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TSH Signaling and Cellular Responses in Preadipocytes and Adipocytes

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TSH SIGNALING AND CELLULAR RESPONSES IN PREADIPOCYTES AND ADIPOCYTES

by

Andrea Bell

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

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ABSTRACT

Thyroid stimulating hormone (TSH) action in adipose tissue remains largely unknown. We demonstrate that TSH activates protein kinase B (PKB/Akt) and p70 S6 kinase (p70 S6K) in a phosphoinositide 3-kinase (PI3K)-dependent manner in 3T3-L1 preadipocytes. TSH had no effect on cAMP levels, suggesting adenylyl cyclase is not involved in TSH activation of the PI3K-PKB/Akt-p70 S6K pathway. 3T3-L1 preadipocyte cell death was reduced by 29 to 76%, in serum-deprived (6 h) preadipocytes treated with 1-20 μM TSH, respectively. In the presence of 20 μM TSH, an 88% reduction in TUNEL-positive cells was observed in serum-starved (3 h) 3T3-L1 preadipocytes, as well as a 93% reduction in the level of cleaved activated caspase 3. TSH acts as a survival factor for serum-deprived preadipocytes, reducing TUNEL-positive cells and caspase 3 activation. A role for TSH may exist in adipose tissue development and remodeling.

Interleukin-6 (IL-6), a pro-atherogenic cytokine, is expressed and secreted by adipocytes, but little is known about its regulation. Since an elevated TSH serum level is a cardiovascular disease (CVD) risk factor, and since thyroid stimulating hormone receptor (TSHR) is expressed in adipocytes, we investigated whether TSH modulates IL-6 secretion in cultured 3T3-L1 and 3T3-F442A mouse adipocytes, and in primary cultures of human abdominal adipocytes differentiated in culture. TSH increased the

secretion of IL-6 by 5-fold in 3T3-F442A adipocytes, by 2-fold in 3T3-L1 adipocytes, and by 3.5-fold in human differentiated adipocytes. TSH is a novel regulator of adipocyte IL-6 secretion, providing a potential mechanism for epidemiological observations identifying an elevated serum TSH level as a CVD risk factor.

DEDICATION

For Jason

My husband, my love, my best friend

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List of Abbreviations

AGC	CyclicAMP-dependent/cyclicGMP-dependent/protein kinase C
aP2	Adipocyte P2
ATP	Adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	3', 5'-cyclic adenosine monophosphate
caspases	Cysteine-dependent aspartate-directed proteases
cDNA	Complementary DNA
CCK-B	Cholecystokinin-B
C/EBP	CCAAT/enhancer-binding protein
CHO	Chinese hamster ovary
$cPGI_2$	Carbaprostacyclin
CREB	CyclicAMP responsive element-binding protein
CRP	C-reactive protein
CS	Calf serum
CVD	Cardiovascular disease
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
Epac	Exchange protein directly activated by cAMP
FBS	Fetal bovine serum
FOXO3a	Forkhead box class O 3a (also known as FKHRL1)
FRTL-5	Fischer rat thyroid line-5
F12	Ham's F12
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GLUT-4	Glucose transporter-4
GPCR	G-protein-coupled receptor
GPDH	Glycerol-3-phosphate-dehydrogenase
GTP	Guanosine triphosphate
HDL	High density lipoprotein
h	Hour(s)
IBMX	Isobutylmethylxanthine
IGF-1	Insulin-like growth factor

IKK	Inhibitor κB kinase
IL-6	Interleukin-6
intra-abdominal	Visceral
IRS-1/2	Insulin receptor substrate-1/2
JAK1	Janus kinase
JNK	c-Jun N-terminal kinases
LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
$MgCl_2$	Magnesium chloride
min	Minute(s)
MOPS	3-[N-morpholino] propanesulfonic acid
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaF	Sodium fluoride
NaPPi	Sodium pyrophosphate
Na_3VO_4	Sodium orthovanadate
NBT/BCIP	Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate
NFκB	Nuclear factor κB
N	Nystatin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1/2	Phosphoinositide-dependent kinase 1/2
PEPCK	Phosphenolpyruvate carboxykinase
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI 4 P	Phosphatidylinositol 4 phosphate
PI 3,4 P_2	Phosphatidylinositol 3,4 bisphosphate
PI 4,5 P_2	Phosphatidylinositol 4,5 bisphosphate
PI 3,4,5 P_3	Phosphatidylinositol 3,4,5 trisphosphate
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB/Akt	Protein kinase B
p(PKB/Akt)	Phospho-PKB/phospho-Akt
PKCθ	Protein kinase Cθ
PKI	Protein kinase inhibitor
PLCβ	Phospholipase Cβ
PPAR	Peroxisome proliferator-activated receptor
PS	Penicillin/streptomycin
PYK2	Proline-rich tyrosine kinase 2

p70 S6K	p70 S6 kinase
rhTSH	Recombinant human TSH
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS	Sodium dodecyl sulfate
ser	Serine
ser/thr	Serine-threonine
SH2	Src-homology 2
SH3	Src-homology 3
Shc	Src homology 2/a-collagen related protein
STAT	Signal transducer and activator or transcription
T_3	Triiodothyronine
T_4	Thyroxine
TMB	3, 3', 5, 5' tetramethylbenzidine
TNFα	Tumor necrosis factor α
TSH	Thyroid stimulating hormone
TSH-NIH	Highly purified bovine TSH
TSHR	Thyroid stimulating hormone receptor
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
tyr	Tyrosine
TZD	Thiazolidinediones
UTRs	5' untranslated regions
VLDL	Very low density lipoprotein
WHO	World Health Organization

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I. INTRODUCTION

A. Obesity

In 2003, the World Health Organization (WHO) reported that over 1 billion adults are overweight, including 300 million that fulfill the definition of obesity. Obesity rates have been increasing steadily since the 1960's. There has also been an alarming rise in the number of people with type 2 diabetes, one of the metabolic consequences of obesity.

The WHO classifies overweight and obesity using body mass index (BMI), which is defined as the weight of an individual divided by height squared (kg/m^2). A BMI ranging from 25-29.9 signifies overweight and a BMI above 30 defines obesity. BMI measurements are easily obtained but they do not represent an ideal assessment of one's fat mass as there is no means to differentiate fat mass from lean mass, or the anatomic deposition of this adipose mass. Waist circumference complements BMI and gives an indication of the degree of visceral (intra-abdominal) obesity.

The increase in weight observed over the past 40 years can be attributed to a general decline in physical activity and an increase in caloric consumption, but there are also strong indications that a number of individuals are genetically predisposed to overweight and obesity (O'Rahilly et al., 2003; Perusse and Bouchard, 1999). Numerous studies have been aimed at delineating the genetic components responsible for the obesity phenotype. Leptin and the leptin receptor held great promise as candidates for the long-

sought-after obesity gene (Tartaglia et al., 1995; Zhang et al., 1994). Leptin has been shown to act as a satiety factor and play a role in energy expenditure. Mice with defective genes encoding leptin or leptin receptor were substantially obese compared to their wild-type littermates. Mutations in the leptin gene are responsible for causing obesity in humans but they are actually quite rare (O'Rahilly et al., 2003). So the search continues for the gene(s) that predispose to obesity. In fact, it appears more likely that multiple genes control the more common form(s) of obesity, and the mechanisms by which they interact will need to be uncovered (Barsh et al., 2000; Flier, 2004).

1. Adipose Tissue Regional Differences

Traditionally, obesity was categorized by gynoid or pear-shaped lower-body obesity versus android or apple-shaped upper-body obesity; in the latter, adipose tissue accumulation is concentrated in the intra-abdominal region (Lafontan and Berlan, 2003; Langeron, 1952). Abdominal adipose tissue deposits can be further subdivided into subcutaneous and omental adipose tissue. Numerous studies indicate that signaling differences exist between subcutaneous and omental adipose tissue depots. For instance, omental adipose tissue lipolysis rates are higher than those observed in subcutaneous adipose tissue in both obese and non-obese subjects (Jensen, 1997; Ostman et al., 1979). Triacylglycerol synthesis on the contrary, is augmented in subcutaneous adipose tissue (Maslowska et al., 1993). Additionally, subcutaneous adipose tissue is known to express and secrete larger quantities of leptin as compared to omental adipose tissue

(Montague et al., 1997; Van Harmelen et al., 1998). Other depot differences include variations in insulin-regulated triglyceride metabolism (Arner, 1995; Wu et al., 2001). For example, the antilipolytic effects of insulin are stronger in subcutaneous adipose tissue compared to omental adipocytes (Bolinder et al., 1983; Montague and O'Rahilly, 2000; Richelsen et al., 1991). Studies continue to be aimed at unraveling the differences between these two sites to give us insight into the particular contribution of each anatomical adipose tissue site towards metabolic complications induced by obesity.

2. Pathophysiology Underlying the Link Between Obesity and Insulin Resistance

Intra-abdominal obesity increases the risk of cardiovascular disease (CVD), type 2 diabetes, dyslipidemia and hypertension (Kopelman, 2000; Macdonald et al., 1997). Overweight and obesity are underlying causes of the metabolic syndrome that has been described as a clustering of hypertension, dyslipidemia, low levels of high density lipoprotein (HDL), and insulin resistance (Grundy et al., 2004; Wilson and Grundy, 2003a; Wilson and Grundy, 2003b). As many as 20-25% of adult Americans are estimated to have this syndrome and its prevalence continues to rise.

Intra-abdominal obesity plays a significant role in the risk of vascular and metabolic health complications, though the precise mechanisms involved are still under investigation. One hypothesis linking obesity to CVD suggests free fatty acids released

from intra-abdominal adipose tissue reach the liver via the portal vein, creating an atherogenic lipid profile, including elevated levels of very low density lipoprotein (VLDL), low levels of HDL, and elevated levels of small, dense low density lipoprotein (LDL) (Howard, 1992).

More recently, the inability to store fat in adipose tissue has led to a related model of metabolic complications. Inappropriate fat deposition in muscle and liver are also detrimental to insulin signal transduction, as demonstrated by studies of genetically engineered fatless mice (Kim et al., 2000). Impaired insulin signaling in muscle and liver of these mice appears to be caused by a significant decrease in insulin receptor substrate (IRS)-1 and 2-associated phosphoinositide 3-kinase (PI3K; described below) activity. A 2-fold increase in muscle and liver triglyceride levels was also observed in the fatless mice. Restoration of adipose tissue in these mice returned muscle and liver triglyceride levels and insulin signaling and action to normal levels. The authors concluded that partitioning of fat between the adipocyte and liver/muscle can lead to insulin resistance and development of diabetes. These studies demonstrate a significant role for adipose tissue in the maintenance of insulin sensitivity.

How muscle and liver regulate IRS-1/2 signal transduction is incompletely known. Several groups have implicated serine phosphorylation of IRS-1 in the inhibition of insulin signaling (Zick, 2003). One mechanism implicates the serine kinase protein kinase C (PKC)θ in IRS-1 phosphorylation. Acutely elevated plasma free fatty acids in rats have led to an increase in PKCθ serine phosphorylation that is associated with an

inhibition of insulin signaling (Griffin et al., 1999). Free fatty acids also appear to play a role in skeletal muscle insulin resistance through phosphorylation of Ser 307, which appears critical for insulin signal transduction (Yu et al., 2002). Inhibitor κB kinase (IKK) is another serine kinase that has been recently implicated in insulin resistance and has been suggested to regulate IRS-1 phosphorylation (Gao et al., 2002; Yuan et al., 2001). An additional mechanism of IRS-1/2 activity implicates c-Jun N-terminal kinases (JNK) in phosphorylation of Ser 307 (Hotamisligil, 2003). Note that not all ser phosphorylation sites are inhibitory; some may be important in insulin signal transduction (Paz et al., 1999).

3. Adipocytokines

Adipose tissue is no longer referred to solely as a storage tissue, as it was once believed to be. Adipose tissue is now being described as an endocrine organ given the numerous hormones and cytokines, including leptin, tumor necrosis factor α (TNFα), adiponectin, resistin, and interleukin (IL-6), that are secreted by adipocytes. Cytokines secreted from adipose tissue are referred to as adipocytokines. The role of adipocytokines in glucose and lipid metabolism, and adipose tissue development is currently being explored by many laboratories. Recently, dysregulated secretion of particular adipocytokines in obese individuals has been implicated in insulin resistance and atherosclerosis.

TNFα is an example of an adipocytokine that regulates insulin sensitivity in rodent models (Hotamisligil et al., 1993). Insulin signaling and action is also inhibited by TNFα in human adipocytes (Liu et al., 1998). One mechanism of TNFα action involves the inhibition of insulin signaling and downstream signaling targets including PI3K (Hotamisligil et al., 1996). TNFα has also been shown to reduce the expression of IRS-1 and the insulin-sensitive glucose transporter-4 (GLUT-4) (Stephens et al., 1997). TNFα messenger ribonucleic acid (mRNA) is found in adipocytes but adipose tissue releases only small amounts of TNFα protein *in vitro* in human cells and *in vivo* in humans suggesting a paracrine effect (Crawford et al., 1997; Hotamisligil et al., 1993; Mohamed-Ali et al., 1997). TNFα expression in subcutaneous adipose tissue is higher than expression in omental adipose tissue (Hube et al., 1999). Macrophages have been shown to infiltrate adipose tissue (discussed below) and may also be a source of TNFα (Weisberg et al., 2003; Xu et al., 2003). TNFα is downregulated by the clinically used insulin-sensitizing agents, thiazolidinediones (TZD) (Hube et al., 1999).

Adiponectin is an insulin sensitizing, anti-atherogenic and anti-inflammatory protein (Berg et al., 2002; Hu et al., 1996a; Scherer et al., 1995). It is the only secreted protein known thus far to be downregulated in obesity (Arita et al., 1999). Adiponectin appears to enhance insulin sensitivity, at least in part, through activation of adenosine monophosphate kinase and subsequent inhibition of acetyl coenzyme A carboxylase (Tomas et al., 2002; Yamauchi et al., 2001). The end result is a decrease in hyperglycemia and increased muscle fatty acid oxidation. Adiponectin is upregulated by

TZD, and decreased by β-adrenoreceptor agonists, glucocorticoids, TNFα, and IL-6 (Fasshauer and Paschke, 2003). Resistin is another recently described adipocytokine. Originally, it was thought to provide the link between obesity and diabetes (Steppan et al., 2001a; Steppan et al., 2001b). The role of resistin in insulin sensitivity appears to be very complex. A number of contradictory results have been published with regard to its action. Low levels of resistin mRNA and protein have been detected in human adipocytes (Janke et al., 2002; Nagaev and Smith, 2001). One study suggests resistin is involved in modulating hyperglycemia in obese individuals (Banerjee et al., 2004). A very recent report demonstrates a strong correlation between human abdominal subcutaneous adipose tissue and serum resistin levels and resistin mRNA levels (Heilbronn et al., 2004). Resistin is downregulated by TZD (Steppan et al., 2001a).

In summary, a lot of exploration is required to further understand the role of adipocytokines in the regulation of adipose tissue metabolism. In this thesis, we examine the expression of one such pro-atherogenic cytokine, IL-6, described below.

B. Adipose Tissue Development

In a state of positive energy balance, energy input (ie. caloric consumption) is greater than energy output (ie. resting metabolic rate and exercise). To store excess energy, adipocytes may increase in size and number (Prins and O'Rahilly, 1997). Adipocytes enlarge through increased lipid storage until the cells reach a finite capacity. At this stage preadipocytes, fibroblast-like cells of mesodermal origin located within the

stromal aspect of adipose tissue, can be induced to differentiate when appropriately cued (Deslex et al., 1987; Hauner et al., 1987). Preadipocytes are differentiated into adipocytes to accommodate the positive caloric load. Adipogenesis is believed to be induced by as yet unidentified adipocyte-derived mitogenic and adipogenic signals, paracrine factors or nutritional factors (Faust et al., 1978; MacDougald and Mandrup, 2002; Marques et al., 1998).

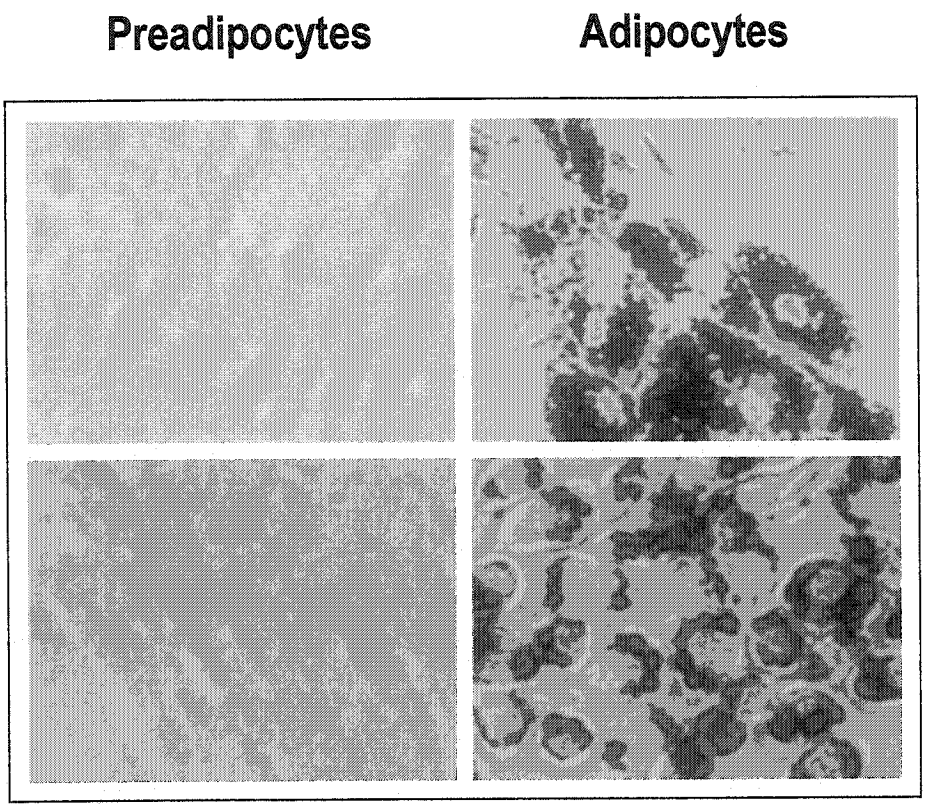
1. Preadipocyte and Adipocyte Cell Models

The 3T3-L1 cell line is a well-established model of adipogenesis that can be induced to differentiate into mature, lipid-laden adipocytes (Rosen and Spiegelman, 2000). 3T3-L1 preadipocytes and differentiated adipocytes in culture are depicted in Figure 1. 3T3-L1 preadipocytes were originally derived from disaggregated Swiss 3T3 day 17-19 mouse embryos (Green and Kehinde, 1976; Green and Meuth, 1974; Ross et al., 2000). The 3T3-F442A cell line was obtained through duplicate subcultivation of a 3T3 clone with low susceptibility to adipogenesis, and eventual selection for a subclone with maximal adipogenic susceptibility (Green and Kehinde, 1976). Subcutaneous injection of 3T3-F442A preadipocytes into Balb-C athymic mice leads to regular fat pad development, suggesting similarities between signal transduction pathways in the cell line and *in vivo* differentiated adipocytes (Green and Kehinde, 1979; Mandrup et al., 1997; Ross et al., 2000). Furthermore, 3T3-L1 preadipocytes undergo rapid proliferation and extensive differentiation, and have the potential to differentiate indefinitely.

Nevertheless, aneuploid immortalized murine cell lines do differ from human preadipocytes in primary culture (Papineau et al., 2003; Ryden et al., 2002; Soukas et al., 2001; Urs et al., 2004). 3T3-L1 murine preadipocytes are embryonal in origin and they must enter a round of mitotic divisions termed clonal expansion for successful differentiation. Human preadipocytes are largely isolated from adults, they are dipoid and do not undergo clonal expansion in culture (Entenmann and Hauner, 1996; Green and Meuth, 1974; Zhang et al., 2004).

Human preadipocytes can be placed in primary culture after isolation from adipose tissue via the most widely used technique involving collagenase digestion and centrifugation (Hauner et al., 2001). Figure 1 depicts human preadipocytes and differentiated adipocytes in culture. Study of human preadipocytes and differentiated adipocytes allows us to gain valuable insight into their mechanisms of action. However, they are not used routinely for the following reasons. The preadipocyte isolation process is labor intensive and tedious, preadipocyte yield is limiting for experiments and variable responses may be observed due to donor heterogeneity. Human preadipocyte growth and differentiation processes are also much slower in comparison to murine preadipocyte cell lines. Currently, human preadipocytes have very limited differentiation capacity once they have proliferated in culture and after they have been subpassaged (Entenmann and Hauner, 1996; Wabitsch et al., 2001). Immortalization of human preadipocytes to overcome many of the disadvantages detailed above is being attempted (Darimont and Mace, 2003). In the meantime, one useful strategy to study adipogenesis is to use

Figure 1. A photomicrograph of 3T3-L1 and human preadipocytes and differentiated adipocytes in culture. Cells were stained with Oil Red O that recognizes neutral lipid.



