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INSULIN AND TSH SIGNAL TRANSDUCTION PATHWAYS IN HUMAN PREADIPOCYTES

by

Andrea Bell

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science.

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa
Ottawa, Ontario, CANADA
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ABSTRACT

Signaling through the rapamycin-sensitive mammalian target of rapamycin-p70 S6 kinase (p70 S6K) pathway was examined in human preadipocytes in primary culture. The inhibitory effect of rapamycin on insulin-induced differentiation of preadipose cell lines has been attributed to a blockade of clonal expansion. We demonstrate that rapamycin inhibits the differentiation of human preadipocytes in primary culture, even though these cells do not undergo clonal expansion. Glycerol-3-phosphate-dehydrogenase (GPDH) activity, an indicator of differentiation, was reduced in omental and subcutaneous preadipocytes to 17±10% (mean±95% confidence limits, n=10) of standard differentiation. Our data suggest rapamycin-sensitive pathways operate independently of clonal expansion.

Our second objective addressed a novel route of p70 S6K activation. We have detected the presence of TSH receptor in human preadipocytes, and have shown it activates PKB and p70 S6K in a wortmannin-sensitive manner.

A model depicting the convergence of insulin and TSH signaling in human preadipocytes is proposed.

DEDICATION

For Mom, Dad, Andrew, Rhonda, Méma and Nana Bell

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I. INTRODUCTION

In 1997, the World Health Organization recognized obesity as a disease, and classified it according to the body mass index (BMI). BMI is calculated by dividing a person's weight in kilograms by height in meters squared. A BMI ≥ 25 indicates "overweight" and BMI ≥ 30 indicates "obesity".

Since the 1960's obesity prevalence rates have been increasing steadily according to National Health and Nutrition Examination Surveys II and III (Taubes 1998). Current trends are likely related to recent lifestyle changes including a general decrease in physical activity and increased consumption of calories. However, there is a complex interaction between genes and environment contributing to the obese phenotype, and it is becoming increasingly evident that a subset of the population demonstrates a genetic predisposition to overweight and obesity (Perusse and Bouchard 1999). A large number of candidate genes for human obesity have been identified with the aid of animal models (Comuzzie and Allison 1998).

One such gene that appears to be associated with human obesity is peroxisome proliferator-activated receptor γ (PPARγ). Several mutations in this gene have been identified (Ristow et al. 1998, Valve et al. 1999, Auwerx 1999), that may affect the rate of new fat cell (adipocyte) formation. The role of PPARγ in adipocyte formation will be discussed in detail below.

A. Risk factors associated with obesity

Increased abdominal obesity is associated with increased risk for developing type 2 diabetes, dyslipidemia, cardiovascular disease and hypertension (Hauner 1995, Bjorntorp and Rosmond 1999, La marche et al. 1999). Exact mechanisms through which excess intra-abdominal fat predisposes to type 2 diabetes and cardiovascular disease are unknown though one hypothesis proposes the release of free fatty acids (FFA) from intra-abdominal adipocytes to the liver via the portal system. The increased lipolytic and decreased antilipolytic responses characteristic of visceral adipocytes lead to increased release of FFA (Bjorntorp and Rosmond 1999). Increased FFA availability and oxidation inhibit glucose metabolism (Groop et al. 1991). This results in increased hepatic glucose output that has been suggested to cause insulin resistance. Recent findings indicate increased FFA availability also leads to insulin resistance through increased hexosamine biosynthesis, which may also impair glucose transport and/or phosphorylation (Hawkins et al. 1997a, Hawkins et al. 1997b). In addition, increased levels of tumor necrosis factorα (TNFα) derived from adipocytes play a role in insulin resistance observed in obesity and type 2 diabetes (Gregoire et al. 1998), via impaired insulin-stimulated glucose transport and/or inhibition of lipolysis. TNFα-induced insulin resistance may be due to reduced tyrosine (tyr) phosphorylation of insulin receptor (IR) and insulin

receptor substrate-1 (IRS-1) (Hotamisligil 1999). In addition, TNFα may directly promote lipolysis (Greenberg 1998). One study suggests that the circulating levels of TNFα are higher in abdominal obesity vs. peripheral obesity in women (Tsigos et al. 1999), suggesting that TNFα might be responsible for the higher lipolytic rates seen in intra-abdominal adipocytes. Increased FFA may also lead to overproduction of atherogenic lipoproteins resulting in cardiovascular disease (Howard 1992). The clustering of hyperinsulinemia, glucose intolerance, dyslipidemia, hypertension, and obesity has been termed the insulin resistance syndrome (Després 1993, Landsberg 1996, Garvey and Hermayer, 1998).

B. Adipogenesis

1. The role of adipocytes

The adipocyte is an integral component in maintaining energy balance in our bodies. In periods of caloric intake, body fuel may be stored as energy in the form of triacylglycerol (TG) in adipocytes. Initially, lipids are stored in previously existing adipocytes. After adipocytes reach a 'critical' size, they may release paracrine factors such as lysophosphatidic acid and prostacyclin that induce proliferation and differentiation (adipogenesis) of the adipocyte precursor cell, the preadipocyte (Hirsch 1989, Marques et al. 1998, Valet et al. 1998). The increase

in adipocyte number results in greater storage capacity (Prins and O'Rahilly 1997). Direct nutritional signals, such as insulin and fatty acids, may also be involved in the induction of preadipocyte differentiation (Amri et al. 1996). Adipose tissue expansion is therefore the result of increases in adipocyte cell number as well as cell size (Prins and O'Rahilly 1997). Preadipocytes play an important role in adipose tissue expansion, given that they can influence adipose tissue via differentiation, apoptosis and proliferation.

2. Preadipocyte experimental models

a. Human preadipocytes

These are fibroblast-like cells of mesodermal origin that are committed to the adipocyte lineage (Deslex et al. 1987, Hauner et al. 1987). Preadipocytes can be isolated from the stromal vascular fraction of adipose tissue by collagenase digestion, centrifugation and size filtration. The floating fat cell layer is removed after centrifugation and the infranatant is filtered to yield the preadipocyte component. Preadipocytes are then seeded in primary culture. Any contaminating cells that may be present in the preadipocyte fraction are not sustained under the culture conditions that are used. Serum-containing media was applied in early studies of preadipocyte differentiation but only low levels of

differentiation was achieved (Hauner and Loffler 1986). Eventually, serum-free differentiation conditions were developed to define the precise components necessary for induction of differentiation (Hauner 1990). Three major components involved in the induction of differentiation are insulin, gluococorticoids and agents that increase cAMP such as isobutylmethylxanthine (IBMX) (Smith et al. 1988, Hauner et al. 1987, Hauner et al. 1989, Hauner 1990).

b. 3T3-L1 preadipocytes

Preadipose cell lines are also used for the study of signal transduction pathways in preadipocytes and adipocytes. Advantages of using a cell line compared to primary culture of human preadipocytes include reproducible and extensive differentiation (≥90% of cells), ease of subpassaging (not limited by cell number), and avoidance of the lengthy process of retrieving human adipose tissue. 3T3-L1 murine preadipose cells were originally isolated from disaggregated mouse embryos and have the potential to differentiate into lipid-laden adipocytes when appropriately triggered with adipogenic agents in culture (Green and Meuth 1974, Green and Kehinde 1976). Transplantation studies reveal that fat pads develop normally after subcutaneous injection of 3T3-F442A cells into Balb-C athymic mice (Green and Kehinde 1979, Mandrup et al. 1997). Nevertheless, the use of these and other preadipose cell lines is not ideal, as

they tend to exhibit aneuploidy due to the immortalization process. Differences also arise between cell models according to the culture conditions employed and the stage at which the various cell lines have been arrested in development. In light of differences associated with the various cell culture models, findings are strengthened when supplemented with studies on cells in primary culture.

3. Anatomic regional differences

Differences exist between the adipose tissue in the intra-abdominal (omental) and subcutaneous abdominal region. Most studies to date have focused on regional differences between adipocytes of the omental vs. the subcutaneous depot. For example, TG metabolism varies between omental and subcutaneous adipocytes. Greater levels of TG hydrolysis (lipolysis) were observed in omental adipocytes (Ostman 1979, Hoffstedt et al. 1997, Arner 1995) whereas TG synthesis was greater in subcutaneous adipose tissue (Maslowska 1993). The antilipolytic effects of insulin are stronger in subcutaneous adipocytes than in omental adipocytes (Bolinder et al. 1983). Omental adipocytes have higher levels of basal cAMP and greater numbers of glucocorticoid receptor (Vikman et al. 1995, Pedersen et al. 1994).

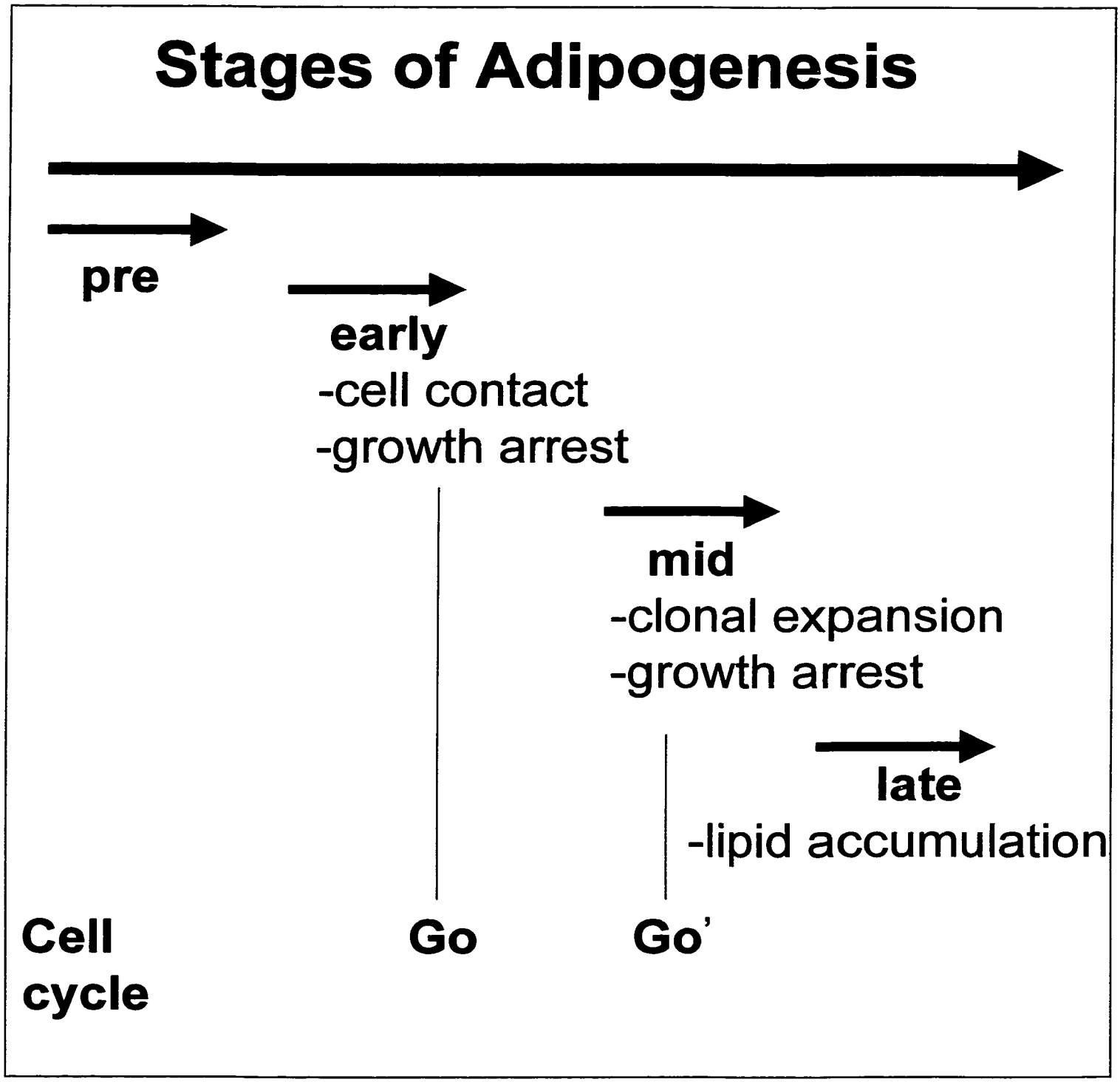
Less is known about preadipocyte regional differences. In one study, PPARγ activation led to greater differentiation of human subcutaneous vs.

omental preadipocytes (Adams et al. 1997). In another study, *in vitro* treated human omental preadipocytes were more prone to apoptosis than subcutaneous preadipocytes (Niesler et al. 1998). Adipogenic and apoptotic rates in these depots may influence the distribution of overall adipose tissue. The molecular mechanisms underlying these regional preadipocyte differences are currently unknown.

4. A temporal sequence of events in adipogenesis

The model of adipocyte differentiation, mainly based on studies of 3T3-L1 cells, consists of various stages as shown in Figure 1. Preadipocytes, grown to confluence, enter a growth arrest phase that primes the cells for differentiation (Smas and Sul 1995). After exposure to adipogenic agents such as insulin, glucocorticoid and IBMX, cells undergo clonal expansion in culture. This entails 1-2 rounds of post-confluent mitoses that are critical for successful differentiation of this cell line (Pairault and Green 1979). Re-entry into the cell cycle is thought to increase accessibility of promoters of adipocyte differentiation-specific genes. This promotes transcriptional activation by trans-acting factors that induce differentiation (Cornelius et al. 1994). After completion of clonal expansion, a second growth arrest phase ensues that is followed by changes in the cytoskeleton, accumulation of lipid, and terminal differentiation. Growth arrest is

Figure 1. Model of adipocyte differentiation based on the murine preadipose 3T3-L1 cell line. Go is growth arrest following cell-cell contact and Go' is growth arrest following clonal expansion.


Stages of Adipogenesis
pre
early
-cell contact
-growth arrest
mid
-clonal expansion
-growth arrest
late
-lipid accumulation
Cell cycle
Go
Go'

a requirement for adipocyte differentiation but it is still unclear whether cell confluence and cell-to-cell contact are necessary. A study in the late 1970's showed that 3T3-F442A cells differentiated when cultured in suspension, lending support to the ability of preadipocytes to differentiate in the absence of cell confluence and cell-to-cell contact (Pairault and Green 1979). In contrast, recent evidence suggests that confluence may be necessary for differentiation. When the expression of Notch, a transmembrane receptor involved in cell-to-cell signaling during development, was blocked, adipogenesis was inhibited (Garces et al. 1997).

In the terminal phase of adipogenesis, GPDH activity and TG mass increase. GPDH and TG may serve as late markers for assessment of differentiation levels (Wise and Green 1979, Cianflone 1994). Studies employing TG assays exclusively may not be entirely representative of differentiation, since overall TG accumulation is also influenced by lipogenesis and lipolysis, processes that are distinct from differentiation.

