimulates secretion of stores of leptin and does not increase leptin expression (43), it seems unlikely that its effects on leptin gene expression are mediated through these pathways. However, it should be remembered that the design of this study differs from the acute, short-term studies showing insulin-mediated inhibition of leptin mRNA (44,45). Indirect effects of insulin on genes important in leptin gene transcription may occur. These could include autocrine growth factors such as fibroblast growth factors, insulin-like growth factors (IGFs), and angiopoietins, which will use some of these signaling pathways. Furthermore, dexamethasone and cortisol are known to increase leptin expression and to interact with the MAPK signaling pathway. Steroid receptors recruit p160 family members whose activity is regulated by MAPKs (46). Inhibition of MAPK may thus inhibit glucocorticoid receptor signaling and inhibit leptin production (43).

Serum leptin has been shown to be an independent risk factor in cardiovascular disease (9,10). Both insulin and rosiglitazone increase differentiation of preadipocytes and increase adipose mass. Inhibiting different signaling pathways resulted in inhibition of leptin secretion, but not always inhibition of adipocyte differentiation. This suggests that there is a redundancy in signaling as far as differentiation is concerned, but not in the regulation of leptin secretion. Rosiglitazone treatment is associated with favorable changes in adipose tissue cytokines, including leptin, adiponectin, and TNF-α (16,47,48). Our data indicate that this is due to stimulatory effects of PPAR-γ agonists on lipogenesis as opposed to leptin secretion.

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