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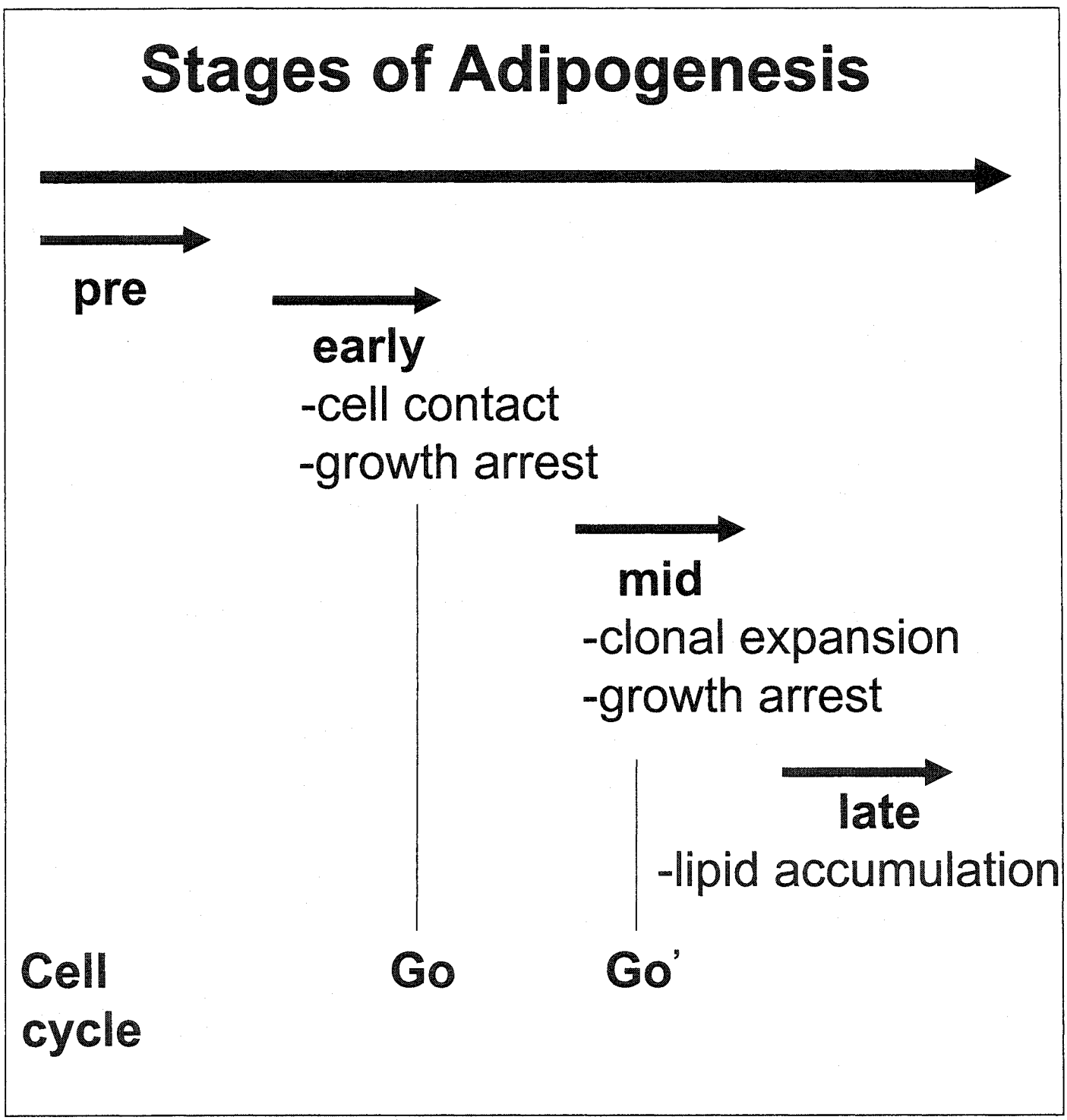
preadipocyte murine cell lines, with supplementary studies on human preadipocytes in primary culture.

2. Adipogenesis

Adipogenesis in cell culture models can be induced upon the addition of insulin, glucocorticoids such as dexamethasone, and 3', 5'-cyclic adenosine monophosphate (cAMP) elevating agents such as isobutylmethylxanthine (IBMX) (Hauner, 1990; Hauner et al., 1989; Hauner et al., 1987; Smith et al., 1988). Hundreds of genes that encode proteins with key functions in the adipocyte are upregulated or suppressed upon differentiation. Figure 2 depicts a model of 3T3-L1 adipogenesis. When preadipocytes are grown to confluence, they reach a growth arrest phase that primes the cells for differentiation (Smas and Sul, 1995). After exposure to the above described adipogenic agents, 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; Patel and Lane, 2000; Tang et al., 2003a; Tang et al., 2003b; Zhang et al., 2004).

In early adipogenesis two major transcription factor families: CCAAT/enhancer-binding protein (C/EBP) and peroxisome proliferators-activated receptor (PPAR) are upregulated. There are 3 isoforms of C/EBP (α, β, δ) and 3 isoforms of PPAR (α, γ, δ). Initially, C/EBPβ and C/EBPδ are expressed after addition of the adipogenic hormone cocktail, and cells begin to enter mitotic clonal expansion. Following clonal expansion,

Figure 2. Model of 3T3-L1 adipogenesis. Preadipocytes are grown to confluence and reach growth arrest. They undergo 1-2 rounds of mitotic divisions termed clonal expansion, followed by a second growth arrest phase. Cells accumulate lipid at the end stage of differentiation.


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

C/EBPβ and C/EBPδ gain deoxyribonucleic acid (DNA)-binding activity and upregulate C/EBPα and PPARγ genes by binding to the C/EBP binding elements located in their promoters (Christy et al., 1991; Clarke et al., 1997; Lane et al., 1999). C/EBPα and PPARγ can induce each other's expression in a positive feedback loop (Hamm et al., 1999; Shao and Lazar, 1997). C/EBPα expression terminates clonal expansion and a concurrent reduction in C/EBPβ and C/EBPδ expression is observed. C/EBPα expression is sufficient to induce 3T3-L1 differentiation even in the absence of adipogenic agents (Freytag et al., 1994; Lin and Lane, 1994). PPARα and PPARγ are predominantly expressed in adipose tissue and liver, whereas PPARδ is expressed ubiquitously but in low levels in the liver. PPARγ is upregulated during adipogenesis, and retroviral expression of PPARγ in fibroblasts is sufficient for successful differentiation (Tontonoz et al., 1994). Studies of PPARγ knockout mice indicate these animals are unable to develop adipose tissue (Barak et al., 1999; Rosen et al., 1999). PPARγ appears to be the master regulatory adipogenic transcription factor as compared to C/EBPα since PPARγ expression in C/EBPα knockout mice promotes adipogenesis but C/EBPα is incapable of inducing differentiation in the absence of PPARγ (Lazar, 2002; Rosen et al., 2002; Wu et al., 1999).

C/EBP and PPAR transcription factors promote the expression of various genes that are involved in proliferation, differentiation, as well as triglyceride and glucose metabolism. C/EBPα binds the promoter region of adipocyte genes and leads to the

transcription of adipocyte P2 (aP2), stearoyl-CoA desaturase, glucose transporter-4 (GLUT-4), phosphenolpyruvate carboxykinase (PEPCK), leptin and the insulin receptor (Gregoire et al., 1998). PPARγ induces expression of many genes involved in adipogenesis, particularly those involved in lipid storage and metabolic control. The genes aP2, PEPCK, acyl CoA synthetase, fatty acid translocase/CD36, fatty acid transport protein 1 and lipoprotein lipase are transcribed by PPARγ (Auwerx, 1999).

In terminal differentiation, glycerol-3-phosphate-dehydrogenase (GPDH) activity and triglyceride mass increase. GPDH and triglyceride levels serve as late markers of differentiation (Cianflone et al., 1994; Wise and Green, 1979). Studies employing triglyceride assays may not be entirely representative of differentiation, since overall triglyceride accumulation is also influenced by lipogenesis and lipolysis, processes that are distinct from differentiation.

In summary, differentiation of preadipocytes into adipocytes affects adipose tissue cell composition. Proliferation of preadipocytes also enhances the potential reservoir of precursor cells for adipose tissue expansion. Other processes appear to be involved, although they are not as well investigated. Apoptosis is one such process that can affect cell turnover in the adipose tissue depot.

3. Apoptosis

The characteristic features of cells undergoing apoptosis include DNA fragmentation, cytoplasmic shrinkage, and membrane blebbing. Apoptosis may occur in

response to external or internal signals. The receptor-mediated or extrinsic pathway involves the binding of a ligand-mediated death receptor such as the Fas receptor with FasL (ligand) leading to activation of apoptotic signaling pathways (Strasser et al., 2000). The second mechanism of apoptosis is the mitochondrial or intrinsic pathway that involves release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol where it binds to apoptotic protease activating factor-1. Both pathways exert their effects through cysteine-dependent aspartate-directed proteases (caspases) that exist as inactive zymogens or procaspases and may be activated via scaffold induction or cleavage by upstream regulatory kinases (Patel et al., 1996). Caspases are responsible for the ultimate cleavage of numerous integral nuclear, cytoplasmic and cytoskeletal proteins. Caspases 8 and 9 are known as the initiator caspases that lead to activation of various effector caspases including caspase 3, caspase 6 and caspase 7 (Grutter, 2000; Nicholson and Thornberry, 1997). Protein kinase B (PKB/Akt; described below) can modulate both initiator and effector caspases as demonstrated by the phosphorylation and inhibition of caspase 9 (Cardone et al., 1998) and through abrogation of caspase 3 activation in fibroblasts (Kennedy et al., 1997).

a. Caspase 3 Signaling in Apoptosis

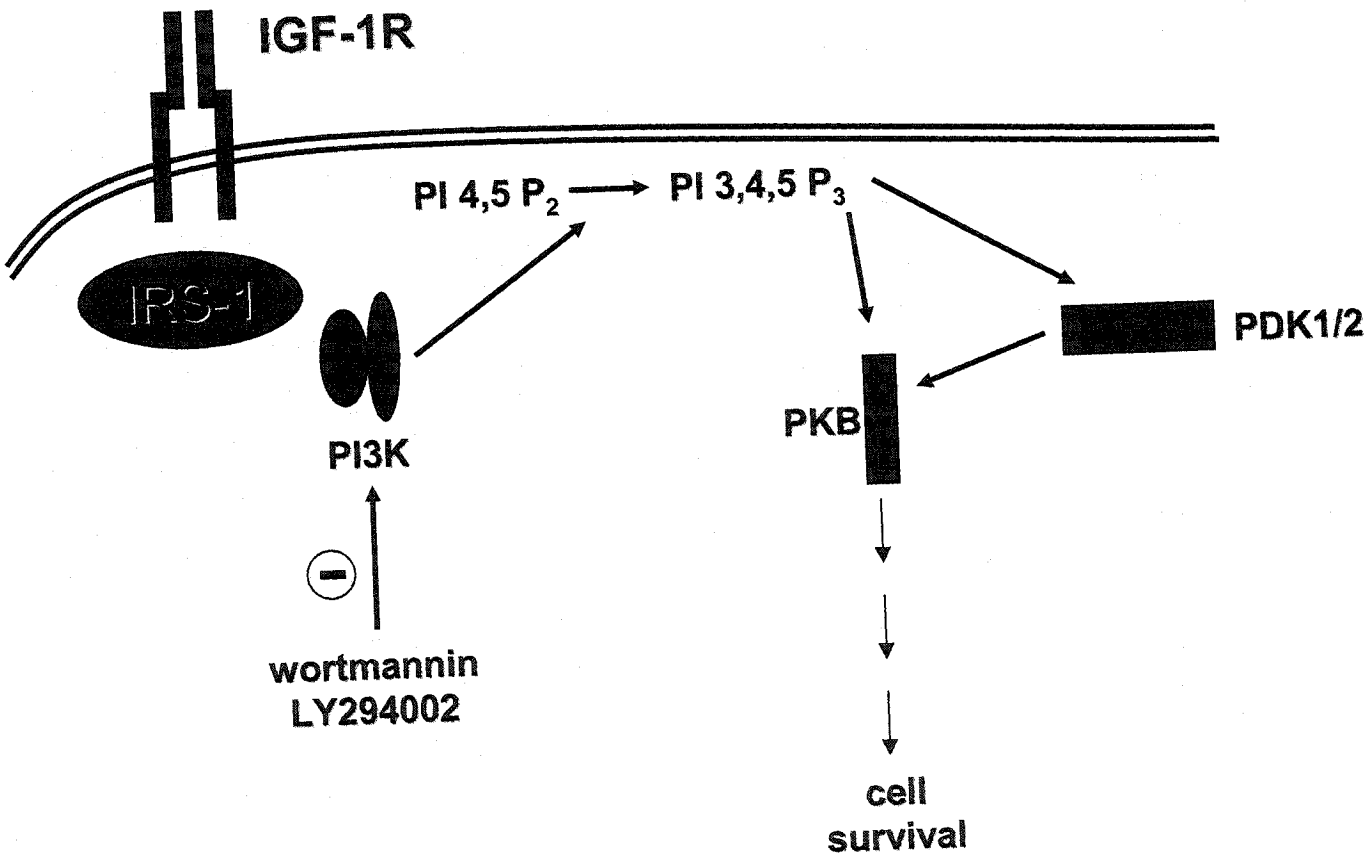
Caspase 3 is one of the most extensively studied caspases involved in apoptosis. Under normal circumstances, caspase 3 exists as an inactive precursor in the cytosol. The

caspase 3 knock-out mouse experiences excessive accumulation of neuronal cells in the brain, due to an inability of the cells to die (Kuida et al., 1996). In one study, the addition of active caspase 3 led to the induction of apoptosis (Enari et al., 1996). These results indicate that caspase 3 is necessary and sufficient for apoptosis. A study of cultured rat adipocytes revealed these cells can be induced to undergo apoptosis in the presence of TNFα or reduced levels of insulin in the media (Qian et al., 2001). The TNFα-mediated apoptosis occurs through activation of caspase 3 and can be abrogated by a caspase 3-specific inhibitor. The PI3K-PKB/Akt pathway has also been implicated in anti-apoptotic signaling pathways (described below), exerting its effects by reducing activation of the pro-apoptotic caspase 3.

b. IGF-1 and Insulin Signaling in Cell Survival

Insulin-like growth factor-1 (IGF-1) was shown to promote neuronal survival through the activation of PI3K and PKB/Akt (Dudek et al., 1997). A model of IGF-1-induced preadipocyte survival is shown in Figure 3. Neuronal survival has also been previously attributed to activation of the guanosine triphosphate (GTP)-binding protein Ras and subsequent activation of mitogen-activated protein kinase (MAPK) (Borasio et al., 1989; Xia et al., 1995). Other studies have indicated that IGF-1 promotes survival through activation of JNK and p38 MAPK (Heron-Milhavet et al., 2001; Krause et al., 2001). One major IGF-1-mediated anti-apoptotic pathway involves PI3K-PKB/Akt and the phosphorylation and inactivation of the Bcl-2 family member Bcl-2 antagonist of cell

Figure 3. Model of IGF-1-induced preadipocyte survival. IGF-1 binds to the IGF-1 receptor inducing a conformational change in the receptor followed by activation of the transmembrane tyrosine kinase domain. IRS-1 which becomes tyrosine-phosphorylated acts as a docking protein to relay the signal to PI3K. Activation of PI3K leads to production of 3-phosphorylated phosphoinositides that recruit PKB/Akt and PDK1/2 to the membrane thereby facilitating their phosphorylation. These events lead to cell survival.


IGF-1R
IRS-1
PI 4,5 P2
PI 3,4,5 P3
PDK1/2
PKB
PI3K
wortmannin
LY294002
cell
survival

death (Bad) (Peruzzi et al., 1999). PKB/Akt can also activate the Forkhead box class O 3a (FOXO3a; also known as FKHRL1) a member of the Forkhead family of transcription factors. PKB/Akt phosphorylation of FOXO3a prevents its nuclear translocation and activation of cell death genes (Brunet et al., 1999). Activation of cAMP responsive element-binding protein (CREB) and nuclear factor κB (NFκB) by PKB/Akt provide additional mechanisms of IGF-1-induced cell survival (Madrid et al., 2000; Pugazhenthi et al., 2000).

Serum-starved 3T3-L1 preadipocytes undergo apoptosis, and our laboratory has demonstrated that the PI3K lipid product phosphatidylinositol (PI) 3,4,5 trisphosphate (PI 3,4,5 P_3) and PKB/Akt play a role in IGF-1 survival signaling in 3T3-L1 preadipocytes (Gagnon et al., 2001).

c. Anti-apoptotic Signaling Factors

i. PI3K

PI3K is the enzyme responsible for phosphorylating phosphatidylinositol at the 3-OH position of the inositol ring. It has been demonstrated to play a critical role in cell division, vesicle trafficking, and cell survival (Cantley, 2002; Vanhaesebroeck and Alessi, 2000; Vanhaesebroeck et al., 2001). Three major classes of PI3K are currently known (Domin and Waterfield, 1997). Class I_A PI3K are heterodimeric proteins composed of an 85 kDa-type regulatory subunit associated with a 110 kDa catalytic

subunit. The regulatory subunits include p85α and p85β in addition to the p85α splice products p50α and p55β. The catalytic isoforms include p110α, p110β and p110γ. The p85 subunit contains two Src-homology 2 (SH2) domains, known to associate with phosphotyrosine residues and one Src-homology 3 (SH3) domain, known to bind proline and hydrophobic residues. The class I_B PI3K is p110γ, a protein with 36% identity to p110α that fails to interact with p85-type regulatory subunits. The putative regulatory subunit for p110γ is p101. Class II PI3K are the largest PI3K ranging from 170-210 kDa. The class III PI3K are unique in that they phosphorylate only phosphatidylinositol. Hormonal-induced activation of class I PI3K in preadipocytes acts on phosphatidylinositol 4,5 bisphosphate (PI 4,5 P_2) and leads to the production of PI 3,4,5 P_3. PI3K can also act on phosphatidylinositol 4 phosphate (PI 4 P) to generate phosphatidylinositol 3,4 bisphosphate (PI 3,4 P_2). However, the main route by which PI 3,4 P_2 is generated involves the dephosphorylation of PI 3,4,5 P_3.

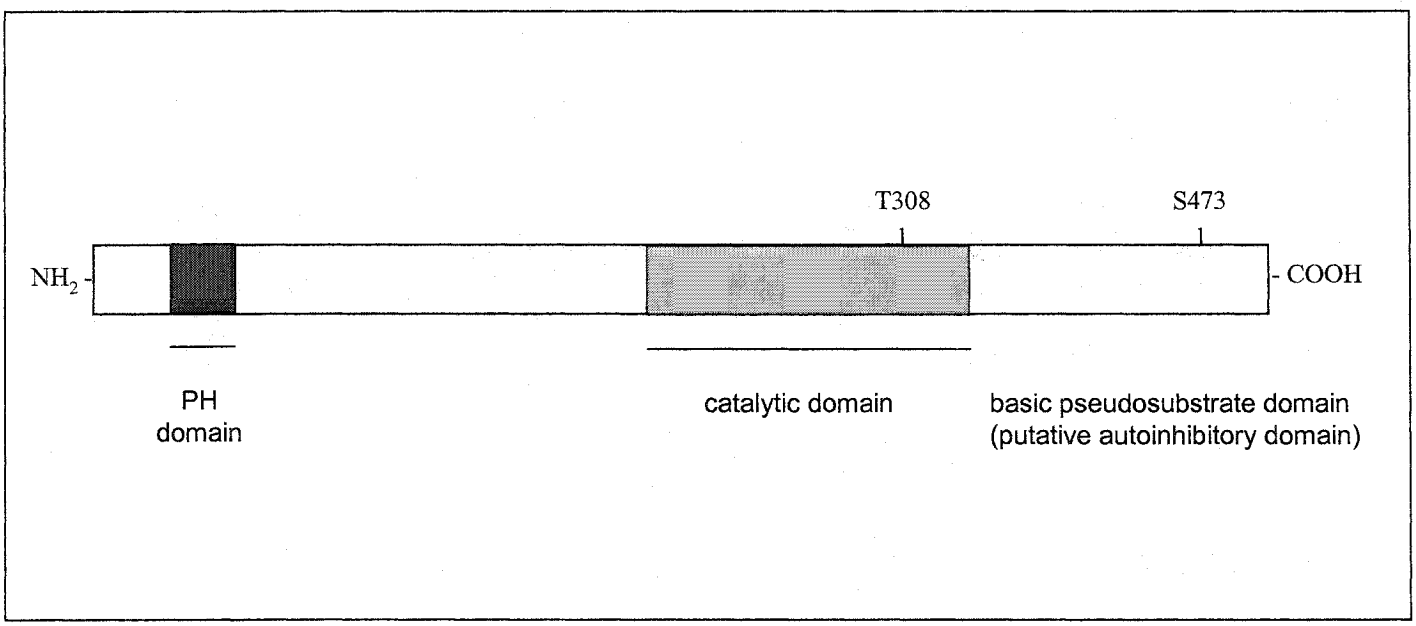
A decade ago, two important PI3K inhibitors were found, that enabled researchers to confirm the previously ascribed PI3K functions and to enhance understanding of the role of PI3K in signal transduction and cellular function (Nakanishi et al., 1995b). The anti-fungal agent wortmannin is known to inhibit PI3K through irreversible binding to the p110 catalytic subunit. The compound LY294002 inhibits PI3K through competitive binding with adenosine triphosphate (ATP). Since the compounds inhibit PI3K through independent mechanisms, application of both inhibitors addresses specificity of findings.

ii. PKB/Akt

PKB/Akt is a member of the cyclic AMP-dependent cyclic GMP-dependent/protein kinase C (AGC) superfamily of protein kinases sharing similar catalytic domains and mechanisms of activation. Three isoforms of PKB/Akt have been characterized (α, β, γ). PKB/Akt is a 60 kDa ser/thr kinase with an N-terminal pleckstrin homology (PH), a central catalytic domain, and a C-terminal hydrophobic motif (Figure 4). It is known to play a significant role in metabolism, growth and cell survival. Activation of PI3K leads to the production of the 3-phosphorylated phosphoinositides that recruit the PH domain containing ser/thr kinases phosphoinositide-dependent kinase 1/2 (PDK1/2) and PKB/Akt to the membrane thereby facilitating PKB/Akt phosphorylation at Thr 308 and Ser 473 (Alessi et al., 1996; Anderson et al., 1998). PDK-1 is a ser/thr kinase containing a C-terminal PH domain. PDK-1 has been shown to phosphorylate Thr 308 on PKB/Akt *in vitro* and *in vivo* by two independent groups (Alessi et al., 1997; Stokoe et al., 1997). Phosphorylation at Ser 473 is much less understood and a search continues for identification of the hypothetical PDK-2 enzyme (Alessi and Cohen, 1998). Conflicting reports indicate that Ser 473 phosphorylation may occur through autophosphorylation or through ser kinases such as integrin linked kinase (Delcommenne et al., 1998; Persad et al., 2001; Toker and Newton, 2000).

PKB/Akt plays an important role in cell survival. As mentioned above, PKB/Akt has been implicated in IGF-1-induced cell survival (Dudek et al., 1997). Constitutively activated PKB/Akt has been shown to inhibit UV-induced apoptosis in Rat-1 and COS-7

Figure 4. Model of ser/thr protein kinase PKB/Akt modular domains. PKB/Akt has a PH domain in the N-terminus that allows it to be recruited to the membrane for phosphorylation and activation. Phosphorylation of Thr 308 in the catalytic domain and Ser 473 in the pseudosubstrate domain are critical for activation of PKB/Akt.


T308
S473
NH2
COOH
PH
domain
catalytic domain
basic pseudosubstrate domain
(putative autoinhibitory domain)

cells (Kulik et al., 1997). PKB/Akt involvement in anti-apoptotic signaling has become more widespread in the field and numerous targets including Bad, FOXO3a, CREB and NFκB, as detailed above, have been implicated in PKB/Akt-mediated cell survival mechanisms.