5. Transcription factors necessary for induction and/or maintenance of differentiation

Induction of differentiation encompasses a variety of responses that lead to changes in morphology and function of the preadipocyte. Many transcription

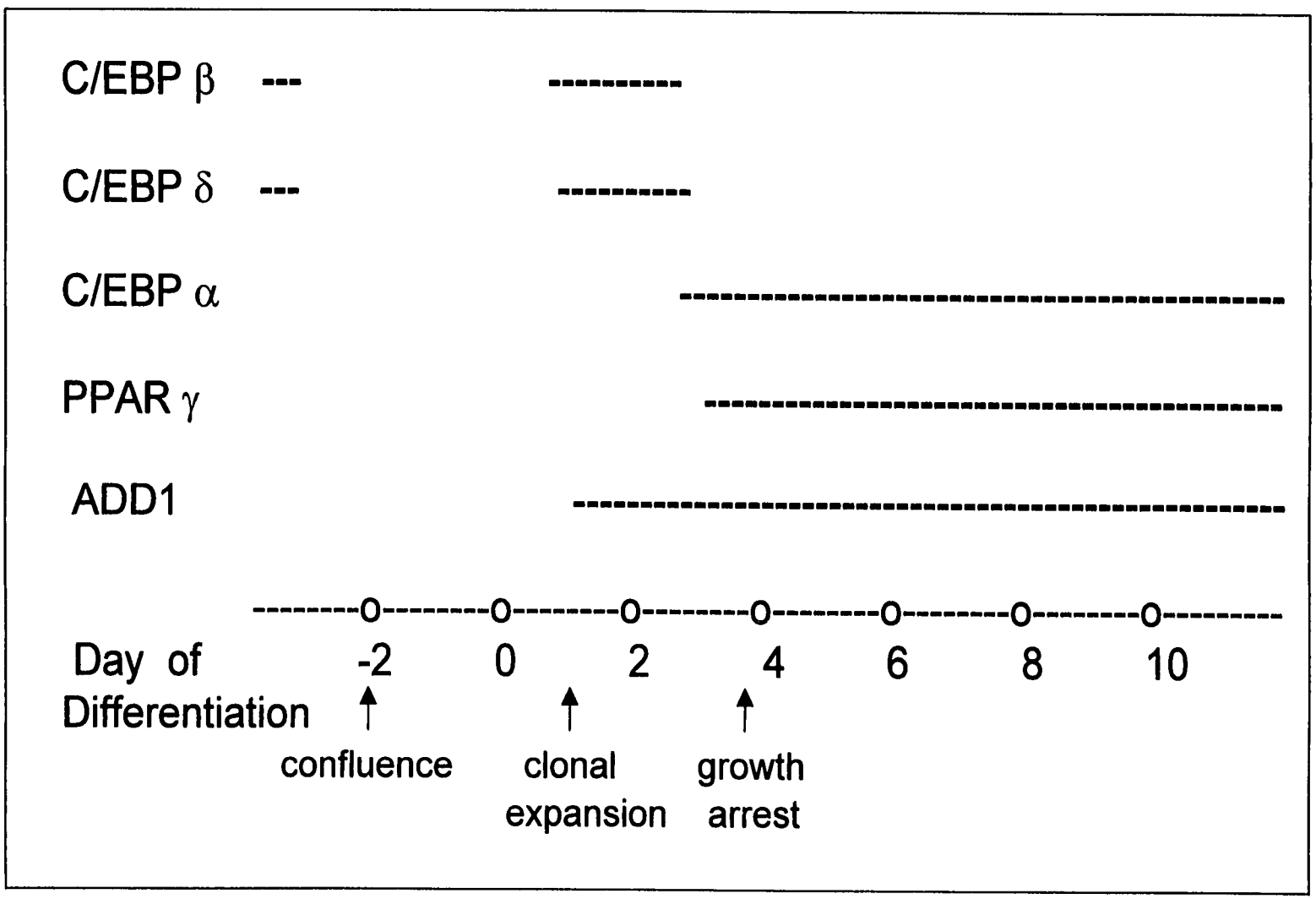
factors are activated to induce adipogenesis. Two major families of transcription factors critical for the induction of differentiation include CCAAT/enhancer binding proteins (C/EBPs) and PPARs (Loftus and Lane 1997, Brun et al. 1996a). C/EBP monomers form homo- or hetero-dimers with other family members. The three major isoforms of C/EBP include α, β, and δ. None of these isoforms are adipose specific. C/EBPβ and δ are present in preadipocytes at low levels and increase transiently during early differentiation. One activator of C/EBPβ appears to be cAMP (Yeh et al. 1995b). Ectopic expression of C/EBPβ leads to induction of PPARγ expression (Wu et al. 1995). Expression of a dominant-negative truncated C/EBPβ inhibits adipocyte differentiation. The overexpression of C/EBPβ in NIH 3T3 cells leads to commitment to the adipocyte lineage (Yeh et al. 1995b). C/EBPδ is activated by glucocorticoids (Yeh et al. 1995b). The differentiation promoting effect of glucocorticoids and insulin was first proposed by Hauner et al. in 1987. Glucocorticoids may regulate adipogenesis by down-regulating preadipocyte factor 1 (Pref-1) gene expression (Wolf 1999). Pref-1 levels are increased in preadipocytes and absent from adipocytes. Prior to transcription of most adipocyte specific genes, C/EBPα is expressed and demonstrates antimitotic activity. C/EBPα is sufficient to induce differentiation of 3T3-L1 preadipocytes even in the absence of hormonal cocktails (Lin and Lane 1994, Freytag et al. 1994). C/EBPα knockout mice failed to accumulate lipid and died within 8 hrs after birth indicating that C/EBPα is essential for neonatal

energy homeostasis (Wang et al. 1995). A temporal sequence of activation of these transcription factors is included in Figure 2.

The three major isoforms of PPAR are located on separate genes and include PPARα, PPARδ, and PPARγ (Auwerx 1999). Natural ligands of PPAR may include derivatives of fatty acid and prostaglandin J2 (Rocchi and Auwerx 1999). PPAR monomers must heterodimerize with retinoid X receptor (RXR) to alter gene transcription. In adipocytes, PPARα is expressed at low levels whereas PPARδ is expressed at high levels (Gregoire et al. 1998). PPARγ is the most adipose specific form and it is activated after C/EBPβ and C/EBP δ, and PPARδ. The three isoforms of PPARγ include γ1, γ2 and γ3. They are products of alternative splicing and alternative translation initiation. No functional differences have been detected but PPARγ2 appears to be the most adipose specific. Ectopic expression of PPARγ induces adipogenesis in response to various PPAR activators (Brun et al. 1996b).

Another family of transcription factors includes the basic helix-loop-helix leucine zipper family. One member of this family, adipocyte determination and differentiation factor 1 (ADD1) plays a role in cholesterol homeostasis and adipocyte development (Tontonoz et al. 1993). The human homologue of ADD1 was cloned independently from that in rat and termed sterol regulatory element binding protein-1c (Yokoyama et al. 1993). ADD1 may be important in ligand-mediated activation of PPARγ, or in directly regulating PPARγ expression (Kim et

Figure 2. Temporal expression of transcription factors necessary for adipocyte differentiation. C/EBP is CCAAT/enhancer binding protein, PPAR is peroxisome proliferator-activated receptor and ADD1 is adipocyte determination and differentiation factor 1.


C/EBP β
C/EBP δ
C/EBP α
PPAR γ
ADD1
Day of Differentiation
-2
0
2
4
6
8
10
confluence
clonal expansion
growth arrest

al. 1998, Fajas et al. 1999). Such studies demonstrate a regulatory role based on interaction between various transcription factors.

These transcription factors are responsible for the expression of various genes whose proteins are involved in TG and glucose metabolism, proliferation and differentiation. C/EBPα binds the promotor region of adipocyte genes and leads to the transcription of the following: adipocyte P2 (aP2), stearoyl-CoA desaturase, glucose transporter-4 (GLUT-4), phosphenolpyruvate carboxykinase (PEPCK), leptin and IR (Gregoire et al. 1998). PPARγ induces expression of many genes involved in adipogenesis, particularly those involved in lipid storage and metabolic control. The transcription of aP2, PEPCK, acyl CoA synthetase, fatty acid translocase/CD36, fatty acid transport protein 1 and lipoprotein lipase (LPL) is induced by PPARγ (Auwerx 1999). ADD1 regulates fatty acid metabolism by increasing mRNA expression of fatty acid synthase and LPL (Kim and Spiegelman 1996).

6. Insulin/IGF-1 signal transduction in preadipocytes

It has been suggested that physiological doses of insulin-like growth factor (IGF-1) or supraphysiological doses of insulin are sufficient to induce 3T3-L1 preadipocyte differentiation (Smith et al. 1988). The IGF-1 receptor (IGF-1R) was thought to be responsible for differentiation since the IGF-1R number was

greater than the IR number in preadipocytes. Further study of the induction of adipocyte differentiation showed that IGF-1R numbers remain the same in preadipocytes and adipocytes at 13000 receptors/cell. In contrast, IR numbers increase dramatically from 6500 to approximately 160000 receptors/cell during differentiation. To determine the importance of the IR in 3T3-L1 adipocyte differentiation, an allele of the receptor was inactivated by homologous recombination (Acilli and Taylor 1991). IR number was reduced to 50-70% of wild type levels, severely blocking the differentiation of the IR-deficient clones. Evidence that the IR domains are required for adipocyte differentiation is also provided by the use of chimeric receptors (Chaika et al. 1997). A chimeric growth factor receptor was constructed combining the extracellular ligand binding domain of the human colony stimulating factor-1 (CSF-1) and the transmembrane and cytoplasmic domains of the human IR. CSF-1 induced 3T3-L1 adipocyte differentiation in cells expressing the chimeric receptor, suggesting that activation of transmembrane and cytoplasmic domains of IR is sufficient to activate the insulin signaling pathway (Chaika et al. 1997).

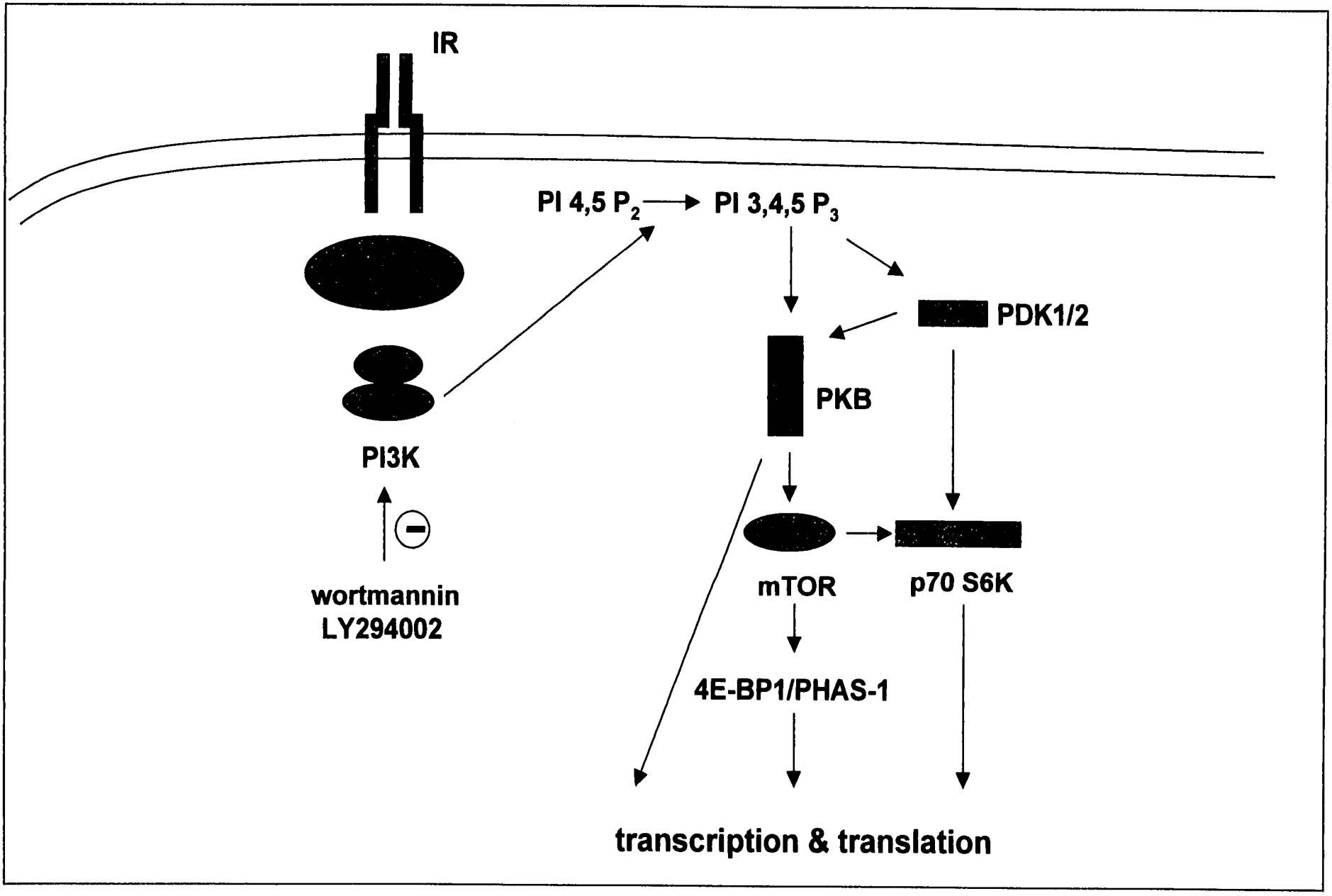
Other evidence suggests that physiological levels of insulin are sufficient to induce differentiation in the preadipocyte (Hauner 1990, Gagnon and Sorisky 1998). Our lab has demonstrated that high glucose concentrations impair insulin-induced preadipocyte differentiation but not that induced by IGF-1 (Gagnon and Sorisky 1998). This adds further evidence for IR involvement in

differentiation, since IR, but not IGF-1R, signaling is affected by glucose concentration. The adipocyte differentiation was enhanced approximately 2-fold in insulin-induced differentiation of preadipocytes treated with 5mM vs. 25mM glucose. In agreement with findings by Hauner (1990), physiological concentrations of insulin (0.1-1nM) in the presence of 5mM glucose were sufficient to induce differentiation in 3T3-L1 preadipocytes (Gagnon and Sorisky 1998). In summary, both insulin and IGF-1, acting via their respective receptors, can induce differentiation.

C. *Insulin signaling in adipogenesis*

The insulin signaling cascade, as shown in Figure 3, begins with the binding of insulin to the α subunits of the heterotetrameric IR (Cheatham and Kahn 1995). A conformational change in the receptor activates the tyr kinase activity of the β subunits, leading to autophosphorylation of the receptor and subsequent receptor-mediated tyr phosphorylation of IRS-1 (Luo et al. 1999). IRS-1 is a large cytosolic protein that contains a phosphotyrosine binding domain (PTB), a pleckstrin homology (PH) and numerous tyrosine (tyr) residues, that when phosphorylated, allow it to interact with various proteins (Cheatham and Kahn 1995). There are 4 known isoforms of IRS, but IRS-1 is the predominant form in 3T3-L1 preadipocytes (Myers and White 1995, Lavan et al. 1997a, Lavan

Figure 3. Overview of the insulin signaling pathway. Upon insulin binding, the insulin receptor (IR) undergoes a conformational change followed by tyrosine kinase autophosphorylation. Insulin receptor substrate-1 (IRS-1) activation causes the docking of phosphatidylinositol 3-kinase (PI3K). Activated PI3K leads to the production of phosphatidylinositol-3,4-bisphosphate (PI 3,4 P2) and phosphatidylinositol-3,4,5-trisphosphate (PI 3,4,5 P3). These 3-phosphorylated phosphoinositides recruit phosphoinositide-dependent kinase 1/2 (PDK1/2) and protein kinase B (PKB) thereby facilitating PDK1/2 phosphorylation of PKB. The ser/thr kinases PDK1/2 and mammalian target of rapamycin (mTOR) subsequently act on p70 S6K. mTOR also phosphorylates eIF4E-binding protein (4E-BP1also known as PHAS-1).


IR
PI 4,5 P2
PI 3,4,5 P3
PDK1/2
PKB
PI3K
wortmannin
LY294002
mTOR
p70 S6K
4E-BP1/PHAS-1
transcription & translation

et al. 1997b, Sun et al. 1997). The phosphotyrosine residues and their surrounding amino acid (aa) residues on IRS-1 serve as docking sites that mediate binding to src-homology 2 (SH_2) domain-containing proteins including phosphatidylinositol 3-kinase (PI3K) and activators of Ras (Myers and White 1994/1995).

PI3K is required for insulin and IGF-stimulated preadipocyte differentiation (Tomiyama et al. 1995, Magun et al. 1996, Suga et al. 1997, Christoffersen et al. 1998, Xia and Serrero 1999). The most well studied PI3K family member is comprised of a regulatory subunit p85 and a catalytic subunit p110. The p85 subunit contains two SH_2 domains and one SH_3 domain. An SH_3 domain binds proline-rich sequences and regulates protein-protein interactions; its precise role in PI3K is not known. The p110 subunit is a dual specificity kinase that acts as both a lipid and protein kinase through a single catalytic domain (Hunter 1995). As a protein kinase, p110 can phosphorylate serine (ser) 608 in the p85 subunit thus turning off PI3K after activation (Carpenter et al. 1993, Dhand et al. 1994). Novel protein substrates are being identified whose roles are not yet known (Rao et al. 1999, Alessi and Downes 1998, Dowler et al. 1999).

As a lipid kinase, p110 can lead to production of 3-phosphorylated phosphoinositides (Rameh and Cantley 1999). The 3-phosphorylated phosphoinositides recruit PH domain-containing proteins to the cell membrane. PI3K may phosphorylate any inositol lipid *in vitro*, though phosphatidylinositol (PI)

4,5 P_2 is the preferred *in vivo* substrate (Divecha and Irvine 1995). The compounds wortmannin and LY294002 inhibit this reaction. Wortmannin selectively inhibits PI3K activity at nanomolar concentrations by covalently binding to the catalytic p110 subunit (Nakanishi et al. 1995). Amino and thiol groups of p110 can nucleophilically attack the chemically active furane ring of wortmannin. The binding of wortmannin with PI3K is irreversible. LY294002 also inhibits PI3K but here the process is reversible since the mechanism involves competitive binding with adenosine triphosphate (ATP) (Nakanishi et al. 1995). These inhibitors block 3T3-L1 differentiation in a time and dose-dependent manner (Tomiyama et al. 1995, Xia and Serrero 1999).

Tyr phosphorylated IRS-1 can also associate with Grb-2 and recruit son-of-sevenless (SOS), a guanine nucleotide releasing protein that activates Ras (Cheatham and Kahn 1995). When 3T3-L1 fibroblasts are transfected with Ras oncogenes, they differentiate into adipocytes. Dominant inhibitory Ras mutants lead to inhibition of differentiation (Benito et al. 1991). GTP-bound Ras leads to activation of the serine-threonine (ser/thr) kinase Raf-1. Raf-1 is thought to play a positive role in adipogenesis since expression of a dominant-negative Raf mutant inhibits insulin-induced differentiation and transfected Raf oncogenes induce adipogenesis in 3T3-L1 cells (Porras et al. 1994, Porras and Santos 1996). In contrast, one of the downstream targets of Raf-1, p42/44 mitogen-activated protein kinase (MAPK) appears to exert a negative influence on

adipogenesis (Font de Mora et al. 1997). MAPK kinase (MEK) is activated by Raf and in turn activates MAPK (Suga et al. 1997). P42/44 MAPK is an important regulator of cell growth and differentiation. It has been shown to negatively modulate adipocyte differentiation by phosphorylating PPARγ (Hu et al. 1996), although one report using antisense strategy indicated it was required for differentiation (Sale et al. 1995). Raf-1 also has the ability to interact with retinoblastoma (Rb) *in vitro* and *in vivo* (Wang et al. 1998). Rb is an important regulator of cell cycle progression (Lipinski et al. 1999). This may provide an important link between Raf, cell cycle regulation, and adipocyte differentiation.

Another MAPK that appears to play an important role in adipogenesis is p38 MAPK (Engelman et al. 1998, Engelman et al. 1999). When 3T3-L1 preadipocytes are treated with inhibitors of p38 MAPK, they are unable to differentiate (Engelman et al. 1998). Inhibition of p38 MAPK is thought to exert its effects through the inhibition of C/EBPβ, a transcription factor involved in differentiation.

1. PDK-1 and PKB

Phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (PKB also known as Akt) are downstream targets of PI3K (Datta et al. 1996, Anderson et al. 1998). PDK-1 and PKB are translocated to the plasma membrane, via their

PH domains, by 3-phosphorylated phosphoinositides (Alessi et al. 1996, Anderson et al. 1998). PDK-1 is a protein ser/thr kinase with a PH domain in the C-terminus. It has been shown to activate PKB both *in vivo* and *in vitro* through co-expression experiments, which demonstrated direct phosphorylation of thr 308 (Alessi et al. 1998). PKB is a protein ser/thr kinase with a PH domain in the N-terminus. It is a member of the cyclic AMP-dependent/cyclic GMP-dependent/protein kinase C (AGC) subfamily based on similarities in the catalytic domain. After initial translocation and first stage activation, maximal activation of PKB is then attained by the phosphorylation of thr 308 and ser 473 (Alessi et al. 1996, Balendran et al. 1999). Phosphorylation of ser 473 has been suggested to occur via the hypothetical PDK-2. However, Balendran et al. suggest that PDK-1 and PDK-2 is the same enzyme. They show that PDK-1 gains the ability to phosphorylate ser 473 after binding to a 77 aa peptide of protein kinase C related kinase 2 (PRK2). This group identified the 77 aa C-terminus of PRK-2 as a potential PDK-1 interacting fragment. Other recent findings suggest that PKB is autophosphorylated at ser 473 (Toker and Newton 2000).

Our lab has shown that expression of constitutively activated PKB induces differentiation in 3T3-L1 preadipocytes (Magun et al. 1996). This work implicates PKB as a regulator of adipogenesis. Recent work by Navé et al. (1999) suggests that PKB is involved in the direct phosphorylation of mammalian target of rapamycin (mTOR), a protein described below. PKB may also phosphorylate

cAMP responsive element binding protein (CREB) a transcription factor that has been shown to be sufficient and necessary for adipocyte differentiation (Reusch et al. 2000).

2. mTOR

mTOR (also known as RAFT and FRAP) is a member of the PI3K-related family of protein kinases that contain a lipid kinase domain in their C-terminus (Peterson 1998, Thomas and Hall 1997). mTOR is capable of autophosphorylation. Phosphorylation levels and kinase activity of mTOR are increased by insulin and high expression levels of mTOR are observed in insulin-responsive cells (Withers et al. 1997, Scott et al. 1998). Upregulation of mTOR occurs during differentiation of preadipocytes into adipocytes (Withers et al. 1997).

The immunosuppressant drug rapamycin is an inhibitor of mTOR. Upon entering the cell, rapamycin binds its intracellular receptor FK506 binding protein 12 (FKBP12). This rapamycin-FKBP12 complex subsequently binds to mTOR with high affinity. This prevents mTOR from functioning in normal capacity. The PI3K lipid kinase inhibitors wortmannin and LY294002 inhibit mTOR indirectly, suggesting that mTOR is positioned downstream of PI3K (Brunn et al. 1996, Withers et al. 1997, Navé et al. 1999).

Translational regulation by mTOR is mediated by its activation of eIF4E-binding protein 1 (4E-BP1 also known as PHAS-1) and p70 S6K (Proud and Denton 1997). These targets are located on separate pathways immediately downstream of mTOR (von Manteuffel et al. 1997, Hara et al. 1997, Heesom and Denton 1999). 4E-BP1 plays a role in the translation of mRNAs with complex 5'UTRs (Beretta 1996). In the non-phosphorylated state, 4E-BP1 remains bound to the eukaryotic initiation factor 4E (eIF4E), resulting in the inhibition of translation. Direct phosphorylation of 4E-BP1 on thr 36 and thr 45 is induced by mTOR (Burnett et al. 1998). Phosphorylation of 4E-BP1 leads to its release from eIF-4E, allowing translation to proceed (Lawrence Jr. and Abraham 1997). A 10-fold increase in 4E-BP1 over p70 S6K is required to obtain similar phosphorylation levels, indicating that the preferred substrate of mTOR is p70 S6K (Burnett et al. 1998). There are no reports suggesting a role for 4E-BP1 in preadipocyte differentiation.

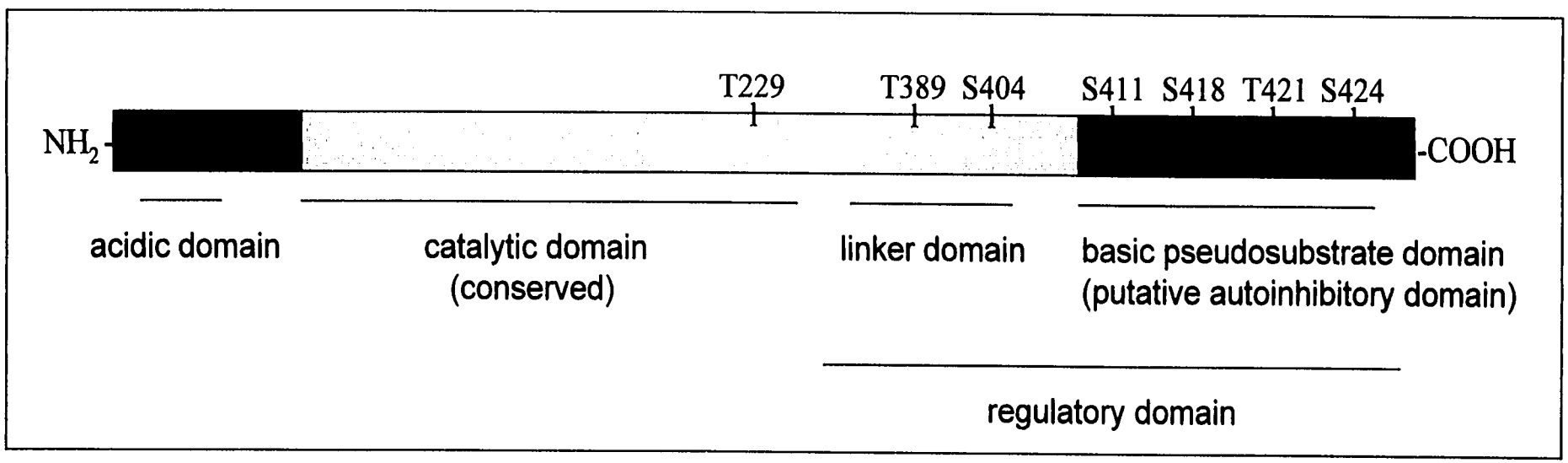
mTOR has been implicated in the activation of p70 S6K through phosphorylation of thr 389 in vitro, and it is thought that mTOR also phosphorylates and activates p70 S6K *in vivo* (Isotani et al. 1999). Others suggest that mTOR controls 4E-BP1 and p70 S6K phosphorylation indirectly by restraining the ser/thr protein phosphatase 2A (PP2A) that would otherwise lead to inactivation of these proteins (Peterson et al. 1999).

3. p70 S6K

a. Structure and function

Two major isoforms of p70 S6K include the 70 kDa αII and 85 kDa αI isoforms that are alternative splice products of a single gene. These two isoforms are known collectively as S6K1 (Dufner and Thomas 1999). The 85 kDa αI differs from the 70 kDa αII in the N-terminal 23 aa sequence that contains a nuclear localization signal. The 85 kDa αI form appears to be expressed exclusively in the nucleus. The 70 kDa αII form is primarily cytoplasmic but nuclear levels reach approximately 32% of cytoplasmic expression levels (Kim and Kahn 1997). p70 S6K contains several domains, as indicated in Figure 4. These include the N-terminal acidic domain, the catalytic domain, the linker region, the autoinhibitory pseudosubstrate domain and the C-terminal basic domain. p70 S6K is a ser/thr kinase implicated in translational control of mRNAs containing 5' terminal oligopyrimidine tracts (5'TOP), such as those encoding ribosomal proteins and elongation factors (Jefferies et al. 1997).

Figure 4. Model of the ser/thr protein kinase p70 S6K.


T229
T389 S404
S411 S418 T421 S424
NH2
-COOH
acidic domain
catalytic domain
(conserved)
linker domain
basic pseudosubstrate domain
(putative autoinhibitory domain)
regulatory domain

b. Activation of p70 S6K

Activation of p70 S6K is accomplished by phosphorylation and subsequent conformational change. A proposed two-stage model involves the phosphorylation of ser/thr residues in the C-terminal autoinhibitory domain that facilitate the second stage: phosphorylation of thr 389 and thr 229, which are critical for enzyme activation (Pullen and Thomas 1997). The C-terminal residues are phosphorylated by as yet undetermined kinases. Thr 389 is the most rapamycin-sensitive site. There is some controversy over whether the site is phosphorylated by mTOR directly or regulated by an mTOR-mediated phosphatase (Scott et al. 1998, Peterson et al. 1999, Randell et al. 1999). Phosphorylation of the C-terminal ser/thr sites and of thr 389 by mTOR appear to facilitate the PDK-1-mediated phosphorylation of thr 229 (Pullen et al. 1998). Activation of p70 S6K leads to phosphorylation of the 40S ribosomal subunit S6. The p70 S6K-regulated translation of mRNAs with 5'TOPs appears to be essential for cell cycle progression from G1 to S phase. p70 S6K has also been implicated in transcriptional regulation. CREB is a family of nuclear factors that regulate transcription. CRE-modulatorτ (CREMτ) is a CREB family member that plays a role in cAMP-induced gene expression. Protein kinase A mediates phosphorylation of ser 117 on CREMτ (de Groot et al. 1994). p70 S6K has been shown to phosphorylate CREMτ at the same ser 117 *in vitro* and *in vivo*. CREB

is also phosphorylated by p70 S6K *in vitro* (de Groot et al. 1994). It is possible that p70 S6K is involved in the regulation of genes involved in adipogenesis as well as mitogenesis. Future studies are needed to investigate this potential role for p70 S6K.

c. Disruption of p70 S6K

Disruption of the p70 S6K gene in murine embryonic stem cells caused a decrease in proliferation rates in vitro (Kawasome 1998). Homozygous disruption of the S6K1 gene did not lead to the expected problems in viability or fertility of mice but it did cause a significant reduction in body size *in vivo* (Shima et al. 1998). There was a significant decrease in size of the S6K1 deficient embryos but no corresponding decrease in S6 phosphorylation was observed. These results prompted the investigation of a compensatory mechanism. S6K2, a new, homologous S6 kinase was identified (Lee-Fruman 1999). S6K2 shares 70% sequence identity with S6K1 and 82% sequence identity with the catalytic domain (Dufner and Thomas 1999, Shima et al. 1998). Increased levels of S6K2 transcripts were observed in the S6K1-deficient mice, consistent with the expected findings.

D. Introduction to Chapter 1

1. Rapamycin inhibits differentiation of 3T3-L1 preadipocytes

In 1995, Yeh et al. reported that 3T3-L1 preadipocytes failed to differentiate in the presence of rapamycin (Yeh et al. 1995a). Decreased levels of the adipogenic transcription factor C/EBPα and decreased lipid droplet accumulation were observed in 3T3-L1 preadipocytes treated with rapamycin over the course of differentiation.

The addition of excess FK506 displaced rapamycin from FKBP12 and reversed the inhibition through competitive-binding. FK506 is an immunosuppressant drug with structural similarity to rapamycin (Cardenas et al 1995). It also binds intracellular FKBP12, but in contrast, it does not act on mTOR and p70 S6K. This indicates that the rapamycin-FKBP12 complex is necessary for rapamycin to exert its effects. They concluded that rapamycin's inhibitory effect on adipogenesis was due to an antiproliferative effect on the clonal expansion phase through the inhibition of p70 S6K.

2. Objective 1

Our first objective was to determine whether rapamycin inhibits differentiation of human preadipocytes in primary culture. If rapamycin inhibits differentiation of 3T3-L1 cells solely based on abrogation of clonal expansion then human preadipocyte differentiation would not be inhibited by rapamycin since these cells do not undergo clonal expansion in primary culture (Entenmann and Hauner 1996). Human preadipocyte differentiation in primary culture in the absence and presence of rapamycin was observed morphologically and quantified by measurement of GPDH.

E. Introduction to Chapter 2

In addition to its involvement in insulin signaling, p70 S6K was recently implicated in thyroid-stimulating hormone (TSH) and cAMP-mediated signaling in a rat thyroid cell line (Cass and Meinkoth 1998). The presence of thyroid stimulating hormone receptor (TSHR) in adipocytes has been suggested (Hart and McKenzie 1971, Roselli-Rehfuss et al. 1992, Endo et al. 1995). We investigated whether the TSHR was expressed in human preadipocytes, and whether it could activate p70 S6K.

1. TSHR expression of mRNA in adipose tissue

Much of the literature on TSHR expression and function is focused on thyroid cells. However, there is evidence pointing to TSHR expression in adipose tissue. Evidence for TSHR in adipocytes dates back to a study by Rodbell in 1964. TSH directly enhanced glucose metabolism, lipogenesis and fatty acid release in free fat cells isolated from rat adipose tissue (Rodbell 1964). The TSH-induced activation of cellular processes in adipocytes suggested the presence of a functional receptor.

More recent studies have focused on TSHR mRNA expression levels. TSHR mRNA expression was detected in rat retroorbital and adipose tissues using polymerase chain reaction (PCR) methods (Endo et al. 1993, Heufelder et al. 1993, Feliciello et al. 1993). TSHR mRNA was also detected in epididymal, subcutaneous and perirenal rat adipose tissue and cAMP levels were increased when adipocytes were stimulated with TSH (Haraguchi et al. 1996). In 1995, the TSHR cDNA was cloned from rat adipocytes (Endo et al. 1995). TSHR mRNA levels increased with induction of differentiation and decreased with the inhibition of differentiation (Haraguchi et al. 1996).

TSHR expression has also been detected in human adipocytes. The first indication of TSHR expression in infant adipocytes was the significant increase in lipolysis that occurred *in vitro* with the addition of stimulatory TSHR

autoantibodies (Janson et al. 1995). In addition to stimulating glycerol release from TG breakdown, TSHR activation also increased cAMP levels in human infant adipocytes, suggesting a role for adenylate cyclase in the TSH pathway (Janson et al. 1995). TSHR mRNA was detected in infant and adult adipose tissue as well as isolated adipocytes using reverse transcriptase-polymerase chain reaction (RT-PCR) and northern blot analysis, confirming the presence of the receptor in human adipocytes (Crisp et al. 1997, Janson et al. 1998).

2. TSHR structure

The structure of TSHR remains controversial. The molecular masses vary from 90 to 500 kDa and subunits vary from 1-3 with masses ranging 15-90 kDa (Misrahi et al. 1994). The TSHR was cloned by several groups in late 1989 and early 1990 (Parmentier et al. 1989, Nagayama et al. 1989, Akamizu 1990a, Misrahi et al. 1990). The TSHR is a G-protein coupled receptor (GPCR) of the seven-transmembrane domain family (Ludgate et al. 1999). Three major classes of G-proteins identified in cell signaling pathways include: Gs, Gi and Gq. Gs and Gi lead to activation and inactivation respectively of adenylate cyclase whereas Gq activates phospholipase Cβ (PLCβ) which cleaves phosphatidylinositol-bisphosphate (PIP_2) into the intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG). This leads to endoplasmic

release of Ca^{2+} stores and activation of protein kinase C (PKC) respectively (Gutkind 1998, Luttrell et al. 1999).

3. TSHR function

TSHR has an established role in the thyroid as an integral component in events leading to thyroid hormone synthesis and release. TSH is involved in the regulation of basal metabolic rate as well as growth and maturation. Activation of TSHR in thyroid cells typically leads to a cascade of events including activation of G-protein and adenylate cyclase, finally leading to an increase in intracellular cAMP levels (Laugwitz et al. 1996).