C. TSH in Cell Survival Signaling

Previous studies have implicated IGF-1 in anti-apoptotic signaling pathways. We hypothesize that thyroid stimulating hormone (TSH) may activate similar intracellular targets and promote survival of 3T3-L1 preadipocytes. As described below, thyroid stimulating hormone receptor (TSHR) has been detected in extrathyroidal tissues including adipose tissue. Serum-starved 3T3-L1 preadipocytes undergo apoptosis, and our laboratory has previously implicated the PI3K lipid product PI (3,4,5) P_3 and PKB/Akt in IGF-1-mediated survival signaling in 3T3-L1 preadipocytes (Gagnon et al., 2001).

1. TSH Signaling in Human Preadipocytes

We have previously demonstrated TSH activation of p70 S6 kinase (p70 S6K) in human preadipocytes using immunoprecipitation and *in vitro* kinase assay as well as gel mobility shifts that detect phosphorylated p70 S6K (Bell et al., 2000). Preincubation with

wortmannin abrogated the TSH activation of p70 S6K in human abdominal subcutaneous preadipocytes, suggesting PI3K involvement.

These data along with other adipose tissue studies suggest adipose tissue is a target of TSH. In infant adipocytes TSH is a strong inducer of lipolysis (Janson et al., 1995; Janson and Marcus, 1997). The adenylyl cyclase-coupled pathway has been implicated in the TSH stimulation of lipolysis. Since preadipocytes do not house triglycerides, TSHR expression may be involved in regulating other cellular responses in these cells. Our data from the studies of human preadipocytes outlined above suggest a novel action of TSH in adipose tissue.

2. TSH Physiology

TSH is a 28-30 kDa glycoprotein with an α-subunit common to all glycohormone receptors and a β-subunit that differs for each individual glycohormone receptor. TSH is secreted by the pituitary gland in response to thyroid releasing hormone secretion from the hypothalamus, and optimal circulating levels of TSH are maintained through a negative feedback loop based on the level of thyroid hormone. TSH has long been known to act on the thyroid gland to induce proliferation, iodine uptake, thyroid hormone synthesis and release, and cell survival in thyrocytes (Grossmann et al., 1997).

3. TSHR Structure

The mature processed form of the TSHR is 84.5 kDa. TSHR is a G-protein-coupled receptor (GPCR) of the seven transmembrane domain receptor family. GPCRs are recognized as regulators of cell homeostasis and sensory signal transduction, cell growth and differentiation. The TSHR contains a large extracellular domain, a transmembrane and an intracellular domain. Posttranslational processing of the receptor produces an extracellular α subunit and a membrane spanning β subunit (Buckland and Rees Smith, 1984; Chazenbalk et al., 2004; Chazenbalk et al., 1997; Ciullo et al., 2003; de Bernard et al., 1999; Szkudlinski et al., 2002). The α subunit is tethered to the membrane bound β subunit by disulfide linkages. The TSHR has been shown to activate G_i, G_s, G_q and G_0 in thyrocyte cell models (Allgeier et al., 1997; Laugwitz et al., 1996; Szkudlinski et al., 2002).

4. Extrathyroidal TSHR Tissue Distribution

TSHR mRNA has been detected in rat retro-orbital and adipose tissues using reverse transcriptase-polymerase chain reaction (RT-PCR) and western analysis (Endo et al., 1993; Endo et al., 1995). TSHR was cloned from rat fat cells and functionally expressed in Chinese hamster ovary (CHO) cells (Endo et al., 1995). Guinea pig adipocytes also express TSHR as evidenced by binding assays and mRNA analysis (Hart and McKenzie, 1971; Roselli-Rehfuss et al., 1992). More recently TSHR mRNA was

found in human infant and adult adipocytes where it appears to play a role in lipolysis (Janson et al., 1995; Janson et al., 1998). Additionally, TSHR protein and mRNA expression has been detected in human orbital fibroblasts (Bell et al., 2000; Stadlmayr et al., 1997; Valyasevi et al., 1999). Findings from our laboratory indicate that TSHR protein is also expressed in human subcutaneous and omental preadipocytes and adipocytes (Bell et al., 2000). The concept of extrathyroidal TSHR expression has gained wide acceptance over the last several years (Davies et al., 2002; Prabhakar et al., 2003).

5. TSH Activation of cAMP-PKA

The classical G-protein effectors include phospholipase C and adenylyl cyclase (van Corven et al., 1993). The GTP-bound G-protein α-subunit ($G_s\alpha$) activates adenylyl cyclase, the enzyme responsible for synthesis of cAMP (Sunahara et al., 1996). Forskolin is a pharmacological activator of adenylyl cyclase that has facilitated understanding of the mechanism of adenylyl cyclase enzyme action. Traditionally, TSH signaling was thought to act solely through adenylyl cyclase elevated cAMP levels and subsequent activation of protein kinase A (PKA). PKA is composed of two regulatory subunits that are bound to two catalytic subunits in the inactive state. When cAMP binds to the regulatory subunits, it decreases the affinity for the catalytic subunits and leads to PKA activation (Taylor et al., 1990). TSH-induced mitogenesis occurs through activation of PKA in Wistar rat thyroid cells, dog thyrocytes, Fischer rat thyroid line (FRTL)-5 cells,

and human thyrocytes (Kimura et al., 2001). Inhibition of PKA does not completely abrogate mitogenesis, so other cAMP-dependent pathways are likely to be operating in these cells to reach the full mitogenic potential. Further study is necessary to delineate the other factors essential for mitogenic pathways in the thyrocyte.

6. TSH Activation of Epac

Increasing evidence supports a role for cAMP-induced direct activation of effectors other than PKA. More recently discovered immediate downstream targets of cAMP include guanine nucleotide exchange factors (GEFs) and ion channels (Sprang, 2001). GEFs act by catalyzing the dissociation of guanosine diphosphate (GDP) and the immediate binding of GTP to G-proteins (Sprang, 2001). Exchange protein directly activated by cAMP (Epac) is a novel downstream target of cAMP that acts as a GEF for the small GTPases Rap1 and Rap2 (de Rooij et al., 2000; de Rooij et al., 1998). Two Epac family members have been characterized: Epac1 and Epac2. Epac1 is broadly expressed though strong expression has been observed in thyroid tissue. Epac appears to play a role in cell adhesion, migration and insulin secretion (Bos, 2003). Early studies suggest the critical Epac effectors include Rap1 and Rap 2. Epac has been suggested to play a role in dog thyrocyte and in FRTL-5 rat thyroid cell signaling pathways (Dremier et al., 2000; Iacovelli et al., 2001).

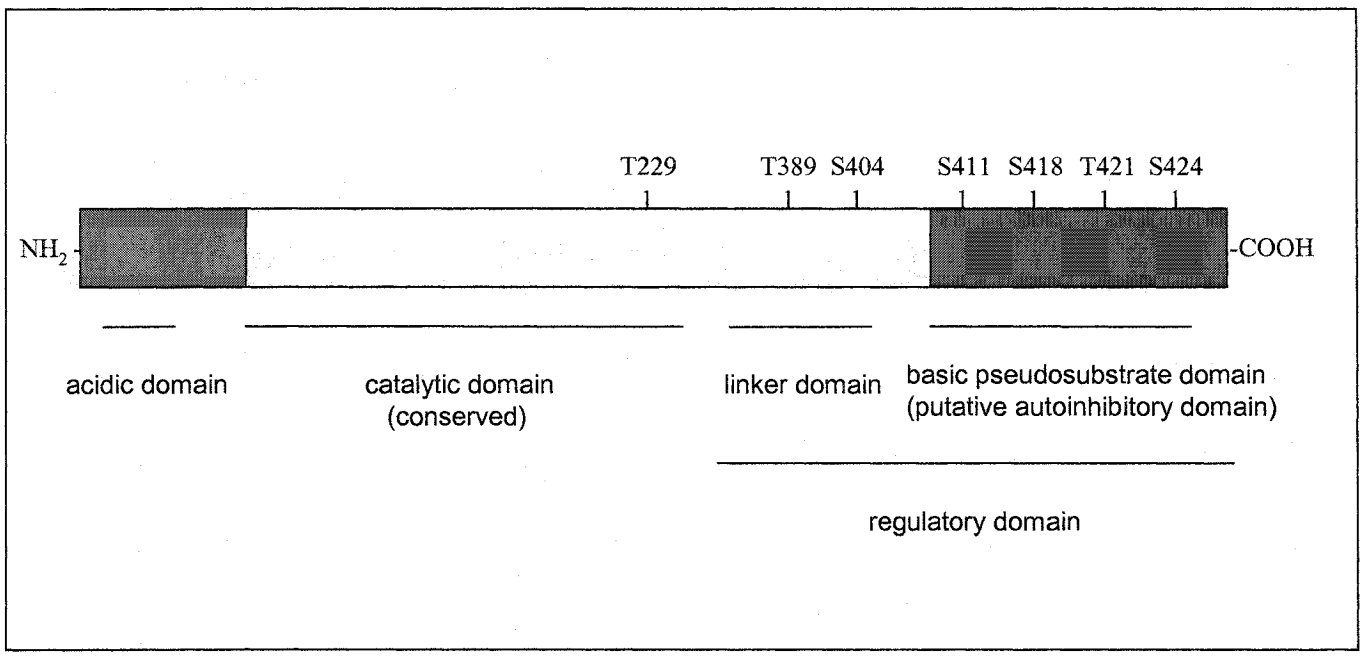
7. TSH Activation of PI3K

Recent findings suggest G-proteins can also activate class I_A and class I_B PI3K. One mechanism by which GPCR activates class I_A PI3K involves the activation of intracellular non-receptor tyrosine kinases. In one case, gastrin/cholecystokinin-B (CCK-B) receptor activates Src-family tyrosine kinases that phosphorylate IRS-1, leading to docking and activation of PI3K (Daulhac et al., 1999). There also appears to be activation of tyrosine kinases in TSH stimulated thyroid epithelial cell lines (Ariga et al., 2000; Nedachi et al., 2000). Alternatively, GPCR activation leading to the dissociation of $G\beta\gamma$ subunits can lead to the direct activation of class I_B PI3K. For example, direct activation of the class I_B p110γ by $G\beta\gamma$ subunits has been observed (Leopoldt et al., 1998).

8. TSH Activation of p70 S6K

p70 S6K is a ser/thr kinase that regulates phosphorylation of the 40S ribosomal protein S6. The structural composition of p70 S6K is described in Figure 5. p70 S6K is required for cell cycle progression through G1 to S phase and it plays an important role in translational regulation of mRNA transcripts containing polypyrimidine tracts in their 5' untranslated regions (UTRs) including those mRNAs encoding ribosomal proteins (Jefferies et al., 1997). The p70 S6K isoform p85 S6K is created using alternative translation start sites on the same transcript and p85 S6K differs only in the N-terminal 23 amino acid nuclear localization signal (Grove et al., 1991; Reinhard et al., 1992).

Figure 5. Model of ser/thr protein kinase p70 S6K modular domains. p70 S6K is phosphorylated at various ser/thr sites in the C-terminus. Next, the critical Thr 389 residue is phosphorylated, followed by the terminal phosphorylation of Thr 229 that results in full activation.


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

Activation of p70 S6K occurs via a multistep phosphorylation sequence leading to various conformational changes (Pullen and Thomas, 1997). Initially, numerous ser/thr sites are phosphorylated in the C-terminus followed by phosphorylation and activation of the critical Thr 389 residue. This leads to the ultimate phosphorylation at the Thr 229 residue resulting in full activation of the kinase.

Recent studies implicate p70 S6K and PKB/Akt as novel downstream targets of TSHR in thyrocytes (Cass and Meinkoth, 1998; Coulonval et al., 2000). Both pathways are cAMP-mediated; however, only p70 S6K signaling appears to be PKA-dependent. Cass et al. have demonstrated that TSH-induced PKA-independent signaling pathways are mediated by PI3K-PKB/Akt (Cass et al., 1999). The authors later reported that TSH mediates PI3K activation through the GEF Rap1A (Tsygankova et al., 2001).

D. Chapter 1 Objectives

Our first major objective was to test whether TSH activates PI3K-PKB/Akt-p70 S6K signaling in 3T3-L1 preadipocytes and whether TSH promotes survival of these cells. We postulated that similarities exist between TSH signaling pathways in 3T3-L1 preadipocytes compared to the previously examined human preadipocytes (Bell et al., 2000). Given the role of PI3K-PKB/Akt in anti-apoptotic signaling, we chose to investigate whether TSH plays a role in preadipocyte survival. First, we tested 3T3-L1 preadipocytes for the presence of TSHR using western blotting. Activation of the PI3K-

PKB/Akt-p70 S6K pathway was examined using western analysis and *in vitro* kinase assay. We used the PI3K inhibitors wortmannin and LY294002 to assess PI3K involvement in activation of PKB/Akt and p70 S6K. The extent of apoptosis was measured via cell enumeration, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), and caspase 3 cleavage and activation.

Elevated TSH levels, as observed in hypothyroidism, may act directly on preadipocytes to promote cell survival and may contribute to weight gain by augmenting the available pool of preadipocytes capable of adipogenesis. The clinical relevance of TSH effects on human preadipocytes is currently unknown and future studies will be necessary to explore this further.

E. Abnormal TSH Physiology

Hypothyroid patients experience low levels of the thyroid hormone thyroxine (T_4). In the presence of low T_4, TSH levels are increased in an attempt to raise T_4. A clinical feature of hypothyroidism is weight gain. This has been attributed to the low metabolic rate resulting from low levels of circulating thyroid hormone. In subclinical hypothyroidism, a milder form of thyroid failure, elevations of TSH can allow compensation, so that T_4 levels are normal. Surprisingly, these patients may also experience weight gain, so it appears that altered thyroid hormone levels are not the only culprits contributing to obesity. Clinical abnormalities in subclinical hypothyroid patients can be attributed to the high circulating TSH since T_4 levels are not frankly low.

Subclinical hypothyroidism has also been referred to, though infrequently, as mild hypothyroidism and it tends to have a high rate of conversion to overt hypothyroidism (Ayala et al., 2000; Cooper, 2001).

F. Elevated TSH Levels as an Independent CVD Risk Factor

Hypothyroidism is known to be associated with CVD and the cause has been attributed to low levels of T_4 (Cappola and Ladenson, 2003). It is less clear whether subclinical hypothyroidism can lead to CVD. A random sample of 1149 postmenopausal women participating in the Rotterdam Study in the Netherlands were studied using a population-based cross-sectional study to assess whether subclinical hypothyroidism and thyroid autoimmunity are risk factors for CVD (Hak et al., 2000). Subclinical hypothyroidism was characterized by circulating TSH levels >4.0 mU/L and normal circulating free T_4 levels ranging from 11-25 pmol/L. Subclinical hypothyroidism was observed in 10.8% of the women sampled and it was associated with a greater age-adjusted prevalence of aortic atherosclerosis and myocardial infarction. This could not be accounted for by traditional CVD risk factors such as blood pressure and cholesterol. Women with subclinical hypothyroidism were 3 times more likely to experience myocardial infarction as compared to euthyroid women. The authors concluded that subclinical hypothyroidism is a strong indicator of risk for atherosclerosis and myocardial infarction.

In a separate study of 2550 subjects including men and women, the association of subclinical hypothyroidism and atherosclerosis was examined (Imaizumi et al., 2004). Subclinical hypothyroidism was defined by circulating TSH levels >5mU/L. Subclinical hypothyroidism was observed in 10.2% of the population, and was associated in a cross-sectional analysis with atherosclerosis in men. In the latter study, a significant association between subclinical hypothyroidism and atherosclerosis was not observed in the female subjects; this may be due to the average age of the women being ~10 years younger than those in the Rotterdam study. There is currently little data on the mechanisms by which subclinical hypothyroidism may predispose to CVD. We propose that TSH elevated IL-6 levels may contribute to the development of CVD.

1. Cardiovascular Disease

Until the last decade, CVD was viewed predominantly as a disorder of lipid metabolism (Libby, 2003). Early studies of atherosclerosis implicated dyslipidemia as the culprit in the pathophysiology of atherosclerotic plaque formation. More recent studies suggest that atherosclerosis is an inflammatory disease from the initiation of the lesion, throughout development, to the birth of thrombotic complications (Libby, 2003; Libby et al., 2002). The reviews listed above suggest CVD is associated with an inflammatory condition in which cytokine levels are disturbed. Interleukins are cytokines that have been shown largely to regulate inflammatory and immune responses. Two

particular cytokines IL-6 and TNFα have been implicated in the development of atherosclerosis (Yudkin et al., 2000).

G. TSH Modulates Adipocytokine Release

Adipose tissue is increasingly being described as an endocrine organ given the numerous hormones and cytokines including leptin, TNFα, IL-6, adiponectin and resistin found to be secreted by adipocytes. A recent role for TSH has been suggested in modulating leptin secretion. Findings concerning the effect of TSH on leptin production and secretion have been contradictory. Shintani et al. found that TSH decreased leptin production at the protein and mRNA level in rat epididymal adipocytes (Shintani et al., 1999). TSH also stimulated lipolysis in the adipocytes, as indicated by increased glycerol release. In contrast, using *in vitro* adipose tissue organ culture models, Menendez et al. revealed that TSH induces leptin secretion from human omental adipose tissue fragments (Menendez et al., 2003). Luteinizing hormone and follicle stimulating hormone, though structurally similar to TSH, were unable to enhance leptin secretion. In addition, the pituitary hormones prolactin and adrenocorticotropic hormone were also incapable of augmenting leptin secretion. These results indicate specificity in the action of TSH on the omental adipose tissue. The conflicting results from these two studies may be due to the possibility that TSH regulates leptin production differently in rat versus human adipose tissue.

H. IL-6 Action

IL-6 has an established role as a proinflammatory cytokine. IL-6 is expressed and secreted by many different cell types including lymphocytes and macrophages (Ray et al., 1989). Posttranslational and postsecretory processing produces multiple isoforms of IL-6 protein ranging in size from 21.5 to 28 kDa. TNFα, IL-1β, leptin and IL-6 are factors known to induce IL-6 expression and secretion (Pang et al., 1994). IL-6 protein acts in both an autocrine and paracrine fashion. Circulating IL-6 induces the hepatic acute phase response leading to elevated C-reactive protein (CRP) and fibrinogen levels. A role for CRP in CVD continues to be actively debated. CRP may play a direct role in CVD, it can directly damage the vasculature, it can be produced by damage to the vasculature, or may serve solely as a valuable marker of CVD (Albert and Ridker, 1999; Danesh et al., 2004; Lagrand et al., 1999; Tall, 2004). IL-6 plays a role in CVD development by acting at the site of the atheroma, increasing basal glucose uptake, inhibiting glycogen synthesis, increasing hepatic fibrinogen release and inducing procoagulation of platelets (Yudkin et al., 2000). Additionally, IL-6 can inhibit lipoprotein lipase and stimulate lipolysis in humans (van Hall et al., 2003). Healthy males infused with high and low dose recombinant human IL-6 (rhIL-6) had fatty acid concentrations 50% greater than males infused with saline control. This study implicates IL-6 in lipid metabolism in humans as well as in the numerous other roles in glucose metabolism and atherogenesis described above.

1. Effect of IL-6 on Atherosclerotic Plaques

IL-6 transcript expression was first shown to play a role in the pathogenesis of atherosclerotic plaques in genetically hyperlipidemic rabbits (Ikeda et al., 1992). Seino et al. later demonstrated that IL-6 transcript levels were 10- to 40-fold higher in human atherosclerotic arteries as compared to the non-atherosclerotic arteries (Seino et al., 1994). Vascular endothelial and smooth muscle cells from normal and aneurysmal arteries secrete IL-6 (Loppnow and Libby, 1989a; Loppnow and Libby, 1989b; Szekanecz et al., 1994). When atherosclerosis-prone mice fed a high fat diet were given weekly injections of IL-6 they developed significantly enhanced fatty lesions as compared to the high fat fed atherosclerosis-resistant mice (Huber et al., 1999).

2. IL-6 Expression in Adipocytes

Over a decade ago, IL-6 mRNA transcripts were detected in early 3T3-L1 preadipocyte differentiation (Stephens et al., 1993). Since then, others have demonstrated that circulating IL-6 levels are positively correlated with obesity (Kern et al., 2001; Mohamed-Ali et al., 1997; Vgontzas et al., 1997). Studies employing arterio-venous drainage assays indicate approximately 30% of circulating IL-6 levels can be attributed to adipose tissue secretion, and these levels are augmented with increased adiposity (Mohamed-Ali et al., 1997). Fried et al. have demonstrated that unstimulated omental adipose tissue releases 2-3 times more IL-6 than subcutaneous adipose tissue (Fried et al.,

1998). To examine potential regulators of IL-6 secretion, human breast adipocyte IL-6 secretion was tested in the presence of the β-adrenergic agonist isoproterenol or the glucocorticoid cortisol (Path et al., 2001). Isoproterenol induced a large increase in IL-6 secretion whereas cortisol induced an inhibitory effect on IL-6 secretion. Additional studies have confirmed that β-adrenergic stimulation with isoproterenol leads to adipocyte IL-6 secretion (Mohamed-Ali et al., 2001). Insulin, isoproterenol, TNFα and growth hormone have all been shown to elevate IL-6 transcripts in mature 3T3-L1 adipocytes (Fasshauer et al., 2003). Furthermore, Vicennati et al. revealed that insulin and catecholamines stimulate secretion whereas glucocorticoids inhibit IL-6 secretion (Vicennati et al., 2002). This study also implicated cAMP as an important modulator of IL-6 release consistent with classic β-adrenergic receptor activation. IL-1β has also been confirmed as another regulator of IL-6 secretion from human adipocytes (Flower et al., 2003). The mechanism of IL-6 secretion from adipocytes is largely unexplored at this time.

I. Chapter 2 Objectives

Our second major objective was to explore the effect of TSH on IL-6 secretion by applying the human and mouse cell models. We hypothesize that TSH stimulates adipocyte IL-6 secretion, thus providing a potential mechanism by which subclinical hypothyroidism may predispose to CVD. We directly tested whether TSH modulates IL-6 secretion from differentiated 3T3-L1, 3T3-F442A and human adipocytes. We examined

whether the classic TSHR coupling to cAMP occurs in TSH-stimulated adipocytes. We also tested whether TSH might exert its effects through PI3K, PKA, and p42/44 MAPK using the inhibitors wortmannin and LY294002, H89, and PD98059, respectively. Controversy exists in the literature concerning the cellular source of IL-6 expression (see Discussion). We tested levels of IL-6 protein secreted from preadipocytes. Lastly, we tested whether IL-6 mRNA is expressed in human preadipocytes and differentiated adipocytes, and whether TSH modulates IL-6 mRNA levels.

In summary, our overall hypothesis is that TSH affects adipose tissue growth and remodeling by modulating preadipocyte survival, as well as function of adipose tissue by controling IL-6 release from adipose cells.

II. CHAPTER 1 TSH Signaling and Survival in 3T3-L1 Preadipocytes

A. MATERIALS AND METHODS

Cell Culture of Rat and Mouse Cell Lines

3T3-L1 preadipocytes (from American Type Culture Collection, Manassas, Virginia, U.S.A.) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (CS). FRTL-5 rat thyroid cells (from Dr. R. Germinario, McGill University, Montreal, Quebec, Canada) were grown in COON's F12 supplemented with 5% newborn calf serum and a 6 hormone cocktail containing 18 nM TSH, 834 nM insulin, 5 μg/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml Gly-L-His-L-Lys and 10 ng/ml hydrocortisone-PO_4. J774 mouse macrophage cells (from Dr. Y. Marcel, University of Ottawa, Ottawa, Ontario, Canada) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and CHO-vector control and human TSHR-transfected CHO-hTSHR cells (from Dr. J.E. Dumont, Erasme University Hospital, Free University of Brussels, Brussels, Belgium) were grown in Ham's F12 (F12) supplemented with 10% FBS, 50 units/ml nystatin (N) and 400 μg/ml G418. All media contained 100 units/ml penicillin and 0.1 mg/ml streptomycin (PS) unless otherwise indicated. 3T3-L1 preadipocytes were stimulated with 0.1, 0.5, 1, 5, 10 or 20 μM bovine TSH (TSH) or 4 μM highly purified bovine TSH (TSH-NIH; AFP8755B, National

Institutes of Diabetes and Digestive Kidney Diseases; National Hormone and Peptide Program, Torrance, California, U.S.A.), as described.

Differentiation of 3T3-L1 Preadipocytes into Adipocytes

Confluent 3T3-L1 preadipocytes were differentiated in DMEM supplemented with 10% FBS in the presence of 1 μM insulin over 8 days, with 0.25 μM dexamethasone and 0.5 mM isobutylmethylxanthine (IBMX) present for the first 2 days. 3T3-L1 differentiated adipocytes were stimulated with 5 μM TSH, when indicated.

Western Analysis

For TSHR and anti-phosphotyrosine immunoblot analysis, cells were lysed in Laemmli buffer (Laemmli, 1970), supplemented with 0.2 mM sodium orthovanadate (Na_3VO_4). For all other immunoblots, cells were lysed in Laemmli supplemented with 5 mM ethyleneglycoltetraacetic acid (EGTA), 5 mM sodium pyrophosphate (NaPPi), 50 mM sodium fluoride (NaF) and 1 μM microcystin. Lysate protein was quantified and equal amounts of solubilized protein (ranging from 15-140 μg across experiments) were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose. Blots were probed overnight at 4°C with mouse monoclonal TSH-R2 (1:60; Novacastra Laboratories), rabbit polyclonal p70

S6K (1:500) (Santa Cruz Biotechnology), rabbit polyclonal phospho-p70 S6K (1:250; New England Biolabs: Cell Signaling Technology), rabbit polyclonal PKB/Akt antibody (1:9000; from Drs. P.J. Coffer and B.M. Burgering, Utrecht University, Utrecht, The Netherlands), rabbit polyclonal phospho-PKB/Akt (Ser473; 0.1 μg/ml; New England Biolabs: Cell Signaling Technology), rabbit polyclonal phospho-PKB/Akt (Thr308; 1:500; New England Biolabs: Cell Signaling Technology), mouse monoclonal phosphotyrosine (1 μg/ml; BD Biosciences: Transduction Laboratories), rabbit polyclonal janus kinase (JAK)1 (1:1000; from Dr. M. Kozlowski, University of Ottawa, Ottawa, Ontario, Canada), or rabbit polyclonal caspase 3 (1:500; PharMingen International) primary antibody. For phospho-PKB/Akt analysis, immunoblots were routinely probed with anti-phospho-PKB/Akt (Ser 473) antibody except when anti-phospho-PKB/Akt (Thr 308) antibody was used, as indicated. Following incubation with the appropriate peroxidase-conjugated secondary antibody, membranes were processed for enhanced chemiluminescence (ECL) detection. When indicated, blots were stripped according to manufacturer's directions and subsequently reprobed.

p70 S6K Immunoprecipitation

To determine p70 S6K activity, preadipocytes were stimulated with TSH and the reaction was terminated by the addition of lysis buffer containing 1% Nonidet P-40, 0.2 mM Na_3VO_4, 0.1 mg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 4 μg/ml benzamidine, 5 mM NaPPi, 50 mM NaF, 1 μM microcystin and 1 mM β-

glycerophosphate in PBS. Lysates were centrifuged at 15000g for 10 min at 4°C and supernatants were precleared for 1 h at 4°C with protein-A-sepharose. Samples were incubated for 90 min at 4°C with 2 μg rabbit anti-p70 S6K antibody preadsorbed to protein-A-sepharose.

p70 S6K *in vitro* Kinase Assay

For measurement of immunoprecipitated p70 S6K activity, the Upstate Biotechnology *in vitro* kinase assay commercial kit was used (Bell et al., 2000). Immunoprecipitates were washed 2x with lysis buffer and 3x with assay buffer (20 mM 3-[N-morpholino] propanesulfonic acid (MOPS) at pH 7, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 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 by the addition of ATP cocktail [75 mM magnesium chloride ($MgCl_2$), 500 μM ATP and 10 μCi/μl [γ-^{32}P]-ATP] in assay dilution buffer. After 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. Following this assay, immunoprecipitated p70 S6K was resuspended in immunoblot lysis buffer and subjected to SDS-PAGE and western analysis to detect gel mobility shifts.

PI3K Immunoprecipitation

To determine PI3K activity, preadipocytes were stimulated with TSH and lysed in the p70 S6K lysis buffer described above, with the following modifications: 1% Triton X-100 was substituted for 1% Nonidet P-40, and NaPPi and microcystin were omitted. Lysates were centrifuged at 15000g for 10 min at 4°C and supernatants were precleared for 1 h at 4°C with protein-G-agarose. Samples were incubated for 90 min at 4°C with 5 μg mouse anti-phosphotyrosine antibody preadsorbed to protein-G-agarose.

PI3K *in vitro* Kinase Assay

For measurement of co-immunoprecipitated PI3K in the anti-phosphotyrosine immunoprecipitates, a PI3K kinase assay was used (Bell et al., 2000). The reaction was initiated with the addition of Mg/ATP cocktail (10 mM $MgCl_2$, 10 μM cold ATP, and 20 μCi [γ-^{32}P]-ATP) to immunoprecipitated phosphotyrosine-containing proteins suspended in PI3K assay buffer [20 mM Tris, 0.1 M sodium chloride (NaCl), 0.5 mM EGTA, 0.2 mg/ml phosphatidylinositol]. After a 3 min incubation period, chloroform:methanol:HCl (50:100:1; v/v/v) was added to terminate the reaction. The lipid product was extracted, spotted onto a K5 silica gel plate previously dipped in 1% potassium oxalate/2 mM ethylenediaminetetraacetic acid (EDTA) in EtOH/H_2O (1:1), and resolved by thin layer chromatography. The silica gel plate was exposed to X-Omat film for autoradiographic detection. The relative intensity of the band was measured

using Molecular Analyst imaging software and data are expressed as integrated optical density [I.O.D.] units.

cAMP Accumulation Assay

3T3-L1 preadipocytes and their differentiated counterparts were labeled overnight in DMEM containing 5% FBS and 2 μCi/ml [^{3}H]-adenine (Iwasiow et al., 1999). The following day, cells were incubated at 37°C in DMEM containing 20 mM HEPES and 1 mM IBMX, and treated with vehicle (H_2O), TSH (5 μM), or forskolin (10 μM) for 30 min. The reaction was terminated upon addition of cold stop solution containing 2.5% (v/v) perchloric acid, 100 μM cAMP and ~5 nCi [^{14}C]-cAMP. After a 30 min incubation period at 4°C, acid-cell lysates were transferred to tubes containing 4.2 M KOH for neutralization. Sequential chromatography on Dowex and alumina columns was performed to determine intracellular cAMP levels. Data are expressed as 1000 times the ratio of [^{3}H]-cAMP formed over the total uptake measured per well.

Cell Enumeration

3T3-L1 preadipocytes were grown to confluence in the absence or presence of triiodothyronine (T_3; 2 nM), as indicated, and either left in serum or serum-deprived in the absence or presence of TSH (1, 5, 10 and 20 μM) for 6 h. Cells were trypsinized and counted in duplicate using a haemocytometer. The following equation was used to calculate % cell death:

$$\frac{\text{\# adherent cells in serum-containing medium - \# adherent cells in sample}}{\text{\# adherent cells in serum-containing medium}} \times 100\%$$

TUNEL Assay

Confluent 3T3-L1 preadipocytes were either left in serum or serum-deprived in the absence or presence of TSH (20 μM) for 3 h. The time period for serum withdrawal was shortened from 6 h to 3 h to optimize the number of adherent cells staining positively for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), thus avoiding cell detachment associated with the final stages of apoptosis. Preadipocytes were subjected to TUNEL according to the In Situ Cell Death Detection Kit (Gagnon et al., 2001). Preadipocytes were grown to confluence on coverslips and then fixed in 4% paraformaldehyde for 30 min. Cell membranes were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, and treated with TdT for 60 min at 37°C. Cells were incubated for 30 min at 37°C with alkaline phosphatase conjugated with anti-fluorescein antibody. Staining was achieved during 20 min incubation at room temperature (RT) using the alkaline phosphatase substrate mix nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). TUNEL-positive cells were counted in ten random fields (field of view using microscope at 400X magnification) by two independent observers (200-300 cells/field), and the average from duplicate samples was calculated.

Statistical Analysis

Data was analyzed by paired t-test or ANOVA with Tukey's post-test, using GraphPad InStat version 3.00 for Windows 98 (Graph Pad Software, San Diego, CA). Values with $p<0.05$ were considered significant.

B. RESULTS

We have detected TSHR protein in 3T3-L1 preadipocytes. Figure 6 is an immunoblot of total cell lysate from FRTL-5 cells (a thyroid cell line), 3T3-L1 preadipocytes and J774 cells (a macrophage cell line) probed with commercially available mouse monoclonal TSH-R2 antibody. The 100 kDa mature processed form of the TSHR was observed in 3T3-L1 preadipocytes and in the positive control FRTL-5 cells but was absent from the negative control J774 cells.

Based on our previous findings in human preadipocytes (Bell et al., 2000), we tested whether TSH activates p70 S6K in 3T3-L1 preadipocytes. Figure 7A is a representative immunoblot demonstrating dose-dependent TSH activation of p70 S6K using rabbit polyclonal anti-phospho-p70 S6K (pp70 S6K) antibody that recognizes phospho-Thr 389. Phosphorylation at this site is indicative of activation (Moser et al., 1997; Pearson et al., 1995). The immunoblot was reprobed with rabbit polyclonal p70 S6K antibody as a loading control. The upward shift in mobility observed in the immunoblot is due to p70 S6K phosphorylation. Figure 7B depicts the densitometric analysis of pp70 S6K levels. The data are derived from 3 separate experiments, each performed in duplicate. Consistent with these data, there was a 20-fold increase in TSH-stimulated p70 S6K activity with the *in vitro* kinase assay (data not shown).

Since PKB/Akt is located upstream of p70 S6K in many cell types (Coffer et al., 1998; Vanhaesebroeck and Alessi, 2000), we tested whether TSH activates PKB/Akt. Figure 8 is a representative immunoblot demonstrating dose-dependent TSH activation of

Figure 6. TSHR protein is expressed in 3T3-L1 preadipocytes. Equal amounts of solubilized protein from FRTL-5 rat thyroid, 3T3-L1 preadipocyte, and J774 mouse macrophage cell lines were resolved on SDS-PAGE and subjected to western analysis using anti-TSH-R2 antibody. As these are different cell types, a loading control could not be used. However, equal amounts of solubilized protein were added in each lane.

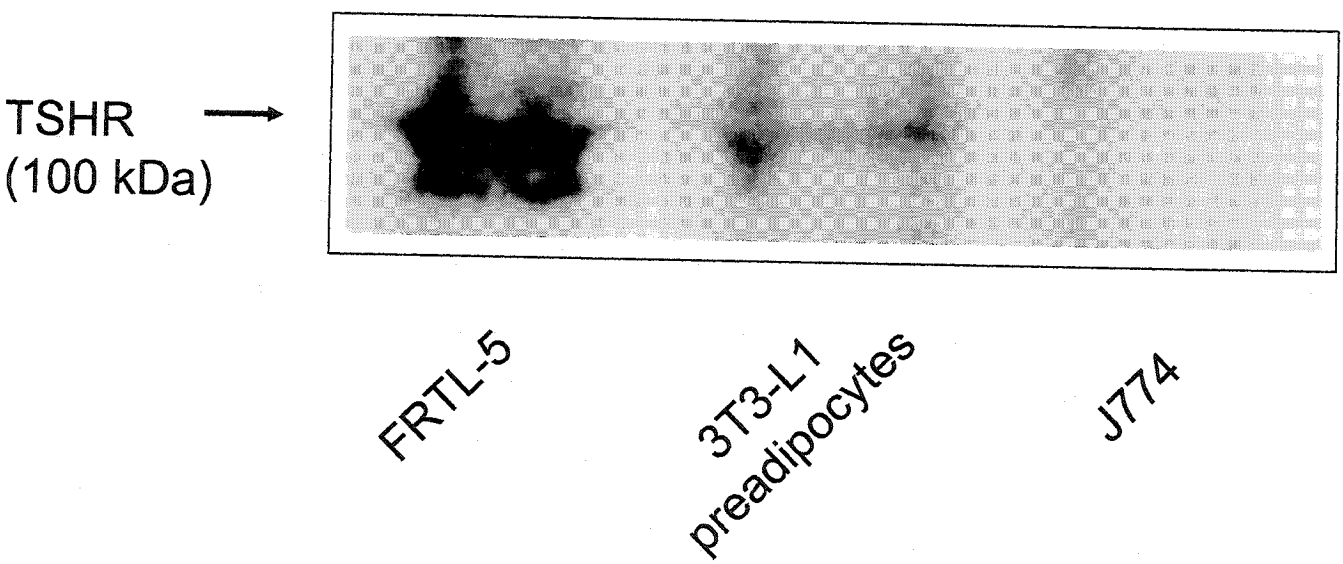
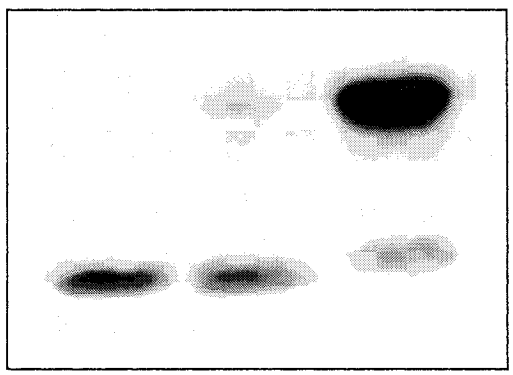

TSHR
(100 kDa)
FRTL-5
3T3-L1
preadipocytes
J774

Figure 7. TSH activates p70 S6K in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were stimulated for 30 min with TSH (1 and 5 μM). Equal amounts of solubilized protein were resolved on SDS-PAGE and subjected to western analysis. (A) A single representative immunoblot was probed with anti-pp70 S6K antibody and reprobed with anti-p70 S6K antibody for loading control. (B) Densitometric analysis was performed on the pp70 S6K immunoblots and data are expressed as integrated optical density units (I.O.D. units). The values are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate.

A


pp70 S6K
p70 S6K
TSH (μM) 0 1 5

B

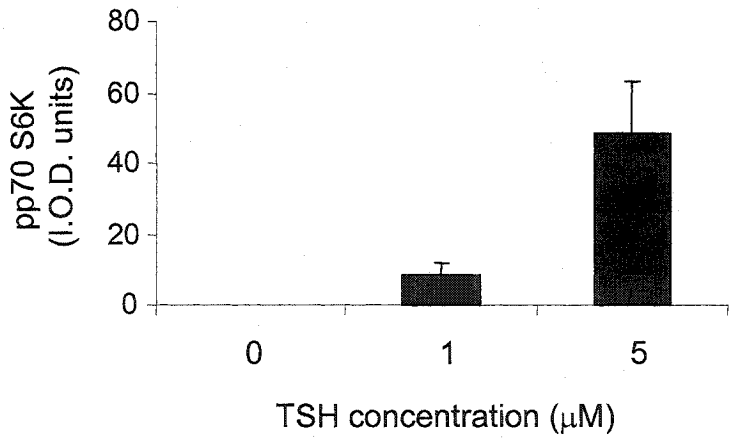
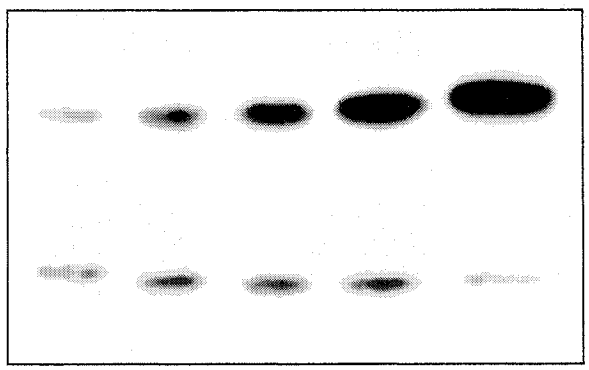

80
60
40
20
0
pp70 S6K
(I.O.D. units)
0
1
5
TSH concentration (μM)

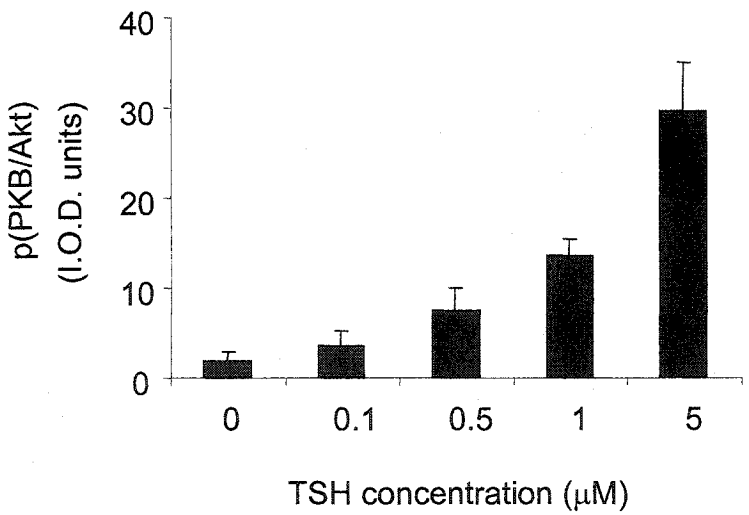
Figure 8. TSH activates PKB/Akt in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were stimulated for 30 min with TSH (0.1, 0.5, 1, and 5 μM). Equal amounts of solubilized protein were resolved on SDS-PAGE and subjected to western analysis. (A) A single representative immunoblot was probed with anti-p(PKB/Akt) antibody and reprobed with anti-PKB/Akt antibody for loading control. (B) Densitometric analysis was performed on the p(PKB/Akt) immunoblots and data are expressed as integrated optical density units (I.O.D. units). The values are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate.

A



TSH (μM) 0 0.1 0.5 1 5

B



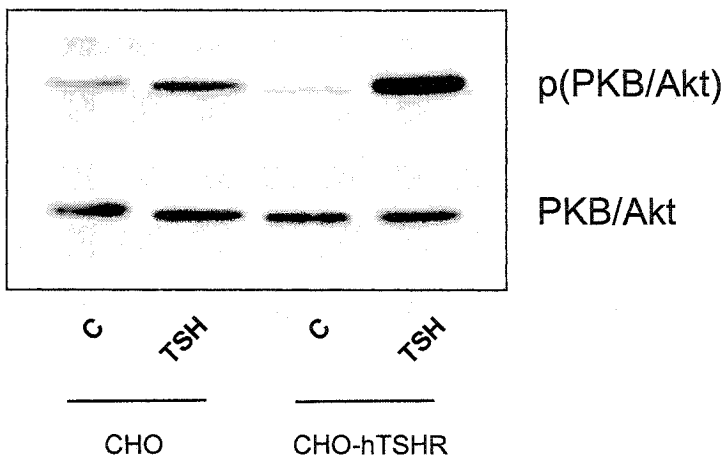
PKB/Akt using rabbit polyclonal anti-phospho-PKB/Akt [p(PKB/Akt)] that recognizes phospho-Ser 473. Phosphorylation at this site is indicative of activation (Alessi et al., 1996). The immunoblot was reprobed with rabbit polyclonal PKB/Akt antibody as a loading control. Figure 8B depicts the densitometric analysis of p(PKB/Akt) levels. The data are derived from 3 separate experiments, each performed in duplicate. A reproducible decrease in PKB/Akt levels was consistently observed at high dose stimulation with TSH. At present we are not sure of the reason for this. Possibilities include proteosomal degradation of PKB/Akt or a decrease in the affinity of the highly phosphorylated protein for the primary antibody.

To address possible concerns that TSH activation of PKB/Akt may not be TSHR-dependent, we tested the effect of TSH on Chinese hamster ovary (CHO) cells transfected with TSHR as indicated in Figure 9A and 9B. CHO cells have previously been shown to have low TSH binding affinity suggesting that little or no TSHR is present in these cells (Chazenbalk et al., 1990). The TSH induced p(PKB/Akt) response was much greater when TSHR was expressed at high levels in TSHR-transfected CHO cells (ANOVA; $p<0.001$, CHO-hTSHR: control vs TSH) in comparison to the low level of p(PKB/Akt) activation in TSH-stimulated vector-control cells (ANOVA; $p>0.05$, CHO: control vs TSH).