4. Objective 2

The second major objective of this thesis was to determine whether functional TSHR protein is expressed in human preadipocytes. TSHR expression was measured by western blot analysis. We determined whether the receptor is functional by measuring the activation of p70 S6K after acute stimulation with TSH. p70 S6K was immunoprecipitated for *in vitro* kinase assay and gel mobility shift analysis. We also investigated other possible immediate downstream targets of the TSHR. An inhibitor of PI3K, wortmannin, was used to

assess the involvement of PI3K in p70 S6K activation. Furthermore, we tested whether PKB, a downstream target of PI3K, was stimulated by TSH.

II. CHAPTER 1 Effect of rapamycin on human preadipocyte differentiation

A. MATERIALS AND METHODS

Subjects. Abdominal subcutaneous and omental adipose tissue samples were obtained from consenting patients undergoing elective abdominal surgery (approved by the Loeb Health Research Institute Ethics Committee). Patients were not acutely ill, and were weight stable. Patients were aged 60.6±1 (mean±SEM) with body mass indexes (BMI) of 29.2±0.7 (mean±SEM). Ten samples from seven patients were used in studies on differentiation (5 males, 2 females). Two samples from two patients were used in studies on [^{3}H]-thymidine incorporation (1 male, 1 female).

Isolation of human preadipocytes. Adipose tissue samples were collected on the day of surgery and transported to the laboratory in Dulbecco's Modified Eagle's Medium (DMEM) : Ham's F12 (F12) (1:1) supplemented with 2xPSN which was 200 units/ml penicillin (P), 0.2 mg/ml streptomycin (S), and 100 units/ml nystatin (N). To isolate the preadipocytes, tissue was placed in sterile phosphate buffered saline (PBS) supplemented with 2xPSN for removal of the fibrous tissue and blood vessels. Finely cut adipose tissue fragments were weighed, and then

placed in 6 mg collagenase A/ g tissue, and 60 mg bovine serum albumin (BSA)/ g tissue and 3 mls/ g tissue DMEM:F12 PSN (1xPSN was 100 units/ml P, 0.1 mg/ml S, and 50 units/ml N) supplemented with 33 μM biotin (B), 17μM pantothenate (P). Collagenase digestion was carried out for 45 min at 37°C, and then 3 mls DMEM:F12 PSN BP / g tissue were added. This suspension was then filtered through a 200 μm filter (Nalgene) using a hand-held pipette. The filtrate was centrifuged at room temperature in a Megafuge 1.0 Heraeus swing-out bucket centrifuge without brake at 1000xg for 20 min. The top layer of floating mature adipocytes was removed and 10% fetal bovine serum (FBS; final concentration) was added to the infranatant to inactivate residual collagenase activity. The pellet was resuspended in DMEM:F12 PSN BP, and filtered through a 200 μm nylon filter. The filtrate was centrifuged as described above. Once again, the resulting cell pellet was resuspended in DMEM:F12 PSN BP, and then subsequently filtered through 100, 50 and 25 μm nylon filters to yield the preadipocyte fraction. A final 20 min centrifugation was followed by resuspension of the pellet and cell counting.

Primary cell culture of human preadipocytes and adipocyte differentiation. Cells were seeded at $5x10^4$ cells/cm^2 and placed in DMEM PSN supplemented with 10% FBS for attachment. The following morning, cells were rinsed 3x with

DMEM:F12 PSN BP and 2x with control medium consisting of DMEM:F12 PSN BP supplemented with 0.1 μM insulin (I), 10 μg/ml transferrin (T) and 0.2 nM triiodothyronine (T_3) for 5 min and 1x DMEM:F12 PSN BP ITT_3 for 1h. After serum removal, control or differentiation medium was added to the cells. Differentiation medium consisted of DMEM:F12 PSN BP ITT_3 supplemented with 1 μM I, 200 nM carbaprostacyclin ($cPGI_2$), 1 μM dexamethasone (Dex) and 0.1 mM IBMX. The media was changed every four days over a period of 15-17 days. On the fourth day, Dex and IBMX were omitted from the differentiation medium. Control preadipocyte cultures were treated as described above.

Differentiation of 3T3-L1 preadipose cells. 3T3-L1 preadipose cells were grown to confluence in DMEM PS supplemented with 10% calf serum (CS). Two days post-confluence, cell cultures were washed once in DMEM PS supplemented with 10% CS, and then placed in DMEM PS supplemented with 10 % CS, 0.25 μM Dex, 0.5 mM IBMX and 100 nM I for 48 h. Following this, the medium was switched to DMEM PS supplemented with 10% CS and 100 nM I for the next six days. Medium was changed every other day. Rapamycin (100 nM) was added to cell lysates from cells differentiated over 8 days.

Analysis of adipocyte differentiation.

Morphology and Oil Red O Staining. Light microscopy and Oil Red O staining were used to monitor the characteristic cell rounding and lipid droplet accumulation in differentiating adipocytes. Images were taken using the Olympus 1X-70 and a dual-color CCD camera.

GPDH Assay. Activity of GPDH, a specific marker of terminal differentiation, was determined in cytosolic fractions. After 15-17 days the cells were washed twice with cold PBS prior to addition of harvesting buffer containing 25 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM 2-mercaptoethanol (2-Me). All solutions and cell culture plates were kept on ice to prevent degradation of the enzyme. The cells were transferred to a chilled microfuge tube, and then vortexed and sonicated at 4°C with the Branson Sonifier 450 for 5 seconds to disrupt the cell membranes. Samples were then centrifuged at approximately 350000xg for 10 min to obtain the cytosolic fraction. The supernatant was transferred to chilled microfuge tubes and kept on ice. Cytosolic protein concentrations were assayed from aliquots of the same cytosolic fractions using the BioRad assay, with BSA as a standard. The BioRad assay is a dye binding assay in which Coomassie Brilliant Blue G-250 binds to

proteins causing a shift in radiation from 465 nm to 595 nm. To assess GPDH activity, the absorbance of reduced nicotinamide adenine dinucleotide phosphate (NADH) at 340 nM was measured on the Beckman DU-50 Spectrophotometer using software on the Soft Pac Module Kinetics cartridge Program 6: Kindata. A linear curve was generated from data recorded every 15 sec over a 2 min period. Lysate volumes were adjusted to achieve adequate substrate levels and avoid saturation of the reaction. NADH is a co-factor required for biosynthesis of TG in which NAD-linked GPDH reduces dihydroxyactetone phosphate (DHAP) to produce L-glycerol-3-phosphate. For human samples, 60 μl cocktail (containing βNADH, 1 M triethanolamine pH 7.7, 5 M 2-Me, and 25 mM EDTA pH 8.0), 20 μl Tris-EDTA-2Me buffer, 80 μl sample, and 40 μl 4 mM DHAP substrate were added sequentially to a quartz cuvette. For 3T3-L1 samples, 10 μl of sample and 90 μl Tris-EDTA-2Me buffer was added. Specific activity was calculated as follows: (unit / ml / min)/ ([mg protein] / ml) = units / mg / min (Wise and Green 1979).

Cell proliferation assay. Subcutaneous preadipocytes were isolated as described and seeded for overnight attachment in DMEM PSN supplemented with 10% FBS at $5x10^4$ cells/cm^2. The following day, preadipocytes were treated for 48h with [^{3}H]-thymidine (1μCi / ml) under each of the following conditions (as described earlier): control medium, differentiation medium, 10% FBS-containing

medium and differentiation medium + cytosine ß-D-arabinofuranoside (Ara-C). The medium was aspirated and cells were fixed in 100% methanol for 10 minutes followed by washing in Hank's Buffered Salt Solution. DNA was precipitated with 10% trichloroacetic acid and cells were scraped in 0.3 N NaOH / 1% SDS for liquid scintillation counting of [^{3}H]-thymidine.

Statistical analysis. For comparison of sample means to maximal differentiation normalized to 100%, data are analyzed as means±95% confidence limits. To compare means±SEM of two experimental groups, a 2-tailed *t*-test was employed.

B. RESULTS

1. Rapamycin inhibits the differentiation of human abdominal omental and subcutaneous preadipocytes.

Addition of rapamycin to the adipogenic medium significantly inhibited adipogenesis of abdominal omental and subcutaneous preadipocytes. Microscopic visualization in Figure 5 indicates fewer than 5% of the subcutaneous preadipocytes acquired the rounded morphology and lipid-filled cytoplasm characteristic of mature adipocytes. As shown in Figure 6, GPDH activity, a terminal marker of differentiation, was severely reduced in the presence of rapamycin. Omental and subcutaneous adipocyte differentiation in the presence of rapamycin, as assessed by GPDH activity, was diminished to 14±25% (mean±95% confidence limits, n=4) and 19±13% (mean±95% confidence limits, n=6) respectively, compared to standard differentiation (normalized to 100%). Cells in control medium alone or in the presence of rapamycin expressed the same levels of GPDH values as cells placed in differentiation medium + rapamycin (Fig. 6). Since the rapamycin-mediated inhibition of omental and subcutaneous adipocyte differentiation was not significantly different, we grouped the 10 samples together. Differentiation in the presence of rapamycin for the combined group was decreased to 17±10%

Figure 5. Rapamycin inhibits the differentiation of human abdominal omental preadipocytes: Oil Red O staining. Photomicrograph (original magnification, X100) of preadipocytes differentiated over the course of 16 days in the presence (A) or absence (B) of 100nM rapamycin.

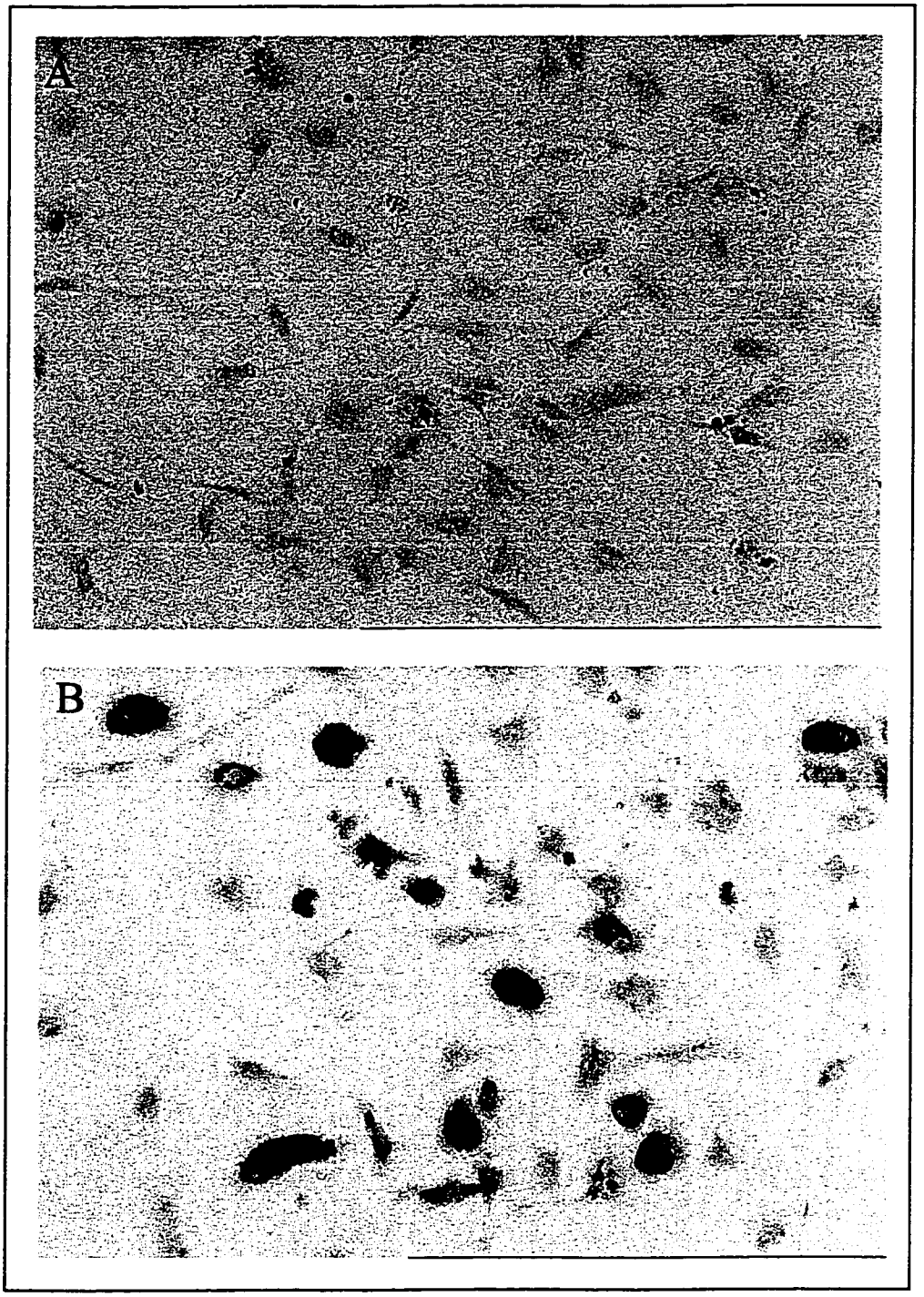
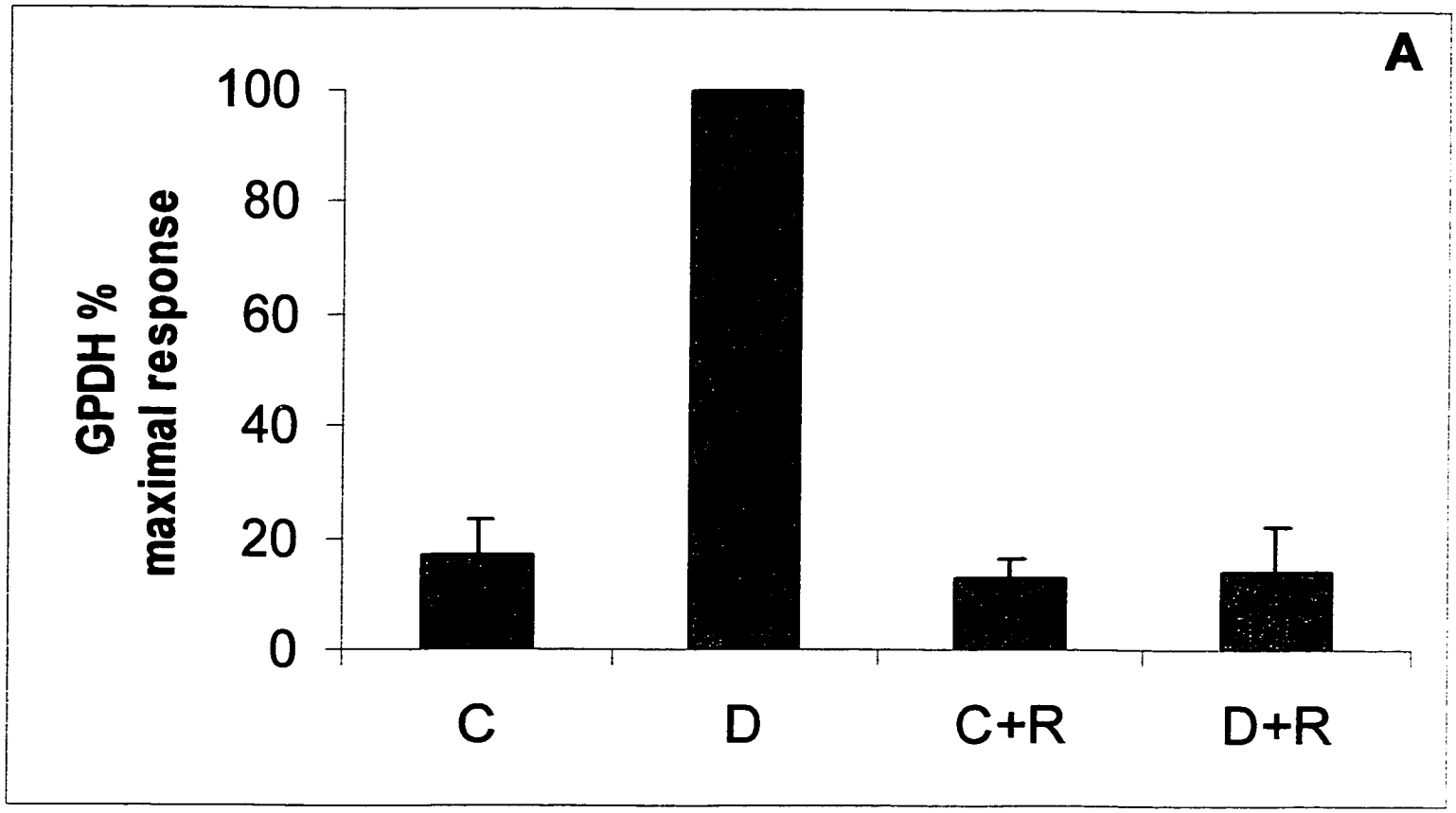
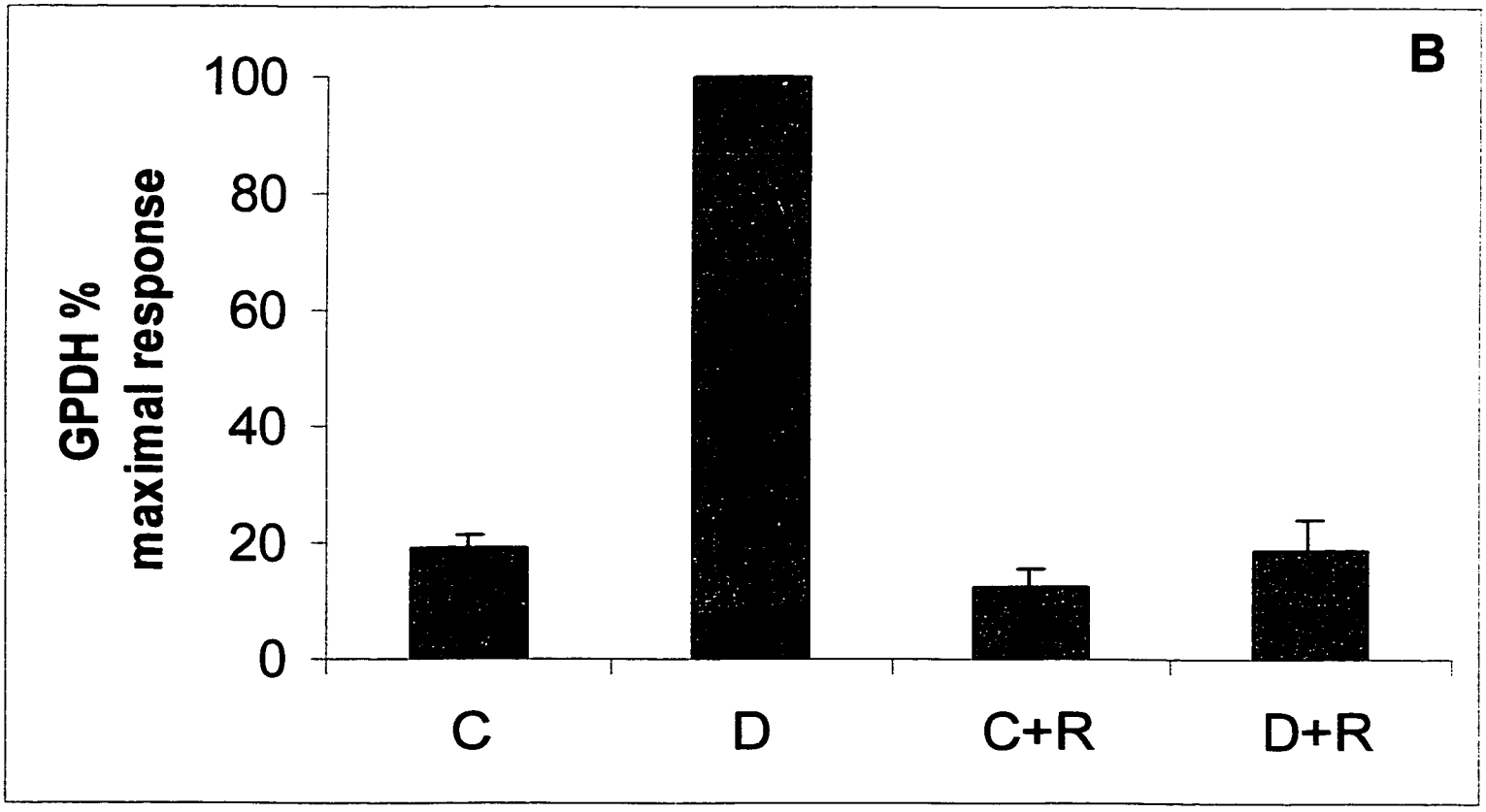