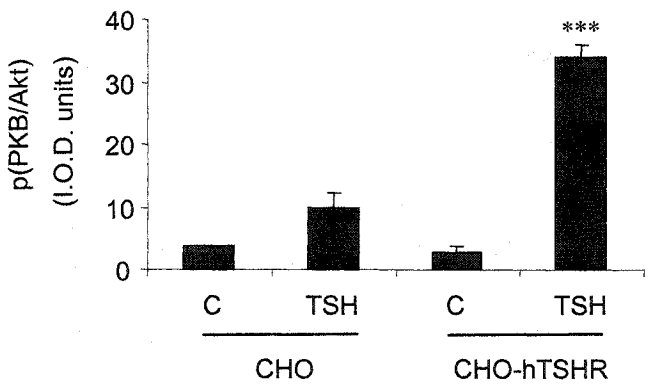
To rule out the possibility that potential contaminants in the bTSH fraction were responsible for PKB/Akt activation, we tested the effects of highly purified bTSH from

Figure 9. TSH-induced activation of PKB/Akt is TSHR-dependent. Confluent CHO-vector control (CHO) and human TSHR-transfected CHO cells (CHO-hTSHR) were stimulated for 30 min with TSH (5 μM). Equal amounts of solubilized protein were resolved on SDS-PAGE and subjected to western analysis. (A) A single representative immunoblot was probed with anti-p(PKB/Akt) antibody and reprobed with anti-PKB/Akt antibody for loading control. (B) Densitometric analysis was performed and data are expressed as integrated optical density units (I.O.D. units). Values are expressed as means±S.E.M. of 3 separate experiments, each performed in duplicate. ***$p<0.001$, CHO-hTSHR: control vs TSH (ANOVA) (C) TSH-NIH activates PKB/Akt. Confluent 3T3-L1 preadipocytes were stimulated for 15 min with TSH-NIH (4 μM). Equal amounts of solubilized protein were resolved on SDS-PAGE and subjected to western analysis. The immunoblot was probed with anti-p(PKB/Akt) antibody.

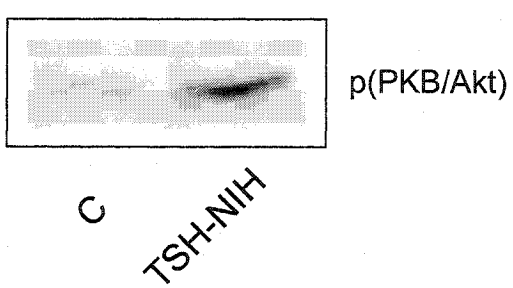
A


p(PKB/Akt)
PKB/Akt
C
TSH
C
TSH
CHO
CHO-hTSHR

B


p(PKB/Akt)
(I.O.D. units)
40
30
20
10
0
***
C
TSH
C
TSH
CHO
CHO-hTSHR

C


p(PKB/Akt)
C
TSH-NIH

the NIH on confluent 3T3-L1 preadipoctyes (Figure 9C). Purified bTSH also induces PKB/Akt activation.

Production of 3-phosphorylated phosphoinositides by PI3K leads to p70 S6K and PKB/Akt activation. The effect of two structurally independent PI3K inhibitors, wortmannin and LY294002, on TSH activation of p70 S6K and PKB/Akt was tested. The first immunoblot in Figure 10 was probed with anti-pp70 S6K antibody, and then reprobed with p(PKB/Akt) antibody, followed by detection with p70 S6K and PKB/Akt antibodies. The addition of 1 μM TSH for 30 min strongly activated of p70 S6K and PKB/Akt. The presence of wortmannin and LY294002 clearly inhibited TSH activation of p70 S6K and PKB/Akt.

Activation of PI3K occurs upon its association with phosphotyrosine-containing proteins following agonist stimulation (Backer et al., 1992; Fry et al., 1992). Increasingly, this is now recognized to occur downstream of G-protein-coupled receptors, as well as receptor tyrosine kinases (Lopez-Ilasaca et al., 1997; Stoyanov et al., 1995). Following TSH stimulation of 3T3-L1 preadipocytes, tyrosine-phosphorylated proteins were immunoprecipitated, then immunoblotted with anti-phosphotyrosine antibody, as shown in Figure 11. A prominent increase in signal intensity is shown in the 125 and 60 kDa regions. Recently, TSH was implicated in the activation of the janus kinase (JAK)1/signal transducer and activator or transcription (STAT) pathway. TSH induced tyrosine phosphorylation of the 125 kDa protein JAK1 in rat thyrocytes and CHO-hTSHR cells (Park et al., 2000). We therefore immunoprecipitated JAK1 from control and TSH-

Figure 10. p70 S6K and PKB/Akt are activated by TSH in a wortmannin or LY294002-sensitive manner. Confluent 3T3-L1 preadipocytes were treated for 30 min with vehicle (C), 1 μM TSH (TSH) with or without 100 nM wortmannin (W) or 10 μM LY294002 (LY). Equal amounts of solubilized protein were resolved on SDS-PAGE and subjected to western analysis with anti-pp70 S6K and reprobed with anti-p(PKB/Akt), and anti-p70 S6K or anti-PKB/Akt antibodies for loading control. The immunoblot is representative of 3 separate experiments each performed in duplicate.

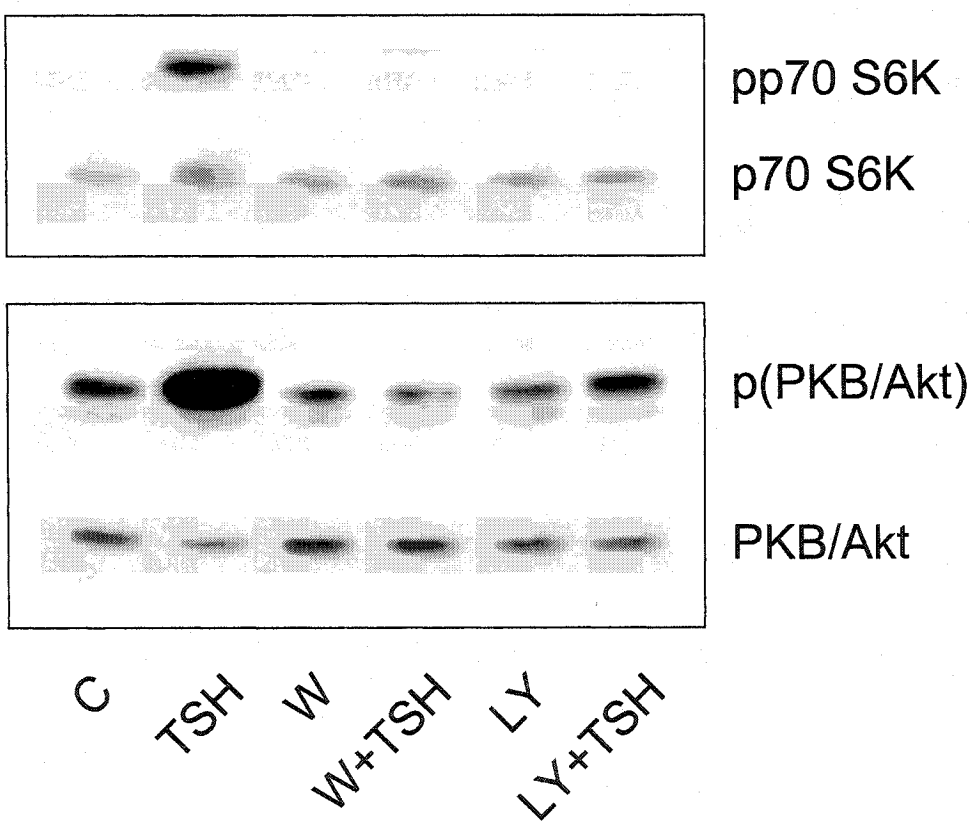
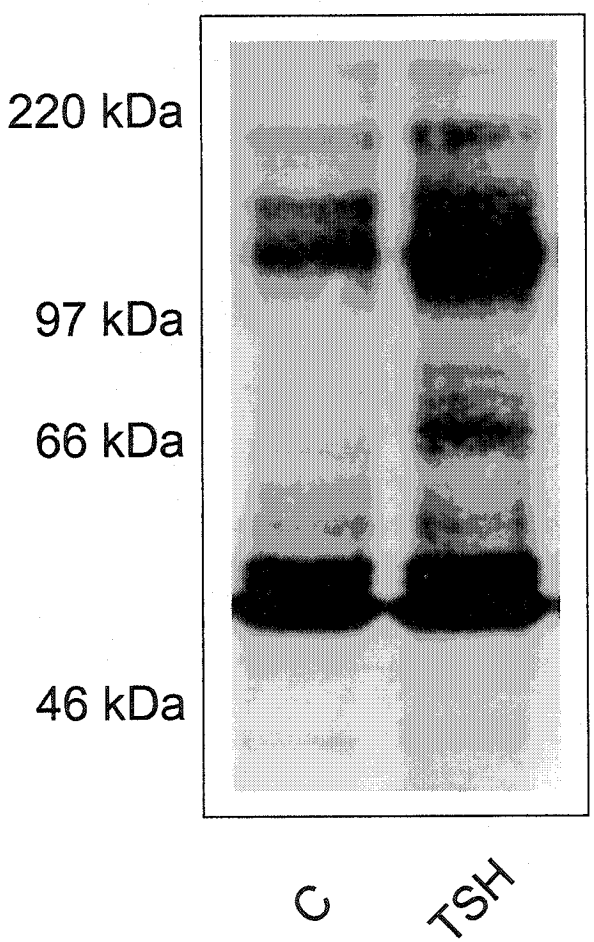

pp70 S6K
p70 S6K
p(PKB/Akt)
PKB/Akt
C
TSH
W
W+TSH
LY
LY+TSH

Figure 11. Tyrosine phosphorylation is induced in TSH-stimulated 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were treated for 30 min with vehicle control (C) or 20 μM TSH (TSH). Equal amounts of solubilized protein were incubated with anti-phosphotyrosine antibody and immunoprecipitated phosphotyrosine-containing proteins were resolved on SDS-PAGE and subjected to western analysis. A single immunoblot probed with anti-phosphotyrosine antibody is representative of 3 separate experiments each performed in duplicate.


220 kDa
97 kDa
66 kDa
46 kDa
C
TSH

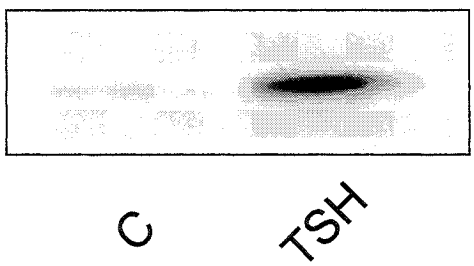
stimulated 3T3-L1 preadipocytes, but did not detect any TSH-stimulated tyrosine phosphorylation of JAK1 (data not shown). Further studies are necessary for identification of this 125 kDa tyrosine phosphorylated protein.

Given that TSH stimulates protein tyrosine phosphorylation, we tested whether TSH promotes the association of PI3K with tyrosine-phosphorylated proteins. Following TSH stimulation, tyrosine-phosphorylated proteins were immunoprecipitated, and the presence of PI3K was evaluated by *in vitro* lipid kinase assay, as shown in Figure 12. The ^{32}P-labeled lipid product PI-3-P was resolved by thin layer chromatography and visualized using autoradiography. A 4-fold increase in PI3K activity, as assessed by the increase in PI-3-P, was observed, and along with the PI3K inhibitor studies, this indicates an important role for PI3K in p70 S6K and PKB/Akt activation.

The activation of p70 S6K by TSH in thyrocytes has been placed downstream of adenylyl cyclase (Cass and Meinkoth, 1998; Cass et al., 1999; Coulonval et al., 2000). We tested whether TSH elevates cAMP levels in 3T3-L1 cells using a cAMP accumulation assay. As shown in Figure 13, TSH did not elevate cAMP levels in 3T3-L1 preadipocytes. However, TSH did increase cAMP levels 4-fold in day 8 differentiated adipocytes (ANOVA; $p<0.05$, adipocytes: control vs TSH). Our data suggest that cAMP is not involved in TSH activation of the PI3K-PKB/Akt-p70 S6K pathway in 3T3-L1 preadipocytes (ANOVA; $p>0.05$, preadipocytes: control vs TSH). Another strategy to address adenylyl cyclase involvement involves the use of inhibitors, but these studies are limited by a lack of readily available specific inhibitors. Future experiments should

Figure 12. PI3K is activated by TSH in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were treated for 15 min with vehicle control (C) or 5 μM TSH (TSH). Cells were lysed, tyrosine-phosphorylated proteins were immunoprecipitated, and PI3K activity was determined by *in vitro* lipid kinase assay, as described. (A) A single representative exposure of PI3P resolved on TLC, as described. (B) Densitometric analysis was performed and data are expressed as integrated optical density units (I.O.D. units). The values are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. Since the amount of tyrosine phosphorylated protein is a function of stimulation, a loading control for protein is not possible. However, equal amounts of solubilized protein were incubated with immunoprecipitating antibody.

A



B

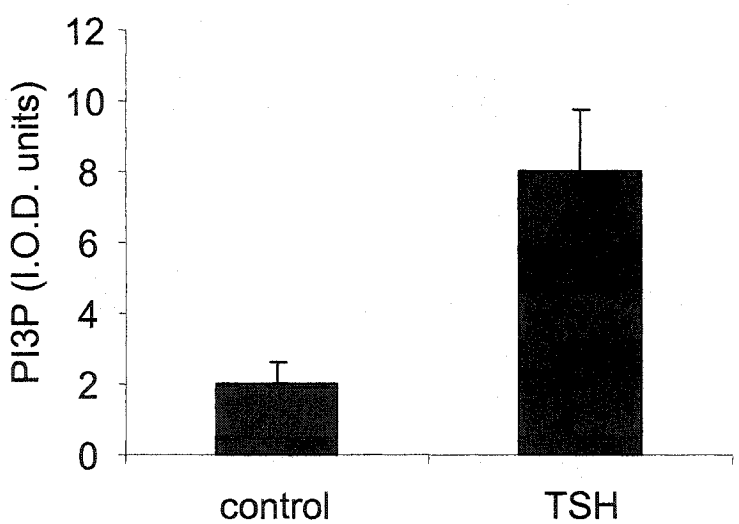
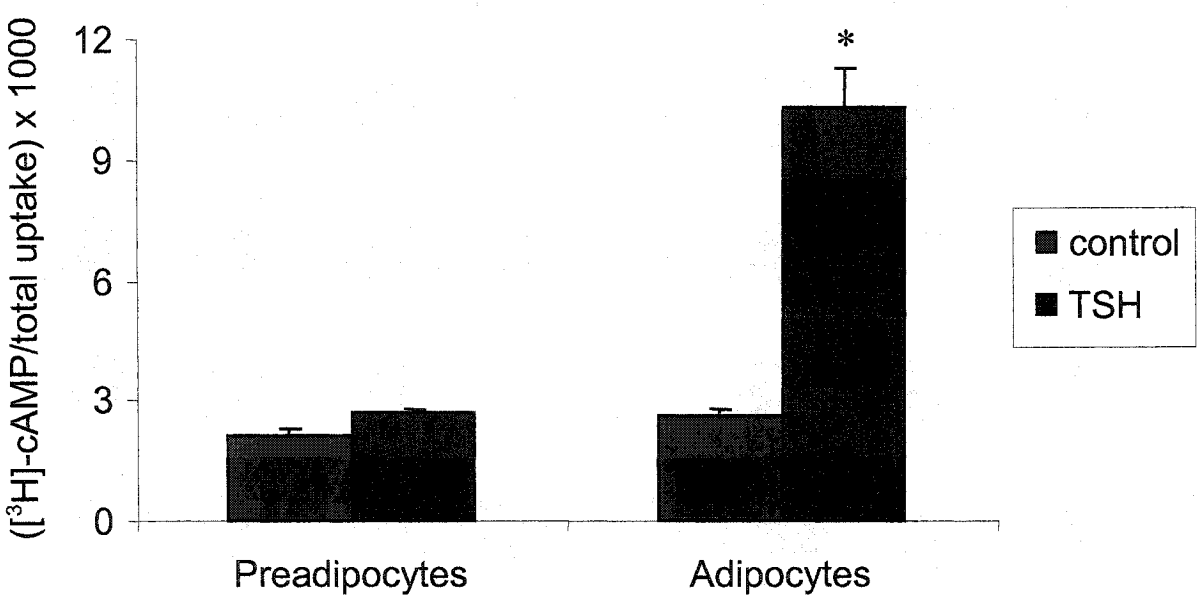


Figure 13. cAMP responsiveness to TSH increases upon differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes maintained 8 days in control medium and adipocytes differentiated in culture were labeled with [^{3}H]-adenine overnight. Cells were treated with or without TSH (5 μM) for 30 min and cAMP was measured. Data are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. *$p<0.05$, versus control (ANOVA).


([3H]-cAMP/total uptake) x 1000
12
9
6
3
0
*
control
TSH
Preadipocytes
Adipocytes

address whether raising cAMP in conjunction with TSH may enhance the response. It is possible that TSH uses two complementary mechanisms to activate PKB/Akt, one being cAMP-dependent. Figure 14 demonstrates that elevating cAMP levels is insufficient for activation of PKB in 3T3-L1 preadipocytes. Although TSH induces strong activation of PKB/Akt, forskolin, a cAMP-elevating agent, had no effect on p(PKB/Akt) levels. Forskolin elevates cAMP levels via direct activation of the adenylyl cyclase catalytic subunit (Insel and Ostrom, 2003; Seamon and Daly, 1986). Forkolin increased cAMP levels in 3T3 L1 preadipocytes and adipocytes as measured by the cAMP accumulation assay (Figure 15).

With the knowledge that PI3K and PKB/Akt play a role in anti-apoptotic signaling, we tested whether TSH promotes preadipocyte survival. As shown in Figure 16, confluent 3T3-L1 preadipocytes were either left in serum or serum-deprived in the absence or presence of 1-20 μM TSH for 6 h. After this incubation, cells were washed and the remaining adherent cells were counted. Our lab has shown that >95% of the adherent cells were viable on the basis of trypan blue exclusion (Gagnon et al., 2001). After 6 h serum-starvation, preadipocyte cell death was reduced 29-76% in cells treated with 1-20 μM TSH (ANOVA; $p<0.05$ at all doses with the exception of 1 μM).

To confirm that TSH promoted preadipocyte survival and did not just result in an increase in preadipocyte number, we applied the TUNEL assay to specifically detect apoptotic cells. In Figure 17, TUNEL-positive cells were counted in ten random fields by two independent observers (200-300 cells/field), and the average from duplicate samples

Figure 14. Elevated cAMP levels have no effect on p(PKB/Akt) levels in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were stimulated for 30 min with vehicle (H_2O), TSH (5 μM), or forskolin (10 μM). Equal amounts of solubilized protein were resolved on SDS-PAGE and subjected to western analysis with anti-p(PKB/Akt) (Ser473), and reprobed with anti-p(PKB/Akt) (Thr308) or anti-PKB/Akt (loading control) antibodies. The immunoblot is representative of 3 separate experiments.

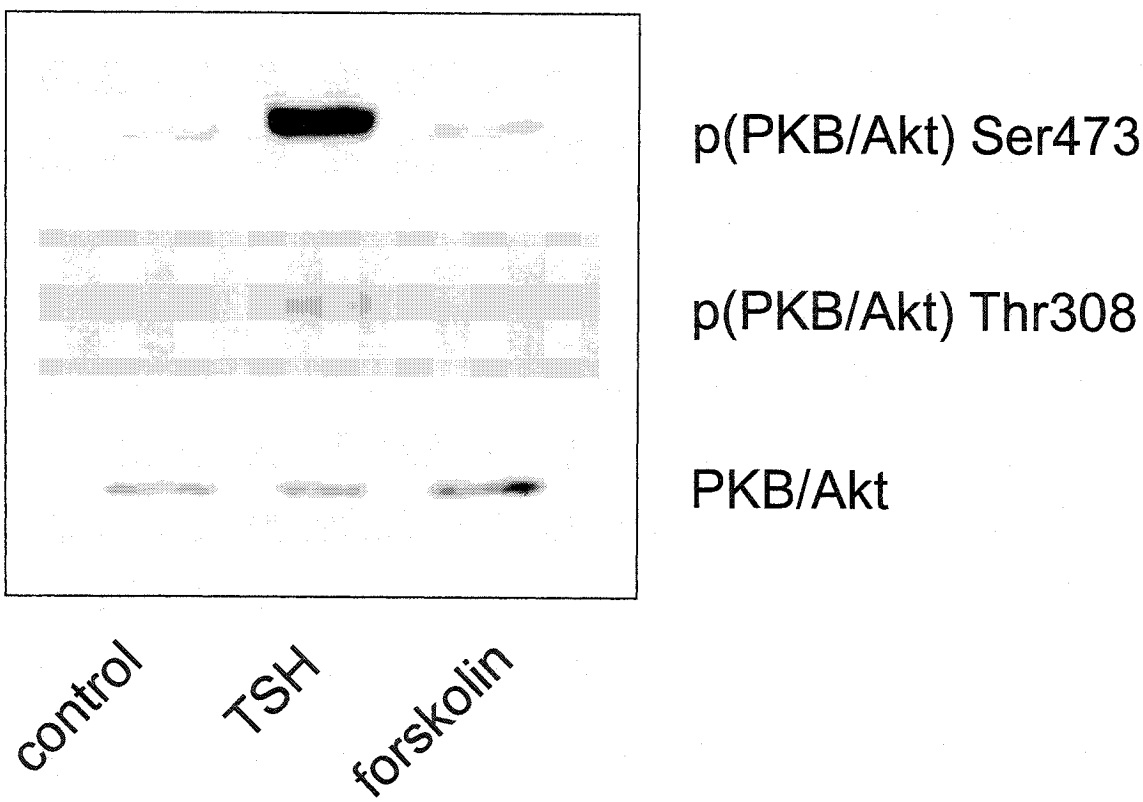

p(PKB/Akt) Ser473
p(PKB/Akt) Thr308
PKB/Akt
control
TSH
forskolin

Figure 15. Forskolin enhances cAMP production in 3T3-L1 preadipocytes and adipocytes. 3T3-L1 preadipocytes maintained 8 days in control medium and adipocytes differentiated in culture were labeled with [^{3}H]-adenine overnight, as described. Cells were treated with or without forskolin (10 μM) for 30 min and cAMP was measured, as described. Data are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate.

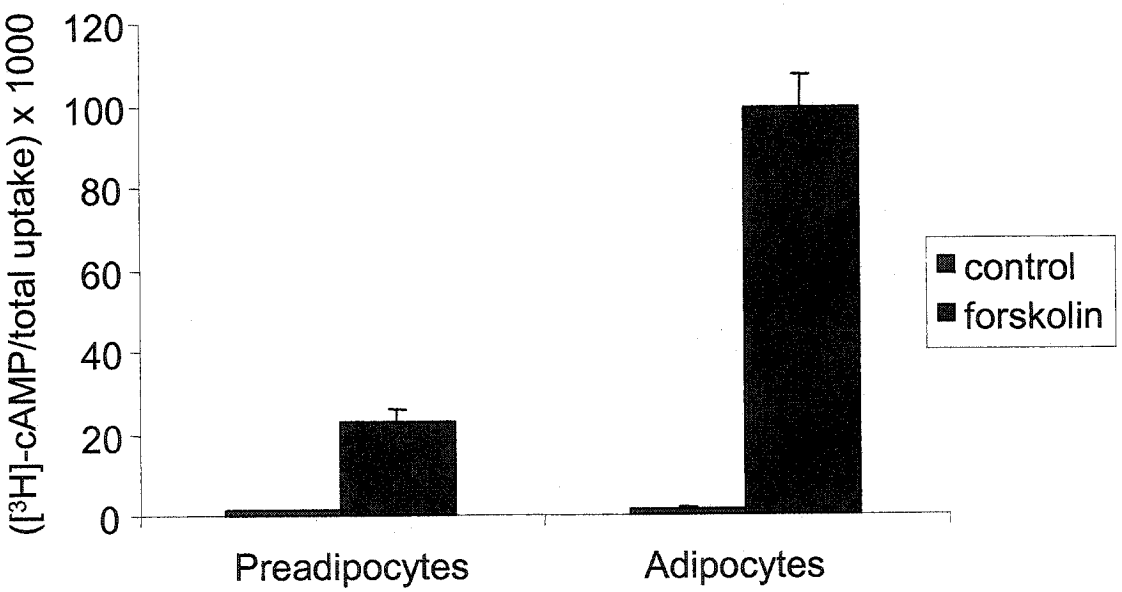

([3H]-cAMP/total uptake) x 1000
120
100
80
60
40
20
0
Preadipocytes
Adipocytes
control
forskolin

Figure 16. TSH reduces 3T3-L1 preadipocyte cell death induced by serum deprivation. Confluent 3T3-L1 preadipocytes were either left in serum or serum-deprived for 6 h in the absence or presence of TSH (1, 5, 10 and 20 μM). Adherent cells were trypsinized and counted in duplicate using a haemocytometer. Data are expressed as means±S.E.M. of 3 independent experiments each performed in duplicate. *$p<0.05$,**$p<0.01$, versus serum-starved (ANOVA).

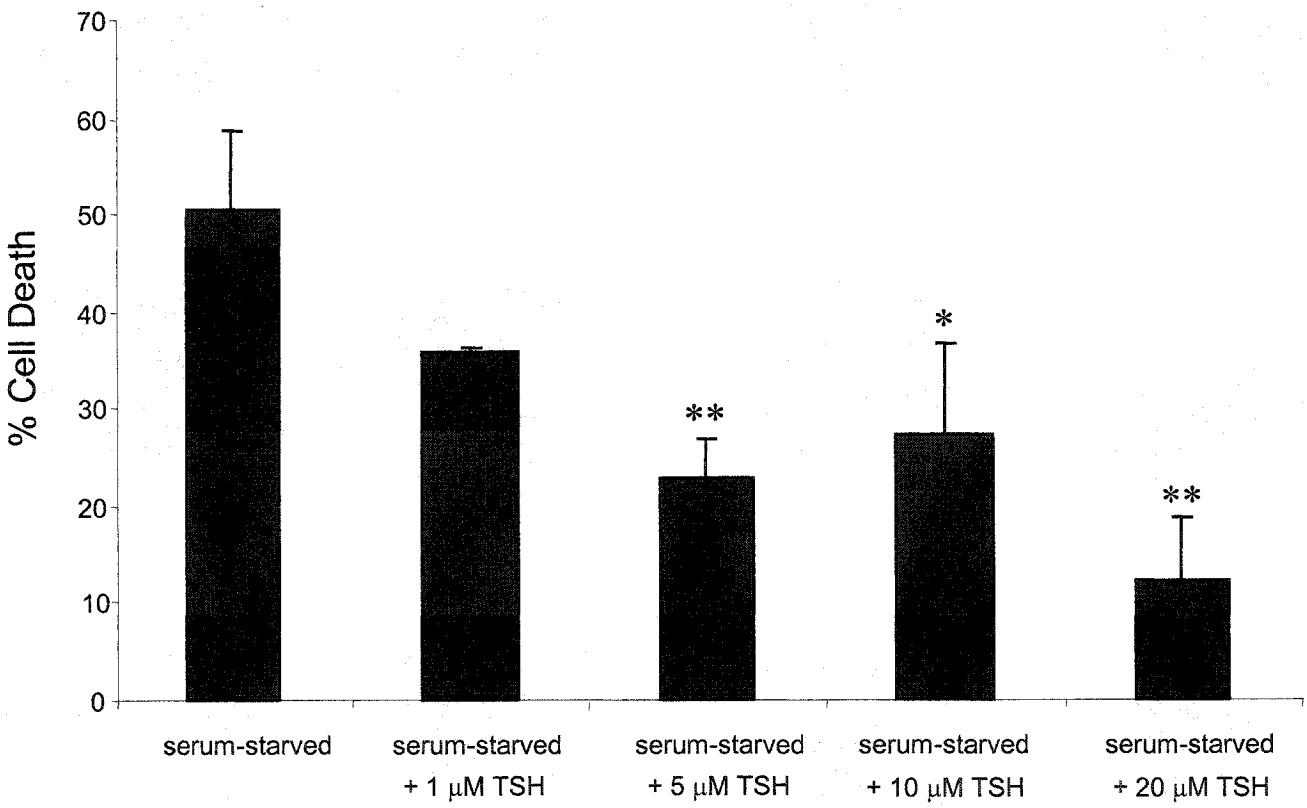
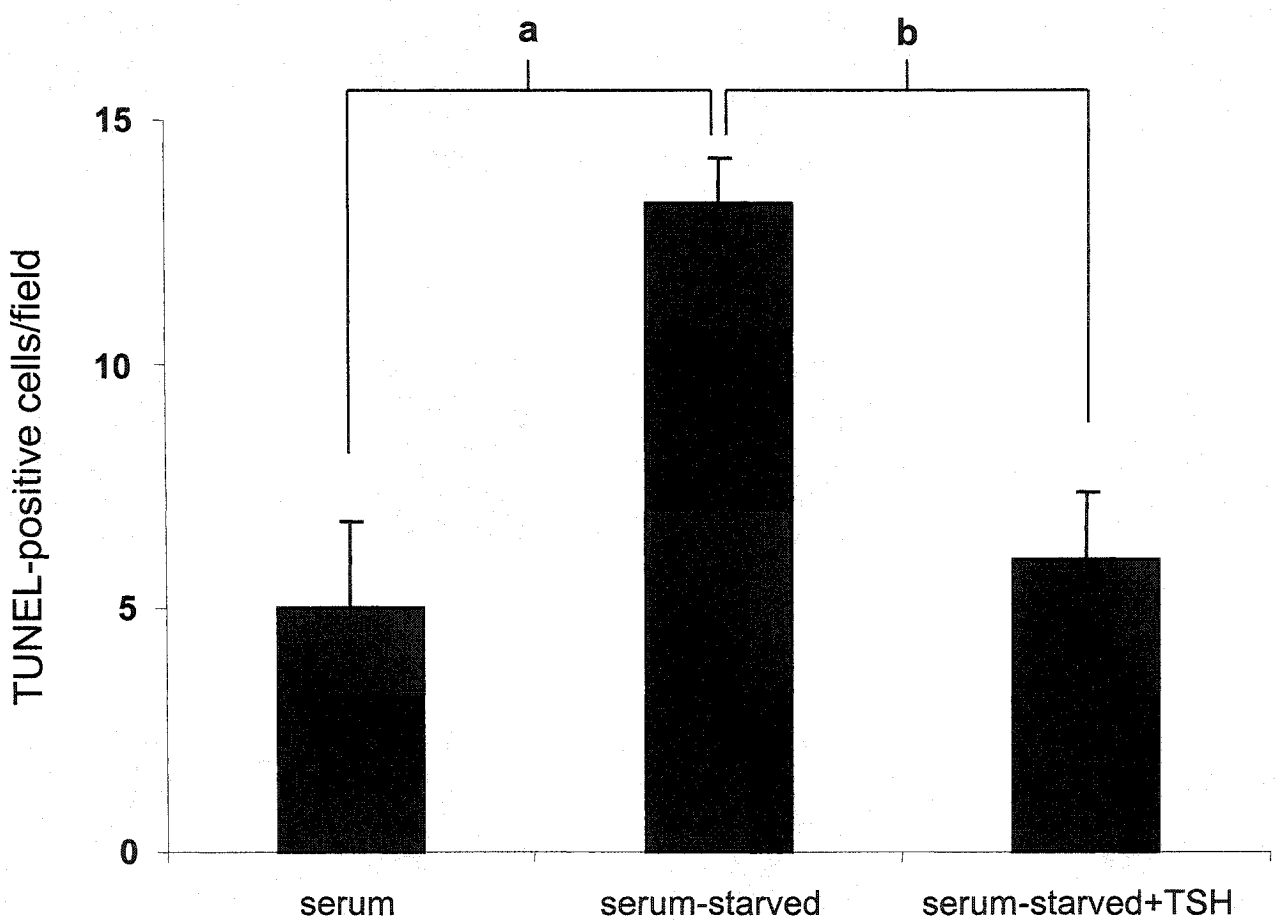

70
60
50
40
30
20
10
0
% Cell Death
**
*
**
serum-starved
serum-starved + 1 μM TSH
serum-starved + 5 μM TSH
serum-starved + 10 μM TSH
serum-starved + 20 μM TSH

Figure 17. TSH reduces TUNEL-positive cells in serum-deprived 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were either left in serum or serum-starved for 3 h in the absence or presence of TSH (20 μM). Following treatment, the cells were subjected to TUNEL and counted. Values are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate.
a, b $p<0.05$, versus serum-starved (ANOVA).


a
b
15
10
5
0
TUNEL-positive cells/field
serum
serum-starved
serum-starved+TSH

was calculated. After 3 h we observed, 5±2 TUNEL-positive cells/field in preadipocytes maintained in serum, 13±1 TUNEL-positive cells/field in serum-starved preadipocytes, and 6±1 TUNEL-positive cells/field in serum-starved preadipocytes in the presence of TSH. The result was an 88% reduction in TUNEL-positive cells with TSH treatment. This indicates that TSH promotes survival, reducing the number of serum-starved apoptotic 3T3-L1 preadipocytes to a level consistent with preadipocytes maintained in serum. A significant difference was observed when the number of TUNEL-positive cells/field from serum-starved preadipocytes were compared to either preadipocytes maintained in serum or serum-starved preadipocytes in the presence of TSH (ANOVA; $p<0.05$).

Since TSH and T_3 act *in vivo* in the same physiological milieu, and T_3 increases lymphocyte apoptosis, we examined whether T_3 regulates 3T3-L1 preadipocyte cell death (Mihara et al., 1999). We examined the effect of T_3 on preadipocyte cell death in the absence and presence of TSH (Figure 18). The presence of T_3 did not alter TSH-induced survival of serum-starved 3T3-L1 preadipocytes.

The majority of apoptosis occurs through caspase 3 (Patel et al., 1996). Activation of caspase 3 during apoptosis involves cleavage of the inactive 32 kDa procaspase into two smaller 17 and 12 kDa fragments. The large 17 kDa and small 12 kDa subunits are both required to form the active enzyme (Krajewska et al., 1997). In Figure 19, 3T3-L1 preadipocytes were serum-starved in the absence or presence of TSH for 24 h and then total cell lysate was resolved on SDS-PAGE and subjected to western analysis. Levels of

Figure 18. T_3 has no effect on TSH-induced 3T3-L1 preadipocyte survival. 3T3-L1 preadipocytes were grown to confluence in the absence or presence of T_3. Confluent preadipocytes were either left in serum or serum-deprived for 6 h in the absence or presence of TSH (20 μM) and/or T_3 (2 nM). Adherent cells were counted, and data are expressed as means±range of 2 independent experiments each performed in duplicate.

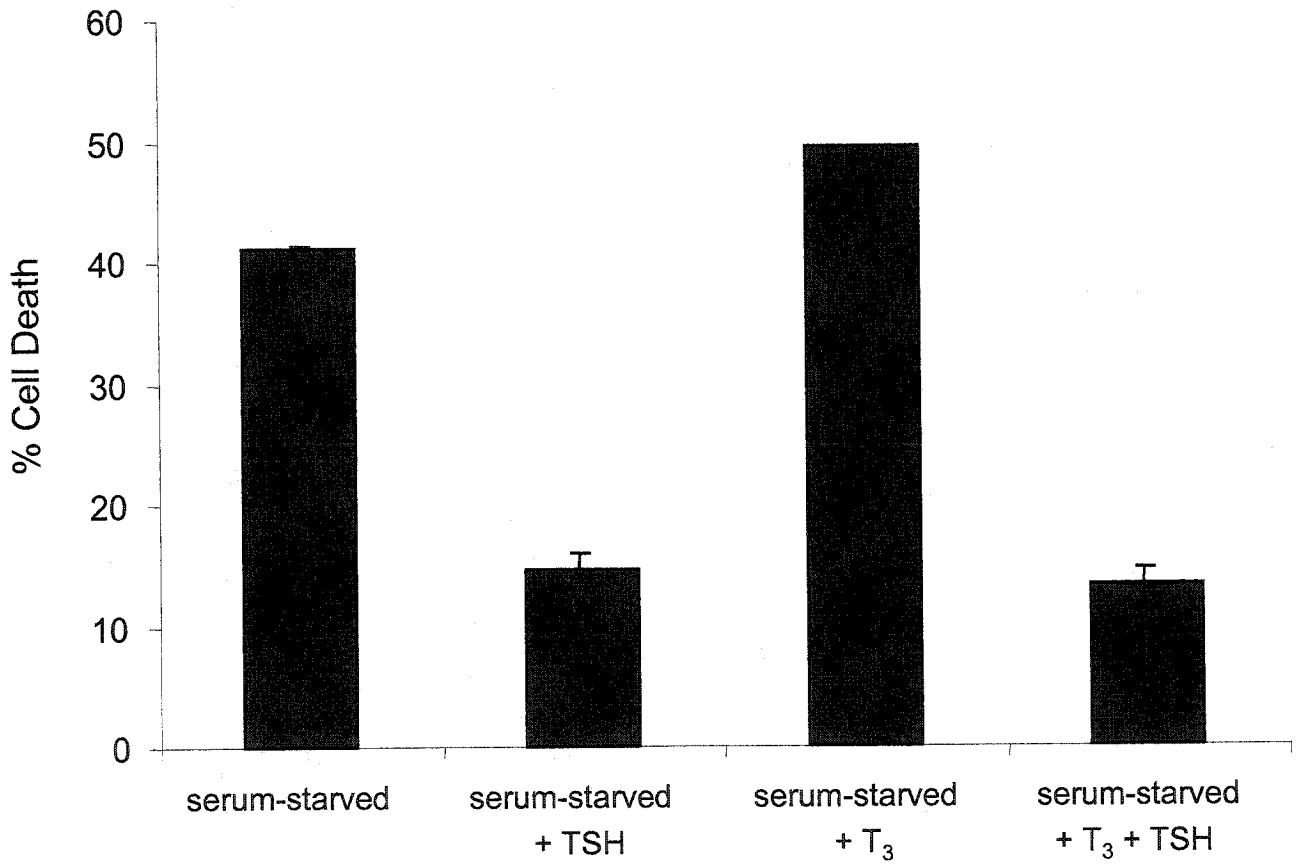
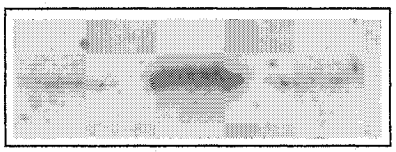

60
50
40
30
20
10
0
% Cell Death
serum-starved
serum-starved + TSH
serum-starved + T_3
serum-starved + T_3 + TSH

Figure 19. TSH reduces the level of cleaved activated caspase 3 in serum-deprived 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were either left in serum or serum-starved for 24 h in the absence or presence of TSH (20 μM). Following treatment, equal amounts of solubilized protein from floating and adherent cells were resolved on SDS-PAGE and subjected to western analysis. (A) A single representative immunoblot was probed with anti-caspase 3 antibody. (B) Values are expressed as means±range of 2 separate experiments each performed in duplicate.

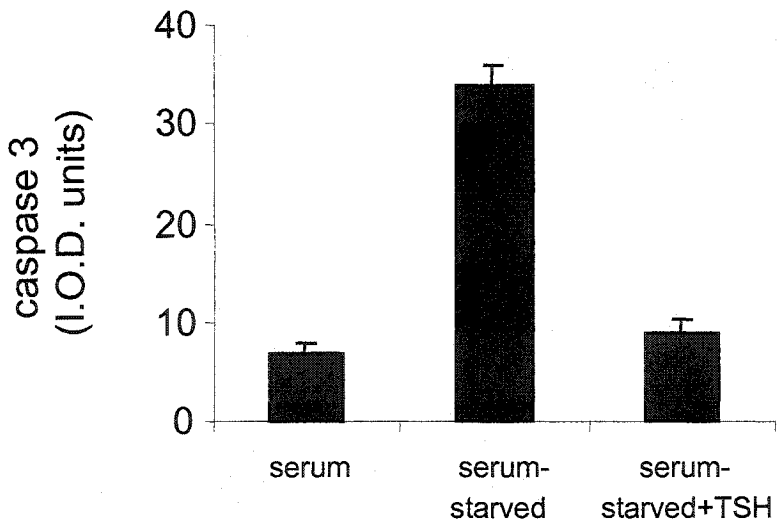
A



caspase 3
(17 kDa cleaved fragment)

serum	+	-	-
TSH	-	-	+

B



the 17 kDa cleaved caspase 3 increased 5-fold when preadipocytes were serum-starved for 24h. TSH reduced the level of cleaved activated caspase 3 by 93%. Future experiments on this topic should include a control immunoblot to ensure equal loading across all lanes.

III. CHAPTER 2 TSH Signaling and IL-6 Secretion from 3T3-L1, 3T3-F442A, and Human Differentiated Adipocytes

A. MATERIALS AND METHODS

Isolation of Human Preadipocytes

Abdominal subcutaneous adipose tissue was obtained from patients undergoing elective abdominal surgery (Ottawa Hospital Research Ethics Committee approval). Adipose tissue samples were collected on the day of surgery and transported to the laboratory in DMEM:F12 (1:1) with 2xPSN, which was 200 units/ml penicillin, 0.2 mg/ml streptomycin, and 100 units/ml nystatin. To isolate the preadipocytes, tissue was placed in sterile phosphate buffered saline (PBS) 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, 60 mg bovine serum albumin (BSA)/g tissue and 3 mls DMEM:F12 PSN supplemented with 33 μM biotin and 17μM pantothenate/g tissue. Collagenase digestion was carried out for 45 min at 37°C, and then 3 mls DMEM:F12 PSN, supplemented with 33 μM biotin and 17 μM pantothenate/g tissue, was added. The suspension was filtered through a 200 μm filter using a hand-held pipette. The filtrate was centrifuged at RT in a Megafuge 1.0 Heraeus swing-out bucket centrifuge without braking at 1000g for 20 min. The top layer of floating mature adipocytes was removed and 10% FBS (final concentration) was added to the infranatant to inactivate residual

collagenase activity. The pellet was resuspended in DMEM:F12 PSN supplemented with 33 μM biotin and 17 μM pantothenate, and serially 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.

Isolation of Mature Human Adipocytes

In one preliminary study, human abdominal omental adipocytes were isolated following the 200 μm filtration step detailed above (Motoshima et al., 2002). Adipocytes (120 μl) were removed from the floating adipocyte layer and left overnight in 800 μl DMEM supplemented with 20% FBS. The next day, adipocytes were stimulated with 1 μM TSH for 4 h and IL-6 levels in the medium were determined, as described below.

Cell Culture of Human Primary Cells and Mouse Cell Lines

Isolated human abdominal subcutaneous preadipocytes were grown in DMEM supplemented with 20% FBS and PSN. 3T3-L1 preadipocytes (ATCC) were grown in DMEM supplemented with 10% CS. 3T3-F442A preadipocytes (from Dr. Howard Green, Harvard University, Cambridge, Massachusetts, U.S.A.) were grown in DMEM supplemented with 10% CS (Green and Kehinde, 1976). PS was added to all media unless otherwise indicated.

Human Preadipocyte Differentiation

Cells were seeded in 24 well plates at $3x10^4$ cells/cm^2 in DMEM supplemented with 20% FBS, grown to confluence, and then placed in serum-free basal or differentiation medium. Differentiation medium consisted of basal medium [DMEM:F12 (1:1), 33 μM biotin, 17 μM pantothenate, 10 μg/ml transferrin, 0.2 nM T_3 and 1 μM insulin] supplemented with 1 μM cortisol, 0.5 mM IBMX, 25 nM dexamethasone, and 5 μM troglitazone (gift from Roche). All media contained 100 units/ml penicillin (P) 0.1 mg/ml streptomycin (S) and 50 units/ml nystatin. The medium was maintained for the first 4 days and was then replaced by basal medium supplemented with 1 μM cortisol every 3 days for 2 weeks.

3T3-L1 Preadipocyte Differentiation

Confluent 3T3-L1 preadipocytes were differentiated in DMEM supplemented with 10% FBS in the presence of 1 μM insulin over 8 days, with 0.25 μM dexamethasone and 0.5 mM IBMX present for the first 2 days. PS was added to all media unless otherwise indicated.

3T3-F442A Preadipocyte Differentiation

Confluent 3T3-F442A preadipocytes were differentiated in DMEM supplemented with 10% FBS in the presence of 1 μM insulin for 8 days. Medium was changed every 2 days. PS was added to all media unless otherwise indicated.

TSH Stimulation Procedure

Differentiated 3T3-F442A and 3T3-L1 adipocytes were placed in DMEM, and treated with 5 μM bovine TSH (Sigma), 1 μM isoproterenol, or vehicle (water) for 4 h. Human adipocytes were placed in DMEM alone or DMEM supplemented with 1% calf serum and/or 10 nM IGF-1 as indicated, and treated with 0.01-5 μM bovine TSH, 1 μM highly purified bovine TSH (TSH-NIH; AFP8755B, National Institutes of Diabetes and Digestive Kidney Diseases; National Hormone and Peptide Program, Torrance, California, U.S.A.), 1 μM recombinant human TSH (rhTSH; Thyrogen), or vehicle (water) for 4 h. When indicated, 100 nM wortmannin, 10 μM LY294002, 10 μM H89, 50 μM PD98059, or vehicle (0.1% DMSO) was added along with the TSH. IL-6 in the medium was measured by enzyme immunometric assay described below (Assay Designs, Inc.).