A
B

Figure 6. Rapamycin inhibits the differentiation of human abdominal omental and subcutaneous preadipocytes. Human omental (panel A; n=4) and subcutaneous (panel B; n=6) preadipocytes were placed in primary culture as described. Preadipocytes were treated with control medium in the absence [C] or presence [C+R] of 100 nM rapamycin, or differentiated into adipocytes, in the absence [D] or presence [D+R] of rapamycin, as described. Cells were then harvested, and cytosolic GPDH specific activity was measured as described. The GPDH activity for differentiated cells in the absence of rapamycin was normalized to 100%, and the data are expressed as means±SEM.


A
GPDH %
maximal response
100
80
60
40
20
0
C
D
C+R
D+R

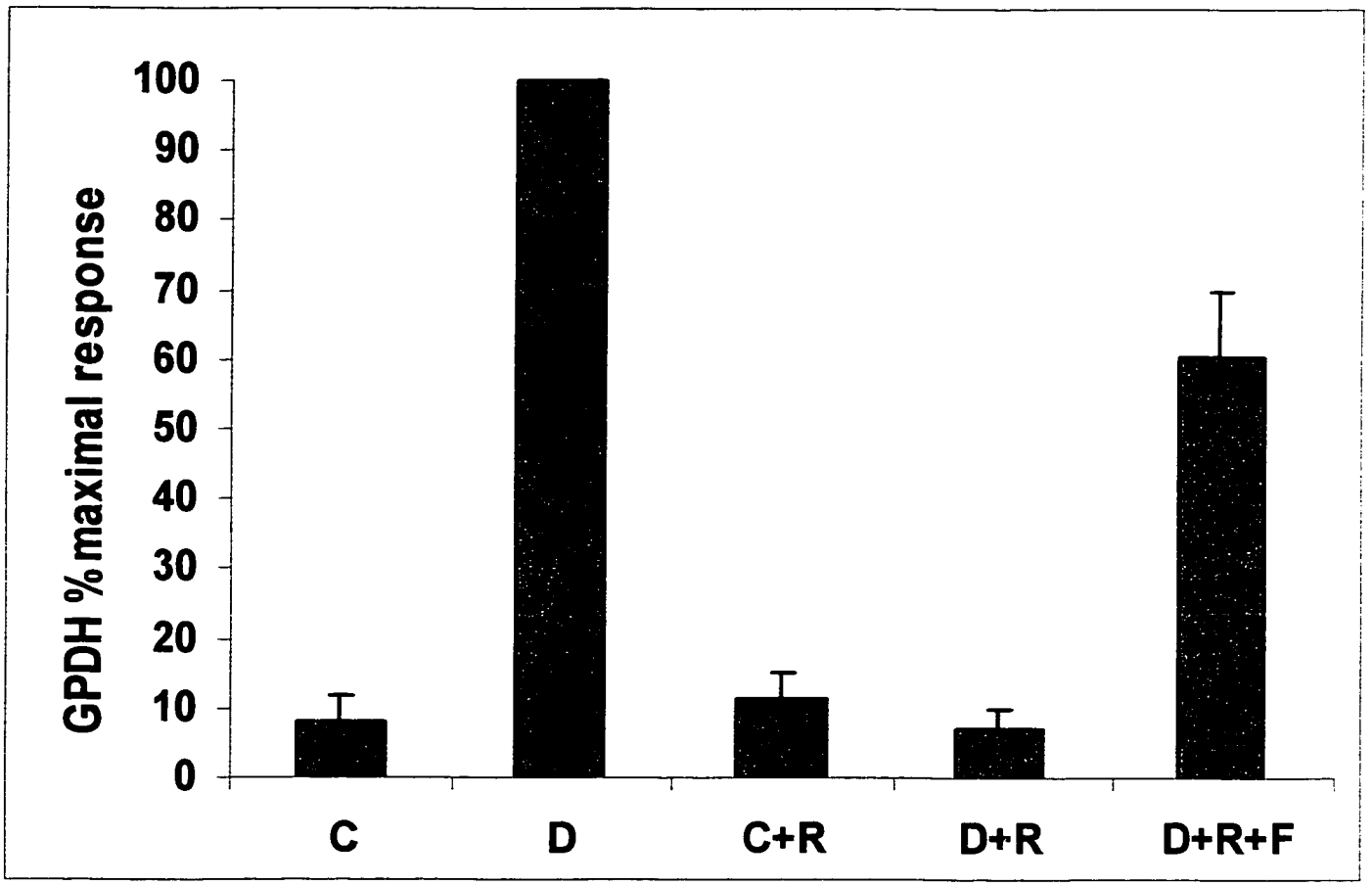

B
GPDH %
maximal response
100
80
60
40
20
0
C
D
C+R
D+R

(mean±95% confidence limits) of standard differentiation (normalized to 100%). The mean value of GPDH activity for all 10 samples differentiated in the absence of rapamycin was 584 units/mg/min.

2. *FK506 reverses the rapamycin-mediated inhibition of differentiation*

We investigated whether the simultaneous addition of FK506 with rapamycin would permit the differentiation of preadipocytes. FK506 is an immunosuppressant drug that also binds to the intracellular receptor FKBP12 but activates distinct cellular signals from those of rapamycin. Downstream targets of rapamycin include mTOR and p70 S6K whereas FK506 inactivates calcineurin (Abraham and Wiederrecht 1996). Yeh et al. (1995a) reported that excess FK506 reverses the inhibitory effects of rapamycin on 3T3-L1 cell clonal expansion and differentiation through competitive binding to FKBP12. They demonstrated that the addition of FK506 itself to the adipogenic cocktail does not inhibit 3T3-L1 adipocyte differentiation over a concentration range from 1-1000 nM (Yeh et al. 1995a). We also observed a reversal of the rapamycin effect on human adipocyte differentiation with the concurrent addition of a 100-fold excess of FK506 as shown in Figure 7. In the presence of 100 nM rapamycin, 10 μM FK506 was sufficient to rescue the differentiation response substantially,

Figure 7. FK506 reverses the effect of rapamycin. Human preadipocytes (2 omental and 1 subcutaneous sample) in primary culture were differentiated into adipocytes as described in the absence [D], or in the presence of 100 nM rapamycin alone [D+R], or 100 nM rapamycin + 10 μM FK506 [D+R+F], as described. Preadipocytes were also treated with control medium in the absence [C] or presence [C+R] of rapamycin. Cells were then harvested, and cytosolic GPDH specific activity was measured as described. The GPDH activity for cells differentiated in the absence of rapamycin was normalized to 100%, and the data are expressed as means±SEM.


GPDH % maximal response
100
90
80
70
60
50
40
30
20
10
0
C
D
C+R
D+R
D+R+F

returning GPDH activity to 60±16% (mean±SEM) of the maximal value, compared to rapamycin-inhibited GPDH activity of 7±9% (mean±SEM) ($p<0.05$). Again, cells in control medium alone or in the presence of rapamycin expressed the same levels of GPDH values as cells placed in differentiation medium + rapamycin. The mean value of GPDH activity for the 3 samples differentiated in the absence of rapamycin or FK506 was 636 units/mg/min.

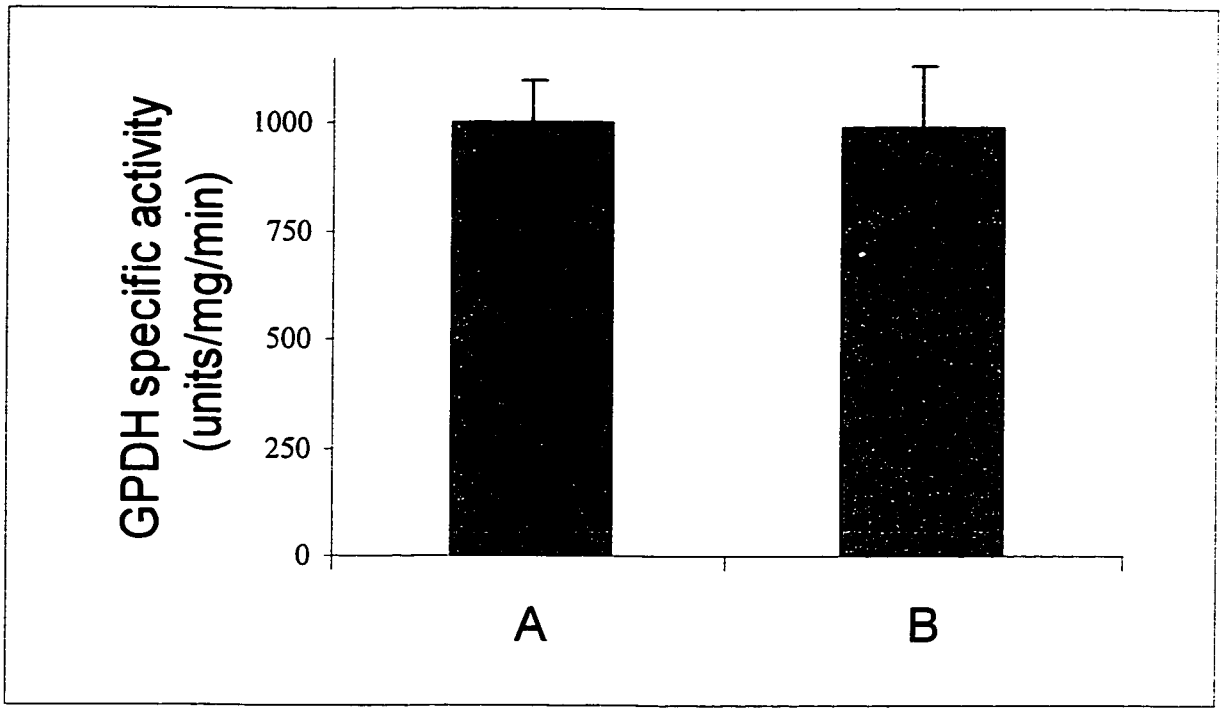
3. Rapamycin does not interfere with the GPDH assay

We tested the effect of rapamycin on the measurement of GPDH activity in cytosolic fractions of differentiated 3T3-L1 adipocytes, to rule out any direct effects of rapamycin on the assay itself. When rapamycin was added to 3T3-L1 cell lysates obtained after the completion of the 8-day differentiation protocol, there was no effect on GPDH activity, as shown in Figure 8.

4. Differentiation medium does not induce proliferation of human subcutaneous preadipocytes

Yeh et al. (1995a) proposed that rapamycin inhibits cell division during the clonal expansion phase of 3T3-L1 preadipocytes resulting in abrogation of

Figure 8. Rapamycin does not affect the GPDH assay when added to cell lysates after completion of differentiation. 3T3-L1 preadipocytes were grown to confluence and induced to differentiate in serum-containing media as described. After 8 days, cells were harvested and 100 nM rapamycin was added to the cell lysates. GPDH specific activity for differentiated cells was determined in the absence [A] and presence of rapamycin [B]. The data are expressed as means±SEM (n=3).


GPDH specific activity
(units/mg/min)
1000
750
500
250
0
A
B

differentiation. However, a previous study by Entenmann and Hauner demonstrated that human preadipocytes differentiated in chemically-defined medium undergo differentiation in the absence of clonal expansion. In addition to the adipogenic components used by Entenmann and Hauner (1996), our adipocyte differentiation medium contains $cPGI_2$. Negrel et al. (1989) have reported that $cPGI_2$ may induce a limited proliferation of the Ob1771 preadipocyte cell line. Studies of $cPGI_2$ on human preadipocytes in primary culture from that same group did show enhanced differentiation but did not demonstrate a clear increase in cell number (Negrel et al. 1989).

To determine if significant proliferation had occurred in our culture system, we first measured cellular protein. The cytosolic protein content of our primary cell cultures of preadipocytes in control medium (8.6±0.6 µg/well) vs. *in vitro* differentiated adipocytes (9.1±0.5 µg/well) was not significantly different, consistent with the expected absence of proliferation.

In addition, we measured the levels of DNA synthesis that occurred under four separate culture conditions, as shown in Table 1. When cells were maintained in serum-containing medium as a positive control, an increase in [^{3}H]-thymidine incorporation was observed, as expected. [^{3}H]-Thymidine values for cells exposed to control medium were low. Similarly, preadipocytes exposed to either differentiation medium alone, or in the presence of Ara-C (inhibitor of DNA synthesis), incorporated low levels of [^{3}H]-thymidine.

Table 1. Incorporation of [^{3}H]-thymidine into DNA of human subcutaneous preadipocytes. Preadipocytes were placed in primary culture and labeled with [^{3}H]-thymidine as described. Data are expressed as means±range of two independent experiments, each performed in duplicate.

Table 1. Incorporation of [^{3}H]-thymidine into DNA of human subcutaneous preadipocytes.

Culture Condition	mean cpm	range
Control medium	77	± 22
Differentiation medium	65	± 17
Serum-containing medium	638	±186
Differentiation medium + Ara-C	73	± 6

C. DISCUSSION

We have shown that rapamycin significantly inhibits the differentiation of human abdominal omental and subcutaneous preadipocytes in primary culture. The rapamycin-mediated inhibition of adipogenesis was assessed visually by morphology and Oil Red O staining as well as biochemically by GPDH activity. Rapamycin-treated omental and subcutaneous preadipocytes cultured in the presence of differentiation cocktail exhibited the same levels of inhibition at the single dose of rapamycin employed.

Anatomic site-specific differences observed in preadipocytes include the greater susceptibility of omental preadipocytes towards apoptosis and the greater responsiveness of subcutaneous preadipocytes to PPARγ agonist-induced adipocyte differentiation. To date, most studies on regional differences in adipose tissue depots have focused on adipocyte differences. Only a few studies compare preadipocyte regional effects. In our studies, using rapamycin at 100 nM, differentiation of omental and subcutaneous preadipocytes was similarly inhibited, indicating no regional differences. Careful dose-response studies should be performed to examine if there is a regional effect before concluding that the rapamycin-sensitive pathway is equivalent in both.

Inhibition of differentiation in human preadipocytes was surprising considering a previous report by Yeh et al. that suggested the inhibitory effect of

rapamycin on 3T3-L1 cell differentiation was dependent on a blockade of clonal expansion (Yeh et al. 1995a). As mentioned previously, 3T3-L1 preadipose cells are grown to confluence and undergo a growth arrest phase, followed by induction of differentiation with an adipogenic cocktail. 3T3-L1 preadipose cells then enter a clonal expansion phase followed by a second growth arrest phase (Pairault and Green 1979). In contrast to 3T3-L1 preadipose cells, Entenmann and Hauner (1996) have demonstrated that human preadipocytes in primary culture differentiate in the absence of a clonal expansion phase. We have shown that rapamycin inhibits human adipocyte differentiation but we needed to rule out possible proliferative effects of our adipogenic cocktail. In addition to the adipogenic components used by Entenmann and Hauner, our adipogenic cocktail contains $cPGI_2$. We assessed proliferation of human preadipocytes in our own chemically-defined serum-free medium. The cytosolic protein content for preadipocytes placed in control and differentiation medium over the course of 15-17 days was essentially the same, indicating absence of proliferation. These results do not preclude the possibility of cell death masking proliferative effects. To measure cell proliferation directly, we measured [^{3}H]-thymidine incorporation levels during DNA synthesis for the first 48 h of differentiation. Our data demonstrate that proliferation (i.e. clonal expansion) does not occur in human preadipocytes differentiating in primary culture when placed in adipogenic

medium containing Dex, IBMX, I and $cPGI_2$.

Our studies on rapamycin-treated human preadipocytes suggest that rapamycin exerts its effects through a mechanism other than abrogation of the clonal expansion phase. It is possible that human preadipocytes obtained from the stromal-vascular adipose tissue component have proceeded through critical cell divisions *in vivo* and are arrested at a later stage than the 3T3-L1 preadipose cells isolated from mouse embryo. These results suggest that rapamycin-induced inhibition of human preadipocyte differentiation occurs through a mechanism other than inhibition of clonal expansion. Yeh et al. (1995a) showed that rapamycin, an inhibitor of mTOR/p70 S6K-mediated signaling, blocked differentiation in 3T3-L1 preadipocytes. They proposed that rapamycin blocked clonal expansion during differentiation since p70 S6K plays a role in mitogenic signaling, causing G1 arrest. We have shown that rapamycin blocks differentiation outside of a blockade on clonal expansion in human preadipocytes. Rapamycin may exert its effects through inhibition of mTOR/p70 S6K-mediated regulation of adipocyte differentiation-specific genes at the level of transcription and/or translation.

We also assessed the effect of concurrent addition of rapamycin and FK506, a structural analog of rapamycin, on differentiation. Addition of 100-fold excess FK506 to the adipogenic cocktail led to a substantial rescue of differentiation. FK506 and rapamycin share the same intracellular receptor

FKBP12, but mediate distinct signaling pathways. The FKBP12-FK506 binds to calcineurin whereas rapamycin-FKBP12 complex binds to mTOR (Abraham and Wiederrecht 1996). This indicates that the rapamycin-FKBP12 complex specifically inhibits differentiation through the inhibition of mTOR.

mTOR has been previously reported to play a role in insulin signal transduction, and mTOR increases with 3T3-L1 adipocyte differentiation (Graves et al. 1995, Lin et al. 1995, Proud 1996, Whithers et al. 1997). These results suggest that mTOR may play a significant role in the differentiation of human preadipocytes. mTOR is involved in the regulation of the translational regulators p70 S6K and 4E-BP1 and cyclin-dependent kinases (Thomas and Hall 1997, Scott et al. 1998). These regulators of translational control may also contribute to adipogenesis. A study by Burnett et al. (1998) suggests that mTOR is a stronger activator of p70 S6K (Burnett et al. 1998). Thus p70 S6K may play a more significant role than 4E-BP1 in the inhibition of differentiation in omental and subcutaneous human preadipocytes cultured in the presence of rapamycin. p70 S6K can also regulate transcriptional events through CREB family members. Studies are needed to compare the significance of p70 S6K, 4E-BP1 and the cyclin dependent kinases for adipocyte differentiation.

Currently, there are two lines of evidence that point to separate roles for mTOR in insulin signaling pathways. Scott et al. (1998) has reported that insulin phosphorylates and activates mTOR directly through a PI3K/PKB-sensitive

pathway. In contrast, Peterson et al. (1999) has suggested that phosphorylation of mTOR regulates p70 S6K indirectly through the inhibition of the ser/thr PP2A. Future studies should focus on the delineation of the precise pathway of mTOR-mediated activation of p70 S6K and the role of mTOR in adipogenesis.

There are presently no studies published on the effects of rapamycin treatment on body weight changes in humans. However, two separate reports on the effects of combination immunosuppressive drug therapy in rodents suggest that rapamycin induces a significant reduction in weight gain (Whiting et al. 1991, Sigalet et al. 1999). Although these studies were not designed to observe the effects of rapamycin specifically on weight gain, the results suggest that this drug may inhibit weight gain in rodents.

III. CHAPTER 2 Investigation of functional TSHR expression in human subcutaneous preadipocytes

A. MATERIALS AND METHODS

Subjects. Abdominal subcutaneous adipose tissue samples were obtained from consenting patients undergoing elective abdominal surgery (approved by the Loeb Health Research Institute Ethics Committee).

Isolation of human preadipocytes. As described in Chapter 1.

Cell Culture

Human abdominal subcutaneous preadipocytes. Isolated human subcutaneous abdominal preadipocytes were grown to confluence in DMEM PSN supplemented with 20% FBS.

Human orbital fibroblasts. Human orbital fibroblasts were from explants of orbital tissue from patients with Graves' disease (from Dr. T. Smith, University of California at Los Angeles School of Medicine, Torrance, California). Cells were grown to confluence in DMEM PS supplemented with 10% CS.

KAT 50 Human thyroid epithelial cells KAT50 cells (from Dr. K. Ain, University of Kentucky, Lexington, Kentucky) were grown to confluence in DMEM PS supplemented with 10% CS.

CHO-K1 Chinese hamster ovarian cells, McA RH7777 rat hepatoma cells, and J774 mouse macrophage cells (from Dr. Y. Marcel, University of Ottawa, Ottawa, Ontario). CHO-K1 Chinese hamster ovarian cells were grown to confluence in F12 PS supplemented with 10% FBS, McA RH7777 rat hepatoma cells were grown to confluence in DMEM PS supplemented with 20% horse serum and 5% FBS, and J774 mouse macrophage cells were grown to confluence in DMEM PS supplemented with 10% FBS.

Jurkat human leukemia cells (from Dr. R. Haché, University of Ottawa, Ottawa, Ontario) were grown to confluence in RPMI PS supplemented with 10% FBS.

Determination of TSHR protein expression. Preadipocytes, in vitro differentiated adipocytes, orbital fibroblasts and KAT 50 thyroid epithelial cells were lysed in 1X Laemmli buffer. Equivalent amounts of protein were added per lane and resolved on 7.5% SDS-PAGE and subjected to western analysis. Proteins were transferred to a BIO-RAD Trans-Blot Transfer Medium and blocked in 5% skim

milk in PBS/0.1% Tween 20. The immunoblot was probed with two separate antibodies. The blot was first probed with a 1:60 dilution of polyclonal TSHR antibody (from Dr. L. Kohn, NIH, Bethesda, Maryland) that recognizes aa region 352-366 of the TSHR. After the addition of secondary donkey anti-rabbit antibody (1:10000) followed by washing and enhanced chemiluminescence (ECL) detection, the blot was stripped and reprobed with a 1:60 dilution of the Nova Castra Laboratories mouse monoclonal TSHR antibody (NCL-TSH-R2) that recognizes aa region 211-414. Secondary sheep anti-mouse antibody (1:1000) was added followed by washes and ECL detection. KAT50 Human thyroid epithelial cells were treated as described above and then probed with mouse-anti hemagglutinin (HA) antibody (from Dr. J. Liu, University of Ottawa, Ottawa, Ontario). Secondary sheep anti-mouse (1:2500) was added followed by washes and ECL detection. KAT50 Human thyroid epithelial cells, CHO-K1 Chinese hamster ovarian cells, McA RH7777 rat hepatoma cells, J774 mouse macrophage cells and Jurkat human leukemia cells were treated as described above and then probed with the NCL-TSH-R2 antibody.

Immunoprecipitation of p70 S6K. Human subcutaneous preadipocytes were grown to confluence as described. Preadipocytes were serum-starved in

DMEM:F12 PSN BP T_3 0.5% BSA for 16 h and then washed 2x in Krebs Ringer Hepes (KRH) buffer containing 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes and 5.6 mM glucose in H_2O at pH 7.4. The KRH buffer was added to the preadipocytes and cells were placed in the incubator at 37°C for 15 min preincubation with 100 nM wortmannin or with vehicle control. Cells were stimulated for 1 h with 20 μM TSH using 2% BSA/KRH as a carrier. After stimulation, ice cold lysis buffer (1xPBS, 1% Nonidet P-40, 200 μM sodium orthovanadate, 100 μg/ml phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1μM microcystin and 1mM β-glycerol phosphate) was added to lyse cells on ice for 15 min. Lysates were centrifuged and supernatants were precleared with 40 μl protein-A-sepharose for 1 h at 4°C. Rabbit anti-p70 S6K polyclonal antibody from Santa Cruz directed against the C-terminus (C-18) was preadsorbed to 40μl protein-A-sepharose in 450 μl lysis buffer/sample for 1 h at 4°C. Precleared supernatant samples were incubated for 90 min at 4°C with p70 S6K antibody preadsorbed to protein-A-sepharose.

In vitro kinase assay. The S6 Kinase Assay Kit from Upstate Biotechnology Incorporated was used for the *in vitro* kinase assay. Immunoprecipitates were washed 2x with lysis buffer and 3x with assay buffer (consisting of 20 mM 3-[N-

morpholino] propanesulfonic acid (MOPS) at pH 7, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) followed by resuspension in assay buffer, 20 μM PKC inhibitor peptide, 2 μM PKA inhibitor peptide, 20 μM calmodulin dependent kinase inhibitor Compound R24571 and 50 μM S6 peptide (AKRRRLSSLRA) substrate. The reaction was initiated with the addition of ATP cocktail containing 75 mM $MgCl_2$, 500 μM ATP and 10μCi/μl $^{32}P\gamma ATP$ in assay dilution buffer. After a 15 min incubation, 25 μl of reaction was spotted onto phosphocellulose disks, followed by three washes in 0.75% phosphoric acid, and one wash in acetone. Dried phosphocellulose disks were each placed in 5 ml scintillation cocktail and phosphorylation of S6 protein was quantified using a scintillation counter.

Gel mobility shift of p70 S6K. Reaction mixtures from the kinase assay were run on 12.5% SDS-PAGE and proteins were transferred to a BIO-RAD Trans-Blot Transfer Medium and blocked in 5% skim milk in PBS/0.1% Tween 20. The immunoblot was probed with primary (as indicated) and secondary antibodies, then washed and detected with ECL.

B. RESULTS

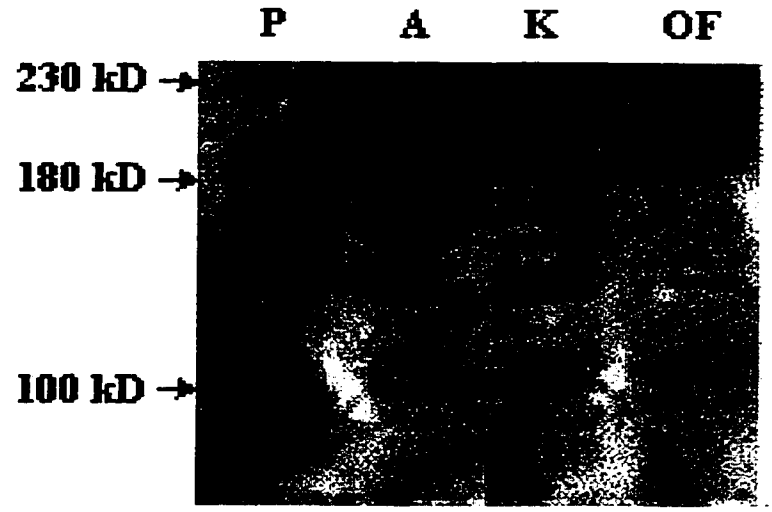
1. TSHR is expressed in human abdominal subcutaneous preadipocytes

Figures 9A and 9B show an immunoblot of total cell lysates from 4 different cell types probed with two separate antibodies to TSHR. Each lane contains solubilized protein from each of the 4 different cell types. Orbital fibroblasts and KAT50 thyroid epithelial cells serve as positive controls (Bahn et al. 1998, Venkataraman et al. 1998). The immunoblot in Figure 9A was probed with polyclonal TSHR antibody that recognizes aa region 352-366 of TSHR. This antibody detects putative higher molecular weight multimers of TSHR as well as what is believed to be the 100 kDa mature, fully processed form of the receptor. The 100 kDa band is present faintly in all 4 lanes.

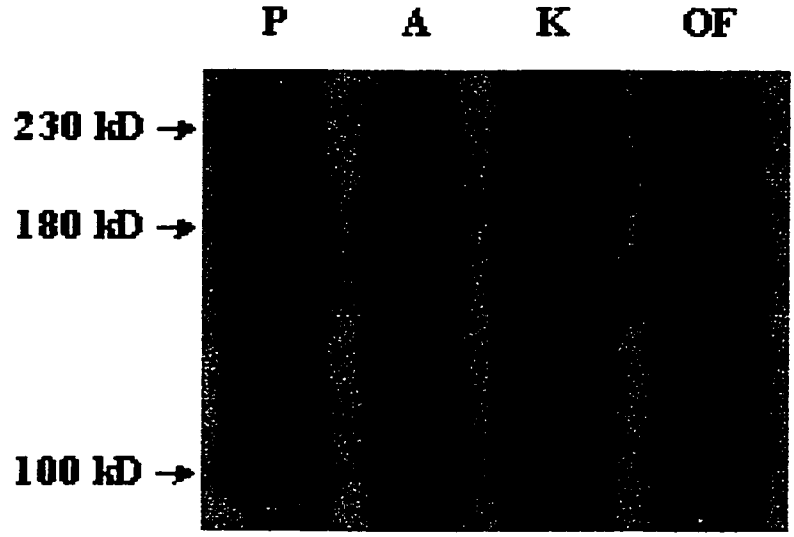
Since the pattern of TSHR detected using western analysis is complex, the blot was stripped and reprobed with a different commercially available monoclonal antibody (Figure 9B). The NCL-TSH-R2 antibody recognizes aa region 211-414 and appears to have a stronger affinity for the 100 kDa band. The doublet observed in orbital fibroblasts and KAT50 thyroid epithelial cells may

Figure 9. TSHR is expressed in human abdominal subcutaneous preadipocytes. Equal amounts of protein from human abdominal subcutaneous preadipocytes (P), adipocytes from in vitro differentiation (A), KAT50 human thyroid epithelial cells (K) and human orbital fibroblasts (OF) was run on SDS-PAGE and subjected to western analysis. A) Immunoblot probed with polyclonal anti-TSHR antibody from Dr. L. Kohn at the NIH that recognizes aa region 352-366. B) Immunoblot probed with NCL-TSH-R2 antibody that recognizes aa region 211-414. C) Immunoblot of KAT50, CHO-K1, McARh7777, J774 and Jurkat cell lysates probed with NCL-TSH-R2 antibody. Lane one contains KAT50 cell lysates probed with anti-HA.