Determination of IL-6 Levels

1. Human preadipocytes, differentiated adipocytes and isolated adipocytes

Following the stimulation procedure, 100 μl of the appropriately diluted samples and recombinant human IL-6 standards were added to a 96 well plate coated with monoclonal antibody specific to human IL-6, and the plate was tapped gently to mix the contents. After sealing the plate, it was incubated at RT for 1 h on an orbital shaker at 250 rpm. The wells were washed after 1 h and 100 μl rabbit polyclonal antibody to human IL-6 was added to each well. Again, the plate was sealed and incubated at RT for 1 h on the

plate shaker at 250 rpm. After washing, 100 µl IL-6 conjugate (donkey anti-rabbit IgG conjugated to Horseradish peroxidase) was added to each well. The plate was sealed and incubated at RT for 30 min on the plate shaker at 250 rpm. Following the washes, 100 µl of substrate [3, 3', 5, 5' tetramethylbenzidine (TMB) and hydrogen peroxide] was added to each well and the plate was incubated at RT for 15 min on the plate shaker at 250 rpm. Next, 100 µl stop solution (1N HCl) was added to each well. The absorbance values were read at 450 nm and 570 nm using a microtitre plate reader (MRX; Dynatech Laboratories) using Revelation Version 2 software.

2. Mouse preadipocytes and differentiated adipocytes

Following the stimulation procedure, 50 µl of the appropriately diluted samples and recombinant mouse IL-6 standards were added to a 96 well plate coated with monoclonal antibody specific to mouse IL-6, and the plate was tapped gently to mix the contents. After sealing the plate, it was incubated at RT for 1 h on an orbital shaker at 250 rpm. The wells were washed after 1 h and 50 µl biotinylated rat monoclonal antibody to mouse IL-6 was added to each well. Again, the plate was sealed and incubated at RT for 1 h on the plate shaker at 250 rpm. After washing, 50 µl IL-6 conjugate (streptavidin conjugated to Horseradish peroxidase) was added to each well. The plate was sealed and incubated at RT for 30 min on the plate shaker at 250 rpm. Following the washes, 50 µl of TMB substrate was added to each well and the plate was incubated at RT for 15 min on the plate shaker at 250 rpm. Next, 50 µl stop solution (1N HCl) was added to each

well. The absorbance values were read at 450 nm and 570 nm using a microtitre plate reader (MRX; Dynatech Laboratories) using Revelation Version 2 software.

cAMP Accumulation Assay

Human preadipocytes and their differentiated counterparts were labeled overnight in DMEM containing 5% FBS and 2 μCi/ml [^{3}H]-adenine (Iwasiow et al., 1999). The following day, cells were incubated at 37°C in DMEM containing 20 mM HEPES and 1 mM IBMX, and treated with vehicle (H_2O), TSH (5 μM), or forskolin (10 μM) for 30 min. The reaction was terminated upon addition of cold stop solution containing 2.5% (v/v) perchloric acid, 100 μM cAMP and ~5nCi [^{14}C]-cAMP. After a 30 min incubation period at 4°C, acid-cell lysates were transferred to tubes containing 4.2 M KOH for neutralization. Sequential chromatography on Dowex and alumina columns was performed to determine intracellular cAMP levels. Data are expressed as 1000 times the ratio of [^{3}H]-cAMP formed over the total uptake measured per well.

RNA Isolation

Ribonucleic acid (RNA) was isolated using guanidine thiocyanate extraction. Equal amounts of RNA (1 μg) were electrophoresed on a 1% agarose gel containing 3% formaldehyde. Equal loading and RNA integrity were verified by ethidium bromide staining of the 18S and 28S rRNAs in the gel and visualized using Gel Doc 1000 and Molecular Analyst Software Version 1.2 (Bio-Rad Laboratories; Hercules, CA).

RT-PCR

RNA was reverse transcribed using the Retroscript kit (Ambion). RNA (1 μg) was treated with DNase I, denatured and annealed to random decamers. Synthesis of complementary DNA (cDNA) was achieved using Moloney Murine Leukemia Virus reverse transcriptase at 42°C for 15 min to 1 h. The cDNA was amplified using the polymerase chain reaction in the presence of 10X PCR buffer, dNTP mix, IL-6 primers or 18S primers/competimers, and 1 unit of Taq polymerase. PCR conditions were denaturing for 30 sec at 94 °C, annealing for 30 sec at 60 °C, and extension occurred for 30 sec at 72 °C for 35 cycles. An assay establishing linearity was previously performed in our laboratory by Dr. AnneMarie Gagnon. PCR was performed using the GeneAmp PCR System 2400 (Applied Biosystems). To analyze the data, relative band density was calculated as % volume using Molecular Analyst Software Version 1.2 (Bio-Rad Laboratories; Hercules, CA).

Statistical Analysis

Data was analyzed by paired t-test or ANOVA with Tukey's post-test, using GraphPad InStat version 3.00 for Windows 98 (Graph Pad Software, San Diego, CA). Values with $p<0.05$ were considered significant.

B. RESULTS

We assessed the effect of TSH (5 μM) on IL-6 secretion in three adipocyte models under serum-free conditions. Figure 20 shows that TSH (5 μM) treatment of 3T3-F442A adipocytes for 4 h increased IL-6 secretion 5-fold, from 6.5±0.8 to 31.1±2.6 (pg/ml/μg protein; means±S.E.M.; ANOVA; $p<0.01$, vs control.). The addition of isoproterenol (1 μM), a known agonist of IL-6 secretion, induced a 6.3-fold rise in IL-6 secretion, up to 41.0±6.1 (pg/ml/μg protein; means±S.E.M.; ANOVA; $p<0.001$, vs control). A similar response to TSH was observed in 3T3-L1 adipocytes in Figure 21. IL-6 secretion was enhanced 2-fold, from 3.2±0.5 to 6.3±1.1 (pg/ml/μg protein; means±S.E.M.; t-test; $p<0.01$, vs control). Figure 22 demonstrates that TSH stimulated IL-6 secretion in human differentiated abdominal subcutaneous adipocytes by 3.5-fold, from 204±32.6 to 690±39.3 (pg/ml; means±S.E.M.; t-test; $p<0.01$, vs control). Results from TSH studies of IL-6 secretion from human differentiated adipocytes are not normalized to protein content, because the limited number of human preadipocytes available to plate precluded measurement of protein at these low levels. Preadipocytes were plated for differentiation in a smaller size well (24 well; surface area 1.88cm^2).

Given that numerous methods exist for culturing adipocytes, we wanted to examine how adipocytes isolated from an *in vivo* context would respond to TSH stimulation. Mature human abdominal adipocytes were isolated after collagenase digestion of omental adipose tissue followed by centrifugation and size filtration. In one preliminary study, we tested whether TSH could alter IL-6 secretion in these isolated

Figure 20. TSH induces IL-6 secretion from mouse 3T3-F442A adipocytes. Differentiated 3T3-F442A adipocytes were stimulated under serum-free conditions with TSH (5 μM) or isoproterenol (1 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 6 separate experiments each performed in duplicate. **$p<0.01$, ***$p<0.001$, versus control (ANOVA).

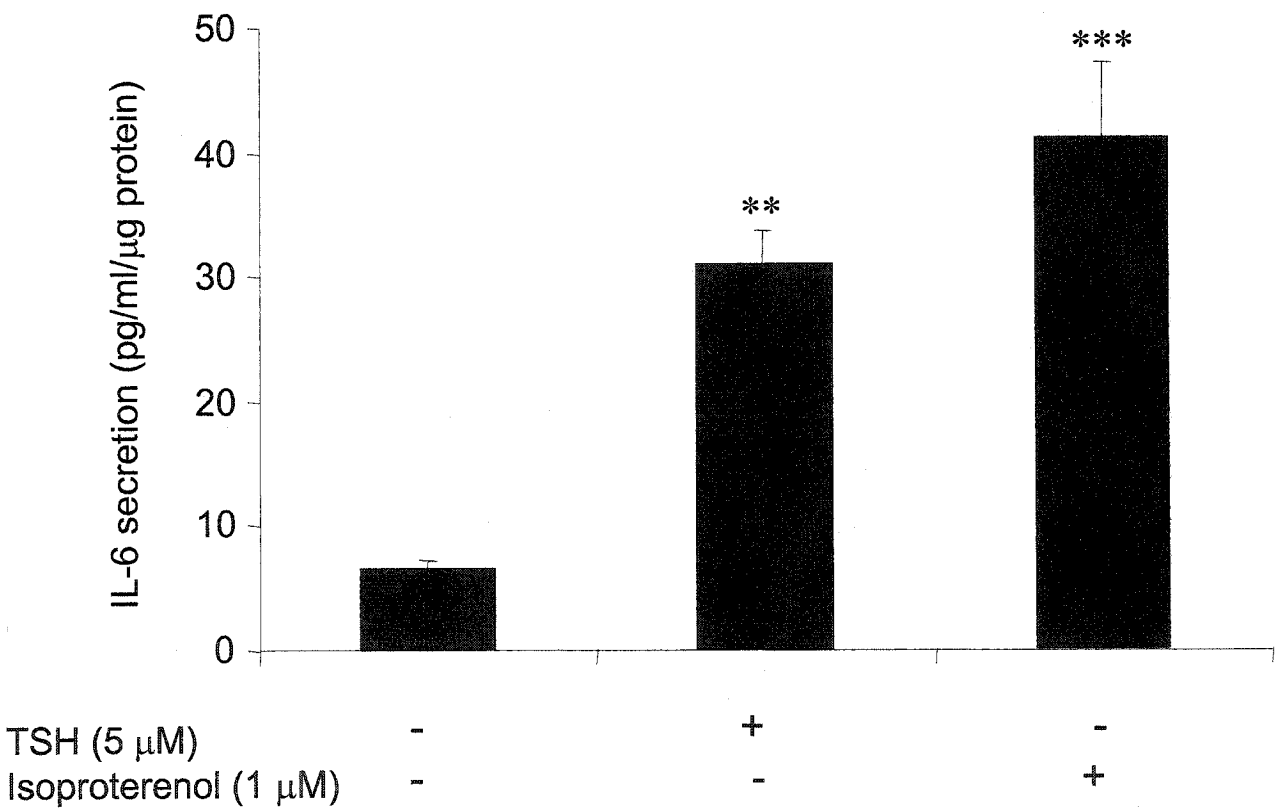

IL-6 secretion (pg/ml/μg protein)
50
40
30
20
10
0
**
***
TSH (5 μM)
Isoproterenol (1 μM)
-
+
-
-
-
+

Figure 21. TSH induces IL-6 secretion from mouse 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were stimulated under serum-free conditions with TSH (5 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 6 separate experiments each performed in duplicate. **$p<0.01$, versus control (t-test).

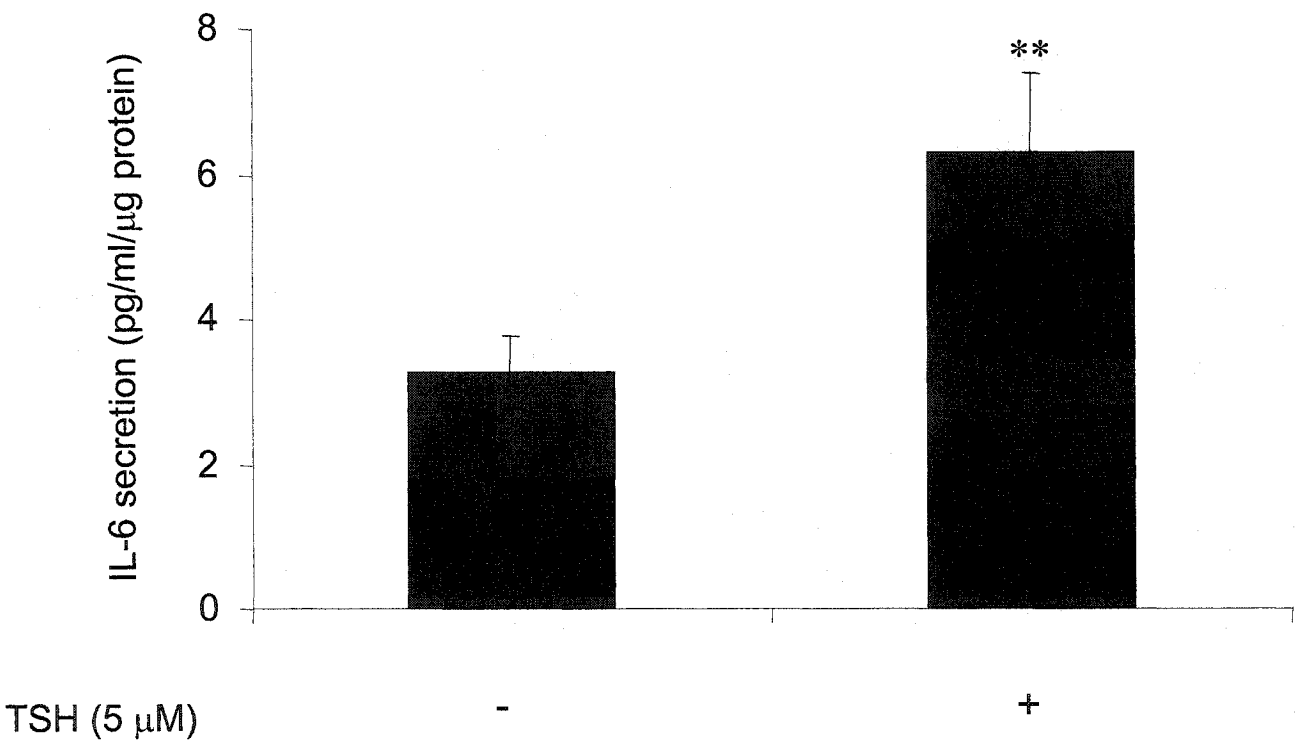

IL-6 secretion (pg/ml/μg protein)
8
6
4
2
0
**
TSH (5 μM)
-
+

Figure 22. TSH induces IL-6 secretion from human abdominal subcutaneous differentiated adipocytes. Differentiated human adipocytes were stimulated under serum-free conditions with TSH (5 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 3 separate patient studies each performed in duplicate. **$p<0.01$, versus control (t-test).

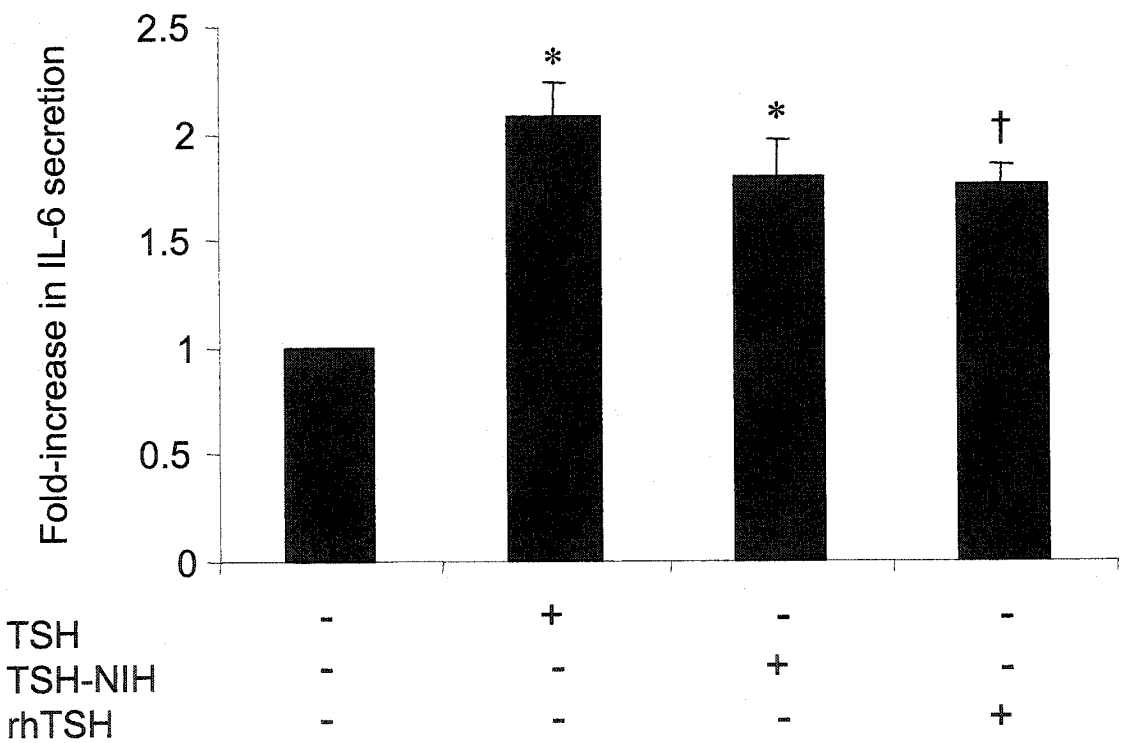

800
600
400
200
0
IL-6 secretion (pg/ml)
**
TSH (5 μM)
-
+

abdominal omental adipocytes. TSH (1 μM) induced a 3-fold increase in IL-6 secretion from the omental adipocytes (data not shown). This positive result confirms that this adipose model is also responsive to TSH. Omental adipose tissue was used for this experiment based on availability.

Some studies have raised concerns about the purity of commercially available TSH preparations, suggesting that contaminants such as pituitary hormones in the TSH preparations could potentially induce signaling pathways independent of TSHR (Correze et al., 2000; Vandeput et al., 2003). To ensure specificity, we added TSH-NIH, a highly purified bovine TSH, or rhTSH, to human adipocytes differentiated in culture (Figure 23). Control IL-6 secretion (pg/ml; means±S.E.M.) was 186±13.9. The adipocyte IL-6 response to either commercial TSH or TSH-NIH was virtually identical and both were statistically significant. There was a 2.1±0.2-fold increase (means±S.E.M.; t-test; $p<0.05$, vs control) in secretion with commercial TSH (1 μM). Stimulation of human adipocytes with TSH-NIH (1 μM) induced a 1.8±0.2-fold increase in IL-6 secretion (means ± S.E.M.; t-test; $p<0.05$, vs control). Addition of rhTSH (1 μM) increased IL-6 secretion by 1.8±0.1-fold (means ± range).

TSH acts in concert with serum and IGF-1 in thyrocyte models (Kimura et al., 2001). When TSH and IGF-1 were added concurrently to FRTL-5 cells, they synergistically enhanced DNA synthesis well beyond the effect of either hormone acting alone. The mechanism by which TSH and IGF-1 act in concert to augment DNA synthesis appears to involve enhanced tyrosine phosphorylation of IRS-2 and subsequent

Figure 23. TSH-NIH and rhTSH induce IL-6 secretion from differentiated human abdominal subcutaneous adipocytes. Following stimulation with 1 μM of either commercial TSH, TSH-NIH, or rhTSH (serum-free conditions) for 4 h, IL-6 secretion was measured as described. Data from 3 separate patient studies, each performed in duplicate, were calculated as fold of basal value and expressed as means±S.E.M. for TSH and TSH-NIH. *$p<0.05$, versus control (t-test). †rhTSH was tested in 2 separate patients in duplicate.


Fold-increase in IL-6 secretion
2.5
2
1.5
1
0.5
0
*
*
†
TSH
TSH-NIH
rhTSH
- + - -
- - + -
- - - +

PI3K activation, as well as upregulation of Src homology 2/a-collagen related protein (Shc) followed by p42/44 MAPK activation (Ariga et al., 2000; Takahashi et al., 1991). We tested the effect of low TSH in the presence of IGF-1 and 1% CS on differentiated human abdominal subcutaneous adipocytes (Figure 24). Medium containing 1% CS supplemented with 10 nM IGF-1 and 0.01 μM TSH significantly enhanced adipocyte IL-6 secretion (ANOVA; $p<0.05$, TSH + IGF-1 + 1% CS vs control or IGF-1 alone). This TSH concentration (0.01 μM TSH), approximately 1 mIU/ml, corresponds with TSH concentrations used for studies on cultured thyrocytes. Future studies examining additional doses of both TSH and IGF-1 in dose response curves would further substantiate this observation.

We explored whether TSH might act through the classic pathway involving the adenylyl cyclase elevation of cAMP levels. The cAMP accumulation assay revealed a 2-fold increase in cAMP levels in TSH-stimulated preadipocytes maintained in control medium as shown in Figure 25. This increase was not considered statistically significant. However, upon differentiation, the responsiveness of adipocytes was enhanced 7-fold (ANOVA; $p<0.05$, vs control).

PI3K has been positioned downstream of cAMP in thyrocyte models (Cass and Meinkoth, 2000; Kimura et al., 2001; Suh et al., 2003). Our preliminary studies suggested that PI3K is not involved in adipocyte TSH-induced IL-6 release as shown in Figure 26. The presence of wortmannin or LY294002 during TSH stimulation of differentiated adipocytes does not appear to influence IL-6 release into the medium.

Figure 24. IGF-1 and low serum levels augment TSH-induced IL-6 secretion from differentiated human abdominal subcutaneous adipocytes. Differentiated adipocytes were stimulated with TSH (10 nM) in the presence of 1% calf serum with or without IGF-1 (10 nM) for 4 h. Following stimulation, IL-6 secretion was measured as described. Data are expressed as pg/ml (means±S.E.M.) of 3 separate patient studies, each performed in duplicate. *$p<0.05$, versus control or IGF-1 alone (ANOVA).