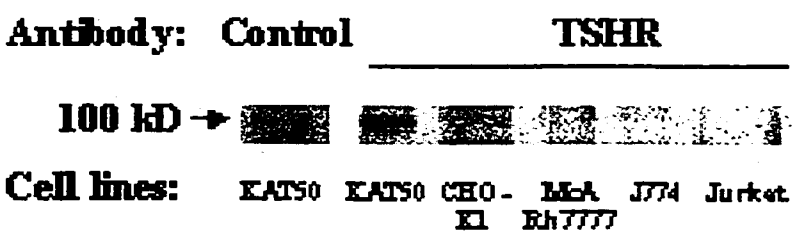
A


P
A
K
OF
230 kD
180 kD
100 kD

B


P
A
K
OF
230 kD
180 kD
100 kD

C


Antibody: Control
TSHR
100 kD
Cell lines:
KAT50
KAT50
CHO-K1
MeA Eh7777
J774
Jurket

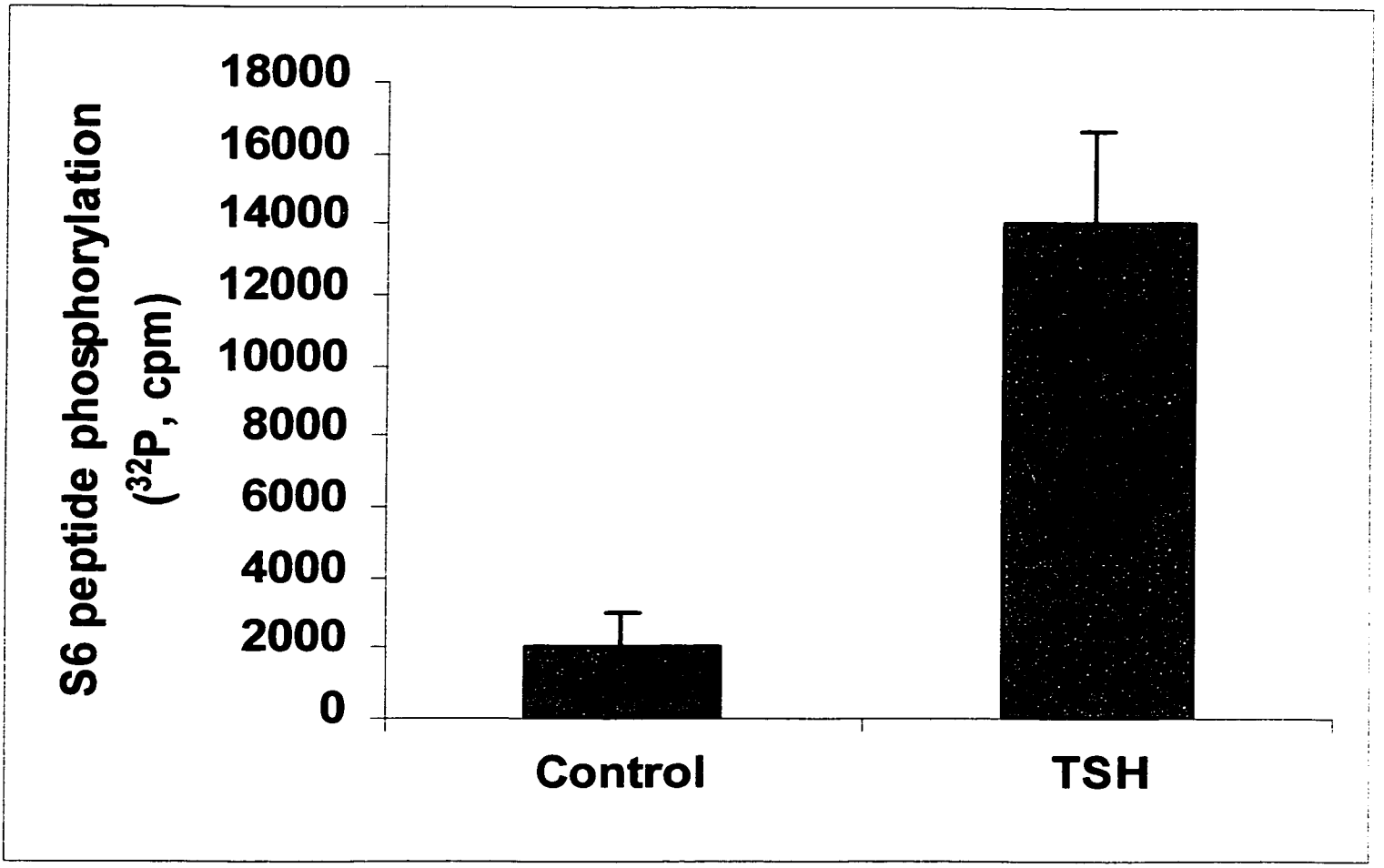
be due to glycosylation (Oda et al. 1999). Figure 9C is an immunoblot of total cell lysates from many different cell lines probed with the NCL-TSH-R2. KAT50 thyroid epithelial cells, probed with mouse anti-HA antibody at the same concentration as the NCL-TSH-R2, did not show a 100 kDa band, indicating the specificity of the anti-TSHR antibody. The 100 kDa band was not detected in the TSHR-negative cells, demonstrating that there is no non-specific binding of primary mouse monoclonal antibody to the cellular protein.

2. *TSH induces activation of p70 S6K in human abdominal subcutaneous preadipocytes*

a. *in vitro kinase assay*

To determine whether TSHR is functional in the preadipocyte, we assessed the activation of p70 S6K, a novel downstream target of TSHR (Cass and Meinkoth 1998). p70 S6K was immunoprecipitated and its activity was measured quantitatively using *in vitro* kinase assay. The results in Figure 10 are representative of 3 independent studies using abdominal subcutaneous preadipocyte samples treated with 20 μM TSH for 1h. Human subcutaneous preadipocytes treated with TSH exhibited a 7-fold increase in phosphorylation of S6 peptide. S6 protein is an *in vivo* substrate of p70 S6K.

Figure 10. TSH activates p70 S6K in human abdominal subcutaneous preadipocytes. Confluent human abdominal subcutaneous preadipocytes were stimulated with 20μM TSH for 1h. p70 S6K was immunoprecipitated for in vitro kinase assay. The results are representative of 3 separate experiments on adipose tissue derived from 3 different donors.


S6 peptide phosphorylation
(^{32}P, cpm)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Control
TSH

b. western analysis of mobility shift

Phosphorylation of p70 S6K serves as another indicator of its activation. Immunoprecipitated p70 S6K was resolved on SDS-PAGE and subjected to western analysis. Figure 11 is an immunoblot of abdominal subcutaneous preadipocytes and orbital fibroblasts (positive control) that were grown to confluence and stimulated with 20 μM TSH for 1h. The phosphorylation and activation of p70 S6K is complex. Phosphorylation at multiple sites leads to a conformational change in the enzyme causing an upward shift in mobility as shown in TSH-stimulated abdominal subcutaneous preadipocytes and orbital fibroblasts. Therefore, based on the *in vitro* kinase assay findings and the mobility shift, p70 S6K is activated by TSH in abdominal subcutaneous preadipocytes.

Confluent Jurkat cells (TSHR-negative, see Figure 9C) were stimulated with 20 μM TSH to confirm the specificity of the TSH effect through the TSHR. Total cell lysates were resolved on SDS-PAGE and subjected to western analysis. The immunoblot in Figure 12 was probed with anti-phospho p70 S6K (pp70 S6K) from New England BioLabs. This antibody only detects p70 S6K when it is phosphorylated on thr 389. As mentioned previously, phosphorylation of thr 389 indicates activation of p70 S6K. TSH-induced activation of p70 S6K is

Figure 11. Confirmation of p70 S6K activation by gel mobility shift. Confluent human abdominal subcutaneous preadipocytes (P) and human orbital fibroblasts (OF) were treated with vehicle (Control) or 20 μM TSH (TSH) for 1h. Immunoprecipitated p70 S6K was resolved on SDS-PAGE and subjected to western analysis with anti-p70 S6K antibody.

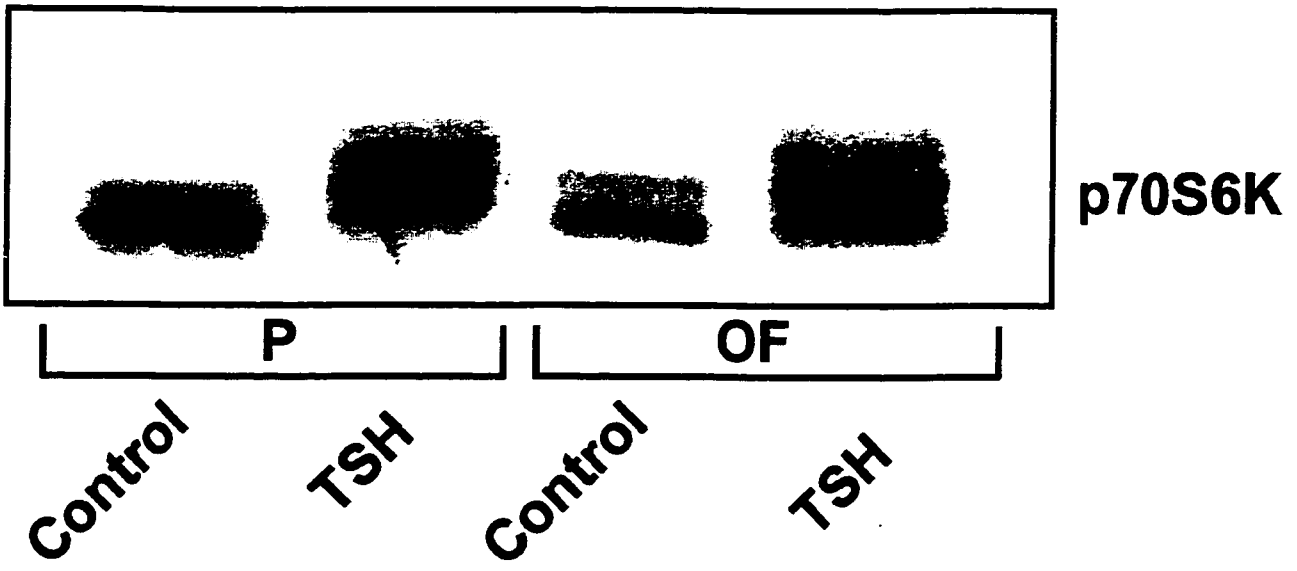
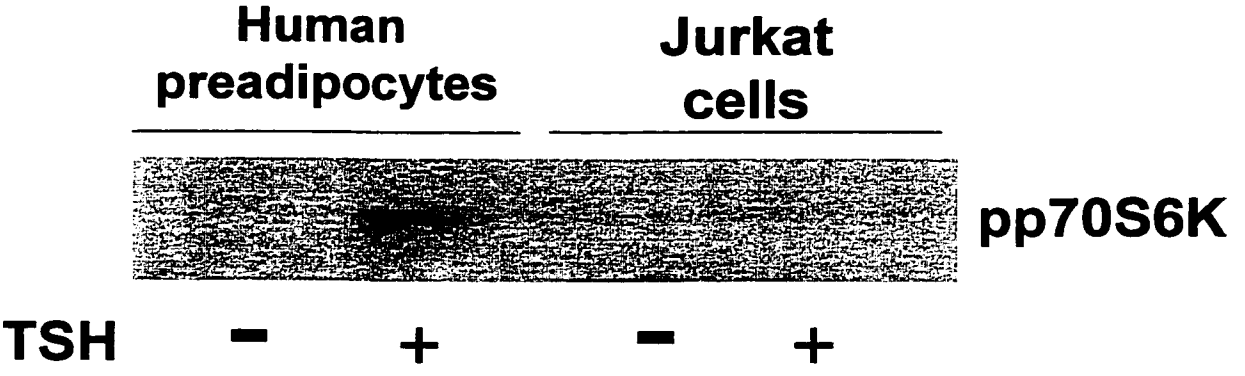

p70S6K
P
OF
Control
TSH
Control
TSH

Figure 12. TSH fails to induce activation of p70 S6K in Jurkat cells. Human abdominal subcutaneous preadipocytes and Jurkat cells were grown to confluence and stimulated with 20μM TSH for 1h. Cell lysates were run on SDS-PAGE and subjected to western analysis with anti-pp70S6K antibody.

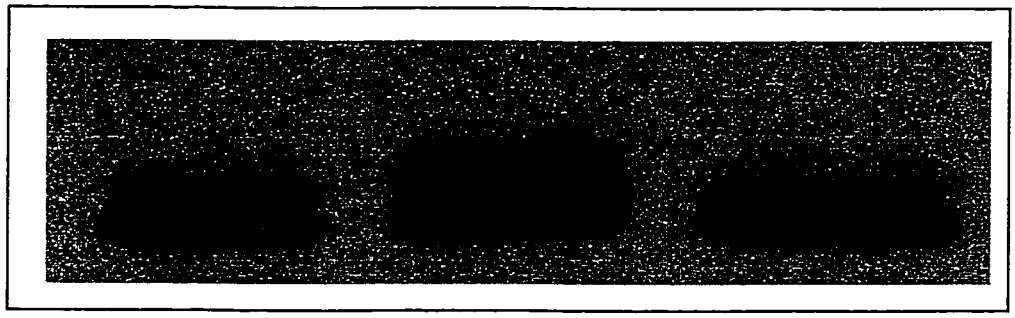

Human preadipocytes
Jurkat cells
pp70S6K
TSH
-
+
-
+

observed in TSH-stimulated human subcutaneous preadipocytes, as seen above. TSH fails to induce activation of p70 S6K in Jurkat cells, as expected.

3. Wortmannin inhibits TSH-induced activation of p70 S6K

Knowing that TSH stimulates p70 S6K activity in abdominal subcutaneous preadipocytes, we decided to look at other signaling proteins positioned upstream of p70 S6K. PI3K is known to activate p70 S6K in other cell types (Reif et al. 1997, Duan et al. 1999, Ballou et al. 2000). To determine whether PI3K is involved in TSH signaling in abdominal subcutaneous preadipocytes, we used wortmannin, a known inhibitor of PI3K (Nakanishi et al. 1995). Human abdominal subcutaneous preadipocytes were grown to confluence and treated for 1h with either vehicle labeled control or 20 μM TSH or 20 μM TSH in the presence or absence of 100 nM wortmannin as shown in Figure 13. The characteristic shifting pattern observed for activation of p70 S6K is seen again in the TSH-treated preadipocytes. The presence of wortmannin substantially blocks the TSH response. This suggests PI3K may be required for TSH to activate p70 S6K in abdominal subcutaneous preadipocytes.

Figure 13. Wortmannin inhibits activation of p70 S6K in human abdominal subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were treated for 1h with vehicle (Control), 20 μM TSH (TSH) or with 20 μM TSH in the presence of 100 nM wortmannin (TSH+W). Immunoprecipitated p70 S6K was resolved on SDS-PAGE and subjected to western analysis with anti-p70 S6K antibody. The results are representative of 2 separate experiments on adipose tissue derived from 2 different donors.



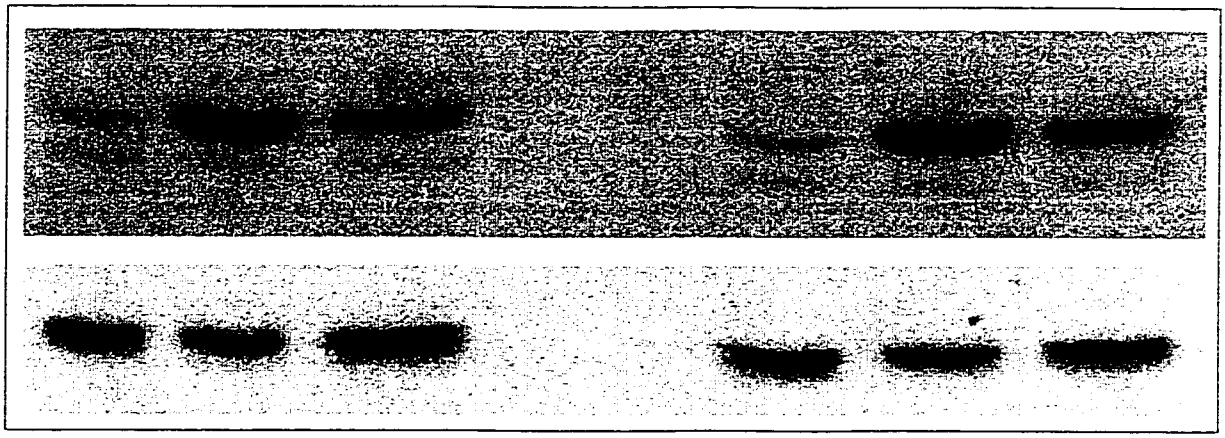
p70S6K

Control TSH TSH+W

4. Activation of PKB in human abdominal subcutaneous preadipocytes

Confluent human abdominal subcutaneous preadipocytes were treated for 15 min and 30 min with control (C), insulin (Ins) and TSH (Figure 1 4). Total cell lysate was resolved on SDS-PAGE and subjected to western analysis. The immunoblot was probed with anti-phosphoPKB (pPKB) and then stripped and reprobed with anti-PKB. The anti-PKB blot is a control for PKB mass to confirm equal protein loading of the gel. In the control lanes, levels of phosphorylated PKB are very low. Levels of phosphorylated PKB are greatly increased with 1μM Ins stimulation (positive control). Lysates from 20 μM TSH-stimulated preadipocytes also show increased levels of phosphorylated PKB over control.

Figure 14. TSH activates PKB in human abdominal subcutaneous preadipocytes. Confluent human subcutaneous preadipocytes were treated for 15 min and 30 min with Control (C), 1 μM Insulin (Ins), or 20 μM TSH (TSH). Total cell lysate (100 μg) was resolved on SDS-PAGE and subjected to western analysis. The immunoblot was probed with anti-phospho PKB (pPKB) and then stripped and reprobed with anti-PKB (PKB). Results at 15 min are from 1 experiment. Results at 30 min are representative of 2 separate experiments.


pPKB
PKB
C
Ins 1uM
TSH 20uM
C
Ins 1uM
TSH 20uM
15′
30′

C. DISCUSSION

We have demonstrated for the first time that TSHR protein is expressed in human abdominal subcutaneous preadipocytes. Using *in vitro* kinase assays and gel mobility shifts of p70 S6K, we have shown that the TSHR is functional in these preadipocytes. Concurrent addition of wortmannin during the TSH stimulation abrogated the activation of p70 S6K in human abdominal subcutaneous preadipocytes, suggesting PI3K involvement. Consistent with this, we have shown that PKB, a known downstream target of PI3K, is activated when human subcutaneous preadipocytes are stimulated with TSH.

Originally, detection of TSHR in adipose tissue was limited to mRNA expression (Roselli-Rehfuss et al. 1992, Endo et al. 1993, Feliciello et al. 1993). Results of two studies using northern analysis suggest that TSHR mRNA exists in human infant and adult adipocytes (Crisp et al. 1997, Janson et al. 1998). We examined the expression of TSHR protein in the human preadipocyte using western analysis. The pattern of TSHR bands recognized by TSHR antibodies is complicated. There are many large molecular weight proteins and low molecular weight subunits (Ban et al. 1992). Post-translational processing of the receptor contributes to the complexity. Work by Kohn's group suggests that bands in the 100 kDa region represent the mature, processed, functional form of the receptor (Akamizu et al. 1990b, Ban et al. 1992). Using the antibody given to us by Dr.

Kohn, we were able to detect the 100 kDa band faintly in human abdominal subcutaneous preadipocytes, *in vitro* differentiated adipocytes, KAT 50 thyroid epithelial cells and human orbital fibroblasts. Using a second antibody, the NCL-TSH-R2, the 100 kDa band detection was even stronger. As expected, we observed TSHR protein in the KAT 50 thyroid epithelial cells and orbital fibroblasts which are known to express TSHR (Venkataraman et al. 1998, Bahn et al. 1998). Our western analysis data with these anti-TSHR antibodies appears to be specific, since TSHR-negative cells did not display the 100 kDa band.

To determine whether TSHR is functional in the preadipocyte, we tested the activation of a novel TSHR target described recently in a thyrocyte cell line (Cass and Meinkoth 1998). Acute TSH stimulation of abdominal subcutaneous preadipocytes led to the phosphorylation and activation of p70 S6K. We have shown that wortmannin, an inhibitor of PI3K, prevents the TSH-mediated activation of p70 S6K. These findings indicate PI3K may be important for TSH signaling in the preadipocyte. Since PKB is a downstream target of PI3K, we also investigated whether PKB is activated upon acute TSH stimulation (Burgering and Coffer 1995). Consistent with the wortmannin data, we observed that TSH stimulates PKB.

Further confirmation of the involvement of PI3K in preadipocyte TSH signaling will be required. Another pharmacological inhibitor of PI3K, LY294002 could be used, as well as expression of dominant-negative PI3K mutants

(Burgering and Coffer 1995). In addition, the direct measurement of cellular 3-phosphorylated phosphoinositides following TSH stimulation will confirm PI3K activation.

Future work should be aimed at the delineation of molecular targets located immediately downstream of the TSHR in these cells. A potential model for TSH signaling in the preadipocyte is discussed in the Conclusion (see Figure 15). Further studies are also needed to elucidate the cellular response of preadipocytes to TSH. With p70 S6K implicated in control of transcription and translation, future studies should be aimed at delineating which genes are regulated by TSH in these cells. Previous studies by Crisp et al. (1997) and Janson et al. (1998) demonstrate TSHR mRNA expression in the adipocyte where it may regulate lipolysis. TSHR in the preadipocyte must serve an alternative role, since lipids are not housed in preadipocytes. A recent study by Haraguchi et al. (1999) suggests that TSH causes proliferation and inhibits differentiation in rat preadipocytes.

The clinical relevance of TSH effects on human preadipocytes is currently unknown. In hypothyroid patients, low levels of the thyroid hormone thyroxine (T_4) result in increased levels of TSH due to the lack of negative feedback on the hypothalamus. Weight gain associated with hypothyroidism is attributed to the low metabolic rate due to low thyroid hormone levels. However, elevations in

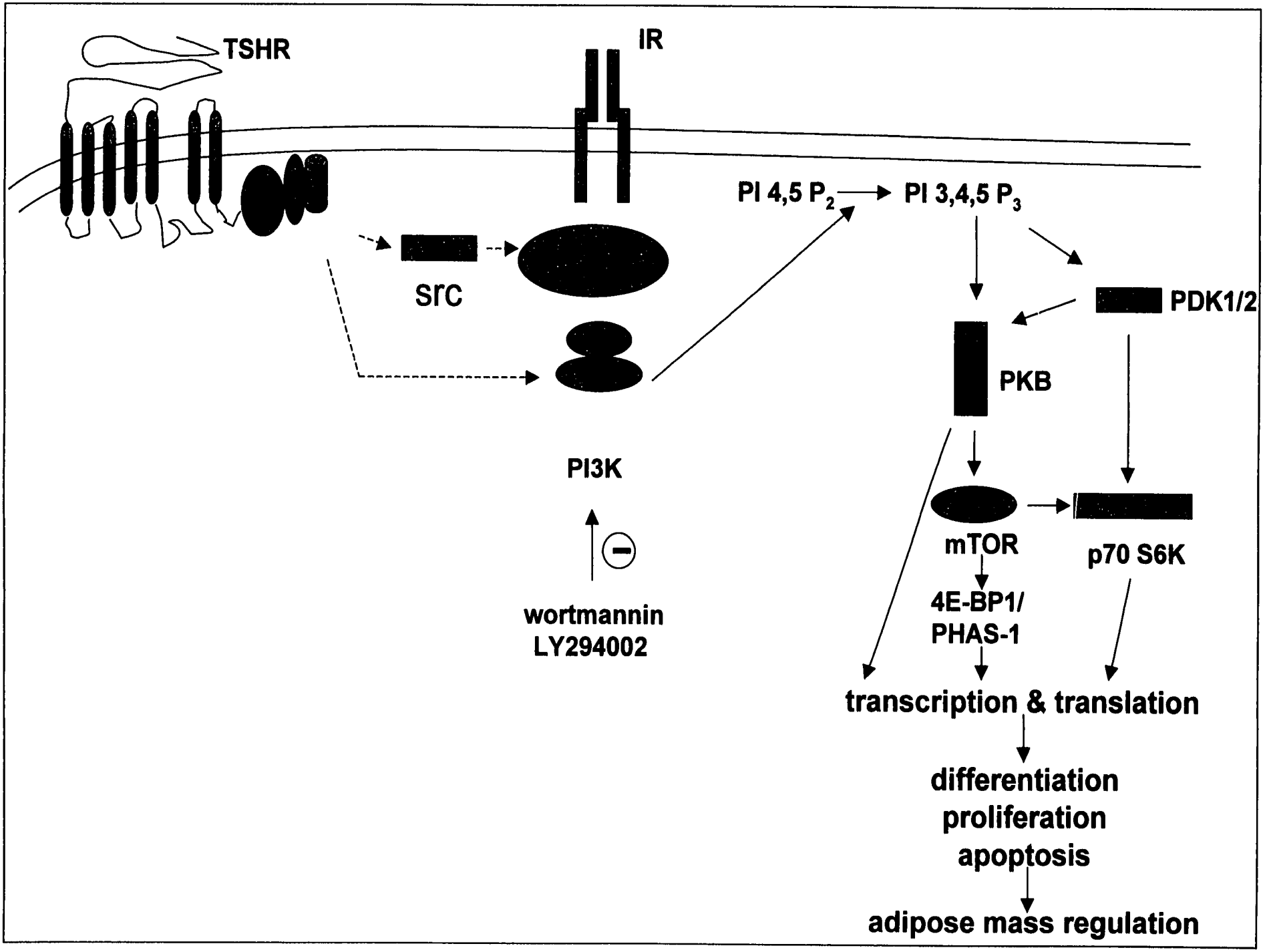
TSH may also act directly on preadipocytes to promote adipose tissue accumulation.

IV. CONCLUSION

The complexities of the insulin and TSH signal transduction networks in human preadipocytes have yet to be unraveled. In Figure 15, we have a potential model for TSH signaling in the preadipocyte that suggests insulin and TSH signaling may converge at the level of PI3K. Insulin signaling in the preadipocyte begins with insulin binding to the α subunits of the IR that leads to a conformational change and autophosphorylation of the β subunits. IRS-1 is recruited to the membrane, followed by the docking of PI3K on IRS-1.

TSH signaling in the human preadipocyte has not been previously characterized. The TSHR in thyroid cells is coupled to Gs proteins (Ludgate et al. 1999). Identification of the G protein that couples TSHR to PI3K will be of interest. G protein-coupled receptors have recently been recognized as regulators of cell growth and differentiation (Dhanasekaran et al. 1995). The classical G-protein effectors include phospholipase C (PLC) and adenylyl cyclase (van Corven et al. 1993). There are a number of reports on the effects of elevated cAMP on adipocyte differentiation. In 3T3-L1 and Ob1771 cells, a rise in cAMP levels appears to be essential for differentiation (Ailhaud et al. 1992, MacDougald and Lane 1995, Gregoire et al. 1998). In contrast, cAMP prevents

Figure 15. Potential model for insulin and TSH signaling in the human preadipocyte. The insulin receptor tyrosine kinase signaling pathway is described in Figure 3. In the TSH signaling pathway, TSH binds to the TSHR, a G protein-coupled receptor. Insulin and TSH signaling appear to converge at the level of PI3K. Steps linking TSHR to PI3K remain to be identified (see text).


TSHR
IR
SrC
PI 4,5 P2
PI 3,4,5 P3
PDK1/2
PKB
PI3K
mTOR
p70 S6K
4E-BP1/
PHAS-1
wortmannin
LY294002
transcription & translation
differentiation
proliferation
apoptosis
adipose mass regulation

the differentiation of porcine preadipocytes and 3T3-F442A cells (Spiegelman and Green 1981, Boone et al. 1999). Future work will be necessary to determine whether TSH stimulates an increase in cAMP levels in human preadipocytes and, if so, whether elevated cAMP levels are connected to PI3K activation. Cass et al. (1998) suggest that p70 S6K is involved in cAMP-mediated proliferation of Swiss3T3 fibroblasts, secondary rat Schwann cells and Wistar rat thyroid cells.

Our work suggests that the TSHR in human preadipocytes signals through PI3K. Two possible mechanisms for G protein-mediated activation of PI3K involve direct and indirect activation of PI3K. The Gastrin/CCK_B receptor is a GPCR that signals indirectly through PI3K. Here, src-family tyrosine kinases phosphorylate and activate IRS-1 followed by docking of PI3K and subsequent activation (Daulhac et al. 1999). The mechanism by which $G\beta\gamma$ activates src is still unclear. Murga et al. (2000) suggest that $G\beta\gamma$ subunits directly activate the PI3K p110 subunit without the need for p85 subunit activity. Signals from the IR and the TSHR appear to signal through the pivotal enzyme PI3K. These pathways may overlap and act in concert to produce an enhanced response or signal crosstalk may cause a dampening affect on either signal. In the state of positive energy balance, combined effects of insulin and TSH may alter overall adipose tissue mass through the processes of differentiation, proliferation and apoptosis in human preadipocytes. Future work is necessary to precisely define components of these molecular pathways.

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VI. APPENDIX A

List of Abbreviations

aa	Amino acid
ADD1	Adipocyte determination and differentiation factor 1
AGC	CyclicAMP-dependent/cyclicGMP-dependent/protein kinase C
aP2	Adipocyte P2
Ara-C	Cytosine ß-D-arabinofuranoside
ATP	Adenosine triphosphate
B	Biotin
BMI	Body mass index
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
C/EBP	CCAAT/enhancer binding protein
$cPGI_2$	Carbaprostacyclin
CREB	CyclicAMP responsive element-binding protein
CREMτ	CyclicAMP responsive element-modulatorτ
CS	Calf serum
CSF-1	Colony stimulating factor-1
DAG	Diacylglycerol
Dex	Dexamethasone
DHAP	Dihydroxyactetone phosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
4E-BP1	eIF4E-binding protein
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic initiation factor 4E
FBS	Fetal bovine serum
FKBP12	FK506 binding protein 12
FRAP	FKBP12-rapamycin-associated protein
F12	Ham's F12
G1 phase	Gap 1 phase
GLUT-4	Glucose transporter-4
GPCR	G protein-coupled receptor
GPDH	Glycerol-3-phosphate-dehydrogenase

Grb-2	Growth factor receptor bound protein
GTP	Guanosine triphosphate
HA	Hemagglutinin
h	hour(s)
I	Insulin
IBMX	Isobutylmethylxanthine
IGF-1	Insulin-like growth factor
IGF-1R	Insulin-like growth factor receptor
IP_3	Inositol trisphosphate
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
KCl	Potassium chloride
KRH	Krebs Ringer Hepes
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
2-Me	2-mercaptoethanol
MEK	Mitogen-activated protein kinase kinase
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sufate
min	minute(s)
MOPS	3-[N-morpholino] propanesulfonic acid
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide phosphate
P	Pantothenate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1/2	Phosphoinositide-dependent kinase 1/2
PEPCK	Phosphenolpyruvate carboxykinase
PIP_2	Phosphatidylinositol-bisphosphate
PLC	Phospholipase C
PH	Pleckstrin homology
PHAS-1	Phosphorylated heat- and acid-stable-1
PI	Phosphatidylinositol
PI 3,4 P2	Phosphatidylinositol 3,4 bisphosphate
PI 3,4,5 P3	Phosphatidylinositol 3,4,5 trisphosphate
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLCβ	Phospholipase Cβ

PPAR	Peroxisome proliferator-activated receptor
Pref-1	Preadipocyte factor 1
PP2A	Protein phosphatase 2A
p70 S6K	p70 S6 kinase
PSN	Penicillan streptomycin nystatin
PTB	Phosphotyrosine binding domain
RAFT	Rapamycin and FKBP-target 1
Rb	Retinoblastoma
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
ser	Serine
ser/thr	Serine-threonine
SH_2	src-homology 2
SH_3	src-homology 3
SOS	Son-of-sevenless
S phase	Synthesis phase
T	Transferrin
T_3	Triiodothyronine
TG	Triacylglycerol
5'TOP	5' terminal oligopyrimidine tract
5'UTRs	5' untranslated regions
TSH	Thyroid-stimulating hormone
TSHR	Thyroid-stimulating hormone receptor
tyr	Tyrosine

VII. APPENDIX B

Curriculum Vitae

NAME: Andrea Dorothy Lee Bell

DATE OF BIRTH: June 25, 1975

PLACE OF BIRTH: Chatham, New Brunswick

CITIZENSHIP: Canadian

EDUCATION:
Cornell University
Ithaca, New York
Bachelor of Arts
Major: Biology
Concentration: Physiology
1993-1997

PUBLICATIONS:

1. Bell A., Grunder L., and A. Sorisky. Rapamycin Inhibits Human Adipocyte Differentiation in Primary Culture. Obesity Research. (in press)

2. *Bell A., *Gagnon AM., Grunder L., Parikh S.J., Smith T.J., and A. Sorisky. Expression of Functional TSH Receptor Protein in Human Abdominal Preadipocytes and Orbital Fibroblasts in Primary Culture. American Journal of Physiology:Cell Physiology. (in press)

*co-first authors

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1. Bell A., Grunder L., and A. Sorisky. Rapamycin Inhibits Human Adipocyte Differentiation. Canadian Diabetes Association Professional Conference and Annual Meetings in Calgary, Alberta. October 14-17, 1998. (Oral Presentation)

2. Sorisky A., Bell A., Grunder L., Gagnon A.M., Parikh S.J., and T.J. Smith. Functional TSH Receptor Expression in Cultured Human Preadipocytes. 72nd Annual Meeting of the American Thyroid Association in The Breakers, Palm Beach, Florida. September 29-October 3rd, 1999 (Oral Presentation: Dr. Sorisky).

3. Bell A., Grunder L., and A. Sorisky. Functional TSH Receptor Expression in Human Subcutaneous Preadipocytes. Canadian Diabetes Association Professional Conference and Annual Meetings in Ottawa, Ontario. October 13-16, 1999. (Poster Presentation)

TEACHING:

Teaching Assistant, 1999
Department of Biochemistry, BCH 2336
University of Ottawa

VIII. APPENDIX C

Contributions of Collaborators

Thanks to Laura Grunder and Dheerja Pardasani for technical assistance in experiments on human adipocyte differentiation. Thanks to Laura Grunder and AnneMarie Gagnon for their contribution in the TSHR expression experiments.