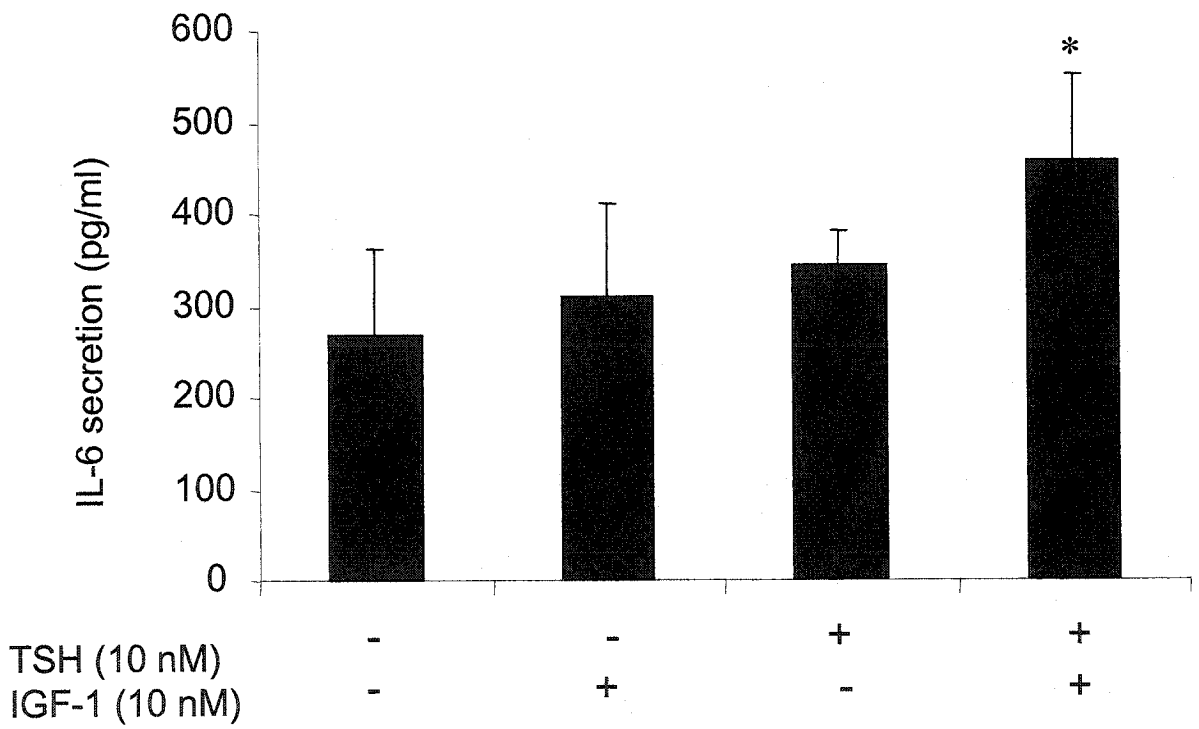

600
500
400
300
200
100
0
IL-6 secretion (pg/ml)
*
TSH (10 nM)
IGF-1 (10 nM)
-
-
+
+
-
+
-
+

Figure 25. cAMP responsiveness to TSH increases upon differentiation of human abdominal subcutaneous preadipocytes. Human preadipocytes and differentiated adipocytes in culture were labeled with [^{3}H]-adenine overnight, as described. Cells were treated with or without TSH (5 μM) for 30 min and cAMP was measured, as described. Data are expressed as means±S.E.M. of 4 separate experiments each performed in duplicate. *$p<0.05$, versus control (ANOVA).

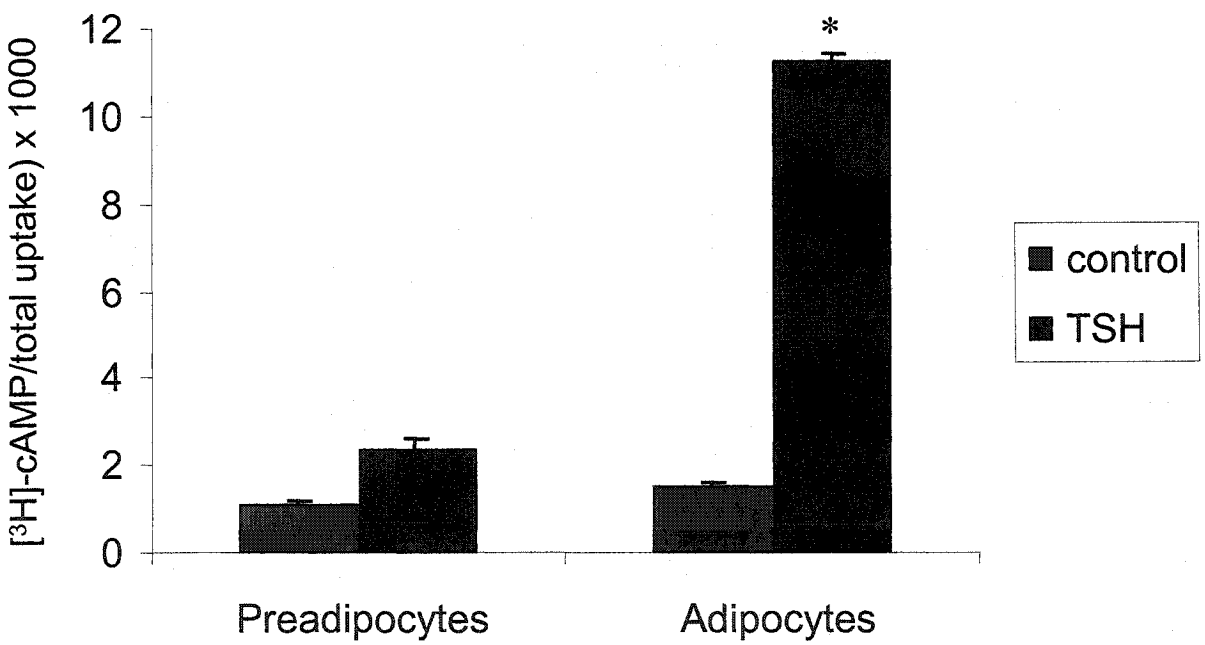
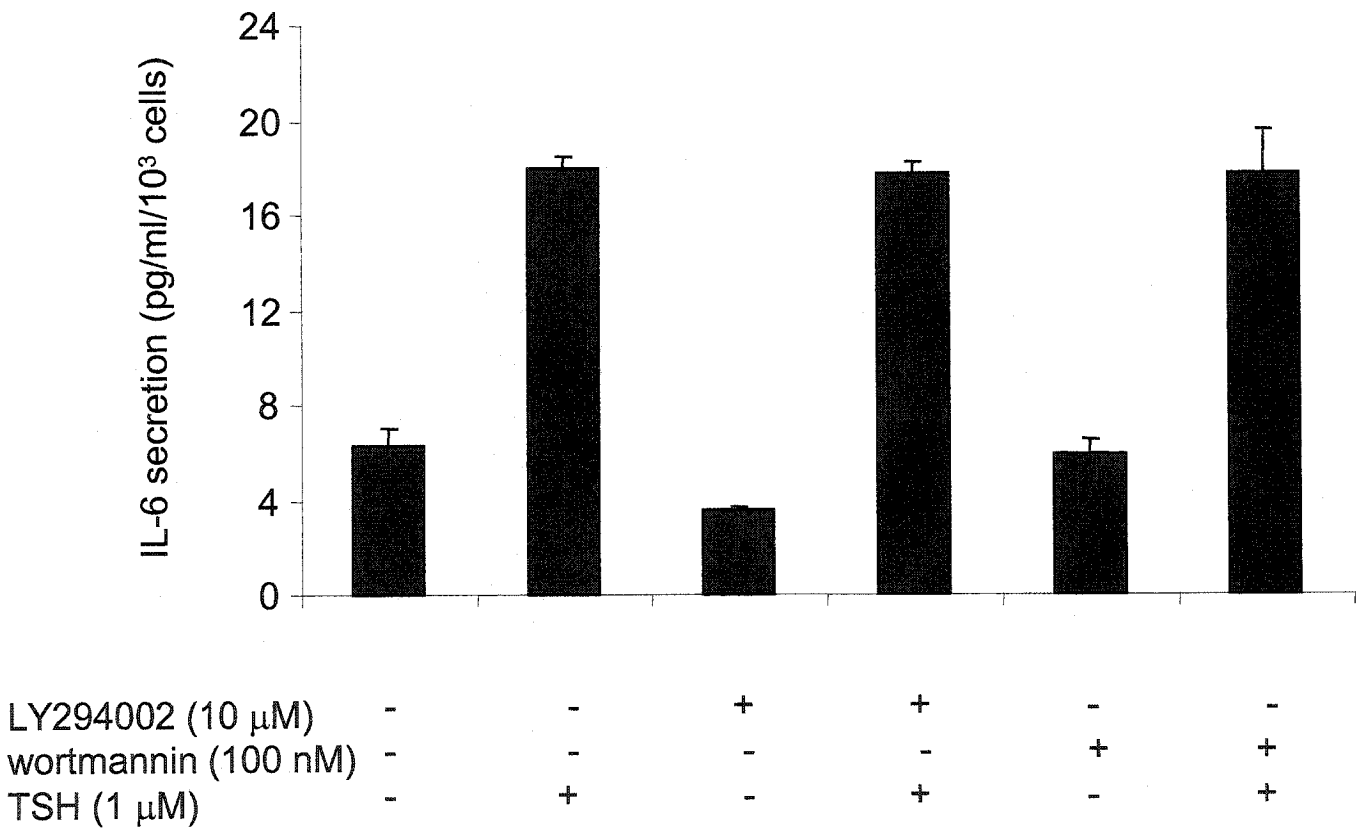

[3H]-cAMP/total uptake) x 1000
12
10
8
6
4
2
0
*
control
TSH
Preadipocytes
Adipocytes

Figure 26. Addition of wortmannin or LY294002 does not alter TSH signaling in human abdominal subcutaneous adipocytes. Day 12 human differentiated adipocytes were stimulated with TSH (1 μM) in DMEM supplemented with 1% CS in the absence or presence of wortmannin (100 nM) or LY294002 (10 μM) for 4 h and IL-6 secretion was measured, as described (n=1). Data are expressed as means±range of 1 experiment performed in duplicate.


IL-6 secretion (pg/ml/10³ cells)
24
20
16
12
8
4
0
LY294002 (10 μM) - - + + - -
wortmannin (100 nM) - - - - + +
TSH (1 μM) - + - + - +

PKA is a well-known downstream target of TSH in thyrocytes. Upon agonist stimulation of adenylyl cyclase, increased levels of cAMP are produced, which in turn have been shown to activate PKA. PKA has long been thought to act as the sole effector of cAMP signaling, but recent studies indicate that cAMP can also directly activate ion channels and GEFs (Kopperud et al., 2003). We tested the effect of the PKA inhibitor H89 on TSH-induced IL-6 release from human subcutaneous adipocytes differentiated in culture. We observed only a modest reduction (15%) in TSH-induced IL-6 release, as shown in Figure 27 (ANOVA; $p>0.05$, TSH vs TSH +H89). TSH signaling in adipocytes does not appear to act through a PKA-mediated pathway.

We postulated that mitogen activated protein kinase (MAPK) might be involved in TSH signaling in adipocytes. Mitogen-activated protein kinase kinase (MEK) is the MAPK kinase situated upstream of p42/44 MAPK. The compound PD98059 is a direct inhibitor of MEK. One study of TSH signaling in the FRTL-5 thyroid cell line revealed that PD98059 led to a significant decrease in TSH-induced DNA synthesis (Iacovelli et al., 2001). In another study, p42/44 MAPK has been implicated in signal transduction leading to IL-6 release from T cells (Dumont et al., 1998). PD98059 significantly inhibited IL-6 production from these cells. We tested the effect of PD98059 on TSH signaling in human adipocytes. In Figure 28, the presence of PD98059 caused a 57% reduction in TSH-induced IL-6 secretion (ANOVA; $p<0.05$, TSH vs TSH + PD98059). MAPK appears to play a major role in human adipocyte TSH signaling. Further studies using *in vitro* kinase assays will be necessary to demonstrate TSH activation of MAPK.

Figure 27. The PKA inhibitor H89 reduces TSH-stimulated IL-6 secretion from human abdominal subcutaneous adipocytes to a minor extent. Differentiated human adipocytes were stimulated with TSH (5 μM) in DMEM supplemented with 1% CS in the absence or presence of H89 (10 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 5 separate experiments each performed in duplicate. $p>0.05$, TSH versus TSH +H89 (ANOVA).

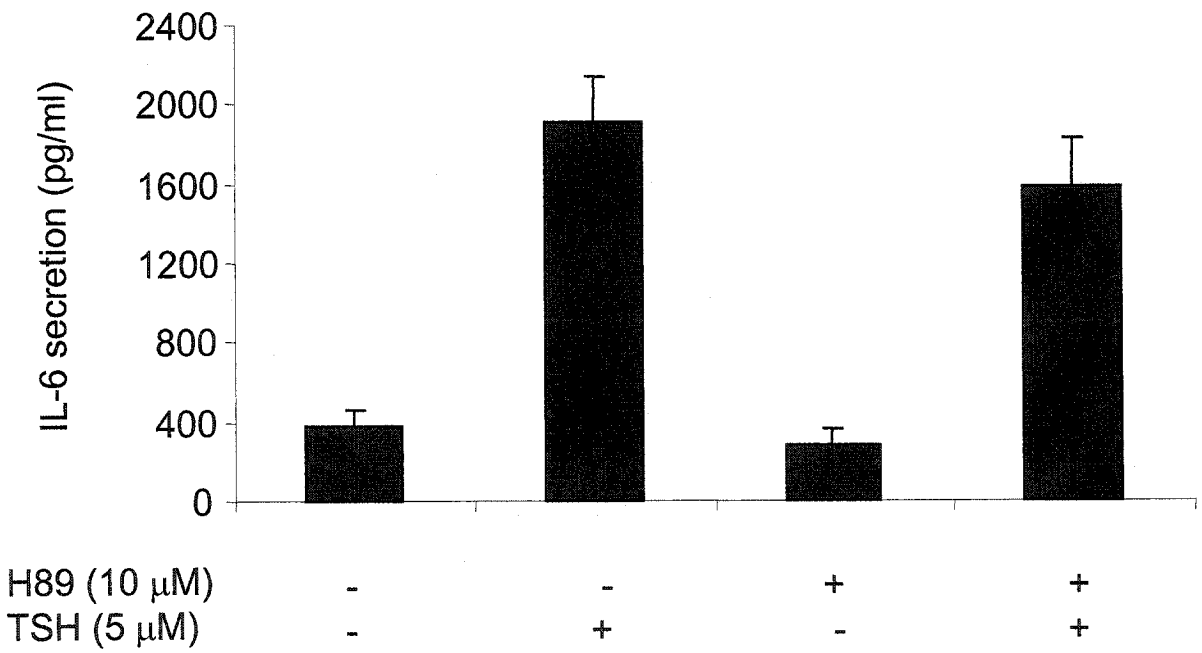
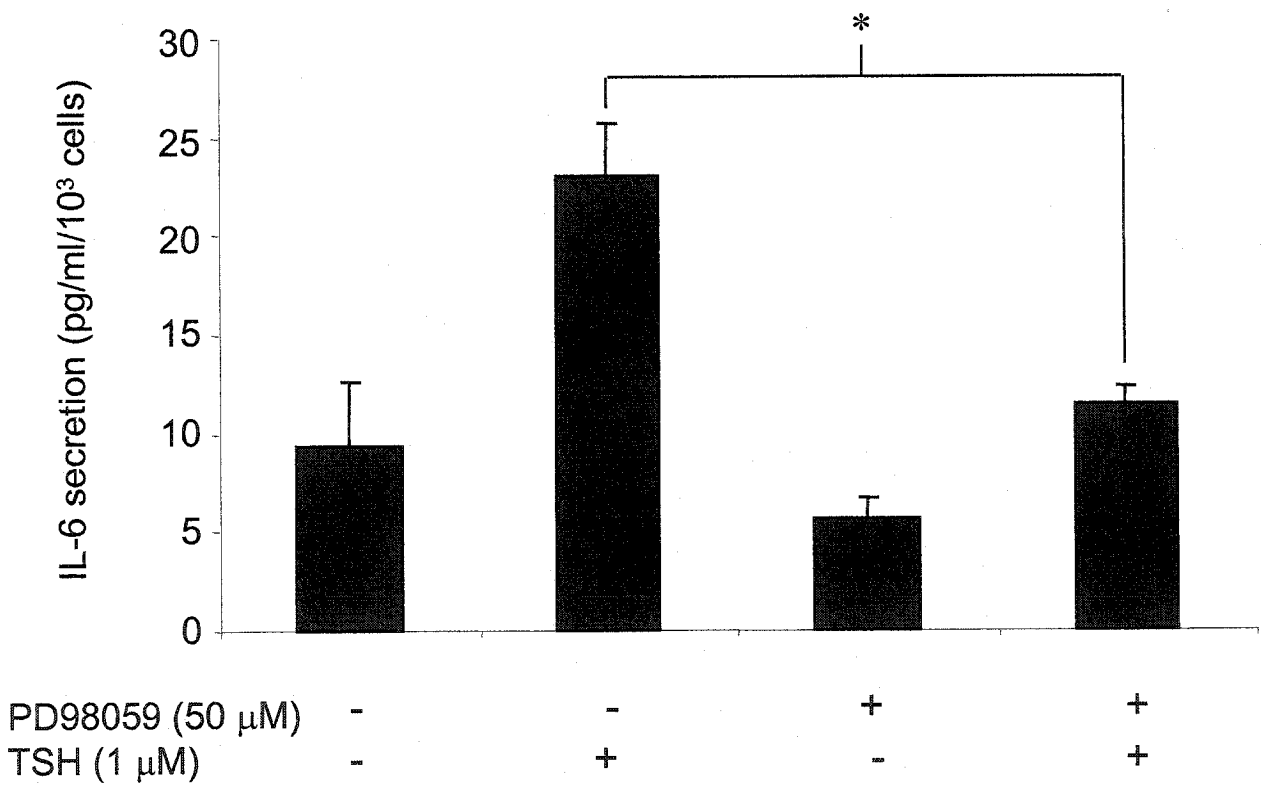

2400
2000
1600
1200
800
400
0
IL-6 secretion (pg/ml)
H89 (10 μM) - - + +
TSH (5 μM) - + - +

Figure 28. The MEK inhibitor PD98059 reduces TSH-induced IL-6 secretion from human abdominal subcutaneous adipocytes. Differentiated human adipocytes were stimulated with TSH (1 μM) in DMEM supplemented with 1% CS in the absence or presence of PD98059 (50 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. *$p<0.05$, TSH versus TSH + PD98059 (ANOVA).


IL-6 secretion (pg/ml/10³ cells)
30
25
20
15
10
5
0
*
PD98059 (50 μM) - - + +
TSH (1 μM) - + - +

To expand our studies of TSH-modulated cytokine secretion, we decided to return to preadipocyte models to examine whether TSH elevates IL-6 secretion from these cells. Several reports indicate that some controversy exists over the exact source of IL-6 release (Clarke and Mohamed-Ali, 2000; Crichton et al., 1996; Fried et al., 1998; Mohamed-Ali et al., 2001; Path et al., 2001; Vicennati et al., 2002; Yudkin et al., 2000). We tested the effect of TSH (5 μM) on 3T3-F442A preadipocytes (Figure 29) and 3T3-L1 preadipocytes (Figure 30). TSH did not induce a significant increase in IL-6 secretion from these cells (ANOVA; $p>0.05$). TSH also failed to induce significant IL-6 secretion from human abdominal subcutaneous preadipocytes (Figure 31; ANOVA; $p>0.05$). For these primary cultures we used a lower concentration of TSH (1 μM) that is closer to physiologically circulating levels. It is possible that at higher supraphysiological doses, TSH may be able to stimulate human preadipocyte IL-6 secretion.

Lastly, we explored whether IL-6 mRNA transcripts are present in human preadipocytes and adipocytes differentiated in culture, as shown in Figure 32. Our data indicate IL-6 mRNA is present in both preadipocytes and differentiated adipocytes. Preadipocyte IL-6 mRNA did not increase with differentiation. IL-6 mRNA levels in preadipocytes stimulated with or without TSH were virtually identical. However, addition of TSH to adipocytes differentiated in culture led to an upward trend in IL-6 mRNA levels. This difference was not statistically significant (ANOVA; $p=0.057$) for data obtained from 3 patients. Increasing patient sample number may highlight this difference

Figure 29. TSH does not effect IL-6 secretion from mouse 3T3-F442A preadipocytes. Confluent 3T3-F442A preadipocytes were stimulated under serum-free conditions with TSH (5 μM) or isoproterenol (1 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. $p>0.05$, versus control (ANOVA).

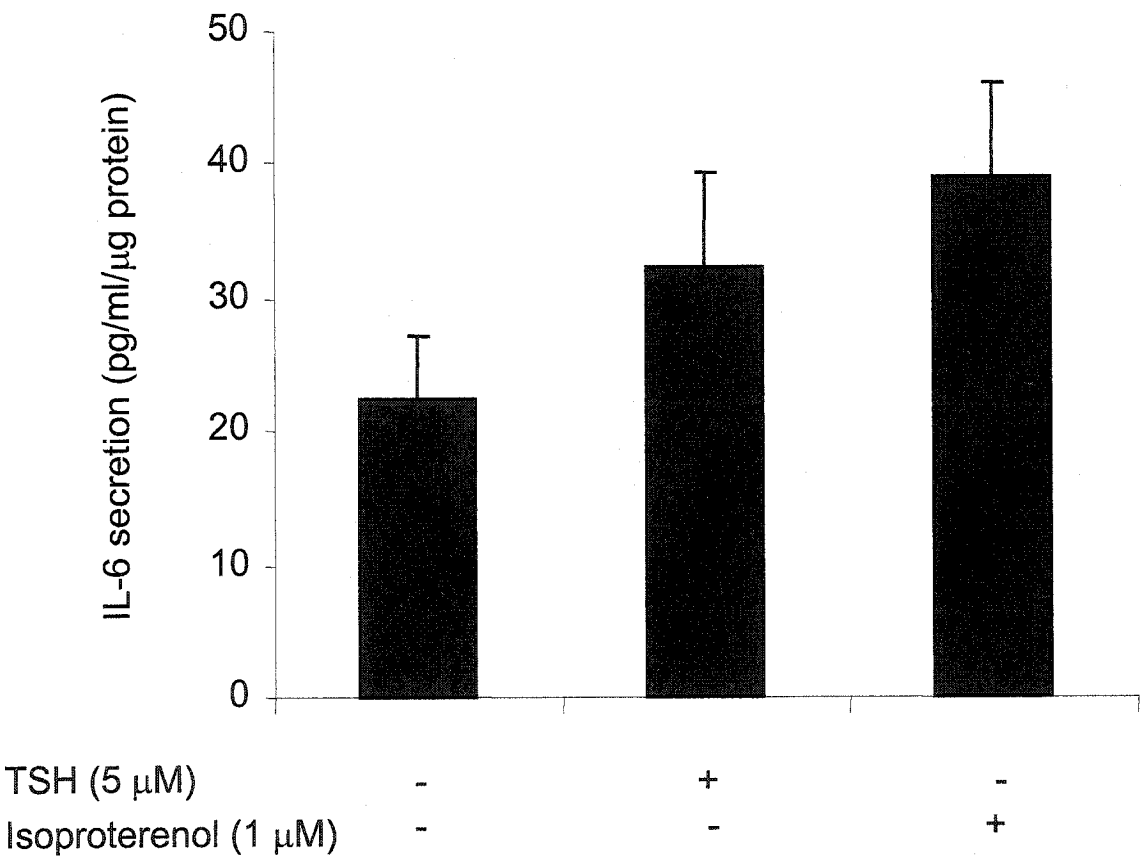

50
40
30
20
10
0
IL-6 secretion (pg/ml/μg protein)
TSH (5 μM)
Isoproterenol (1 μM)
-
+
-
-
-
+

Figure 30. TSH does not effect IL-6 secretion from mouse 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were stimulated under serum-free conditions with TSH (5 μM) or isoproterenol (1 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. $p>0.05$, versus control (ANOVA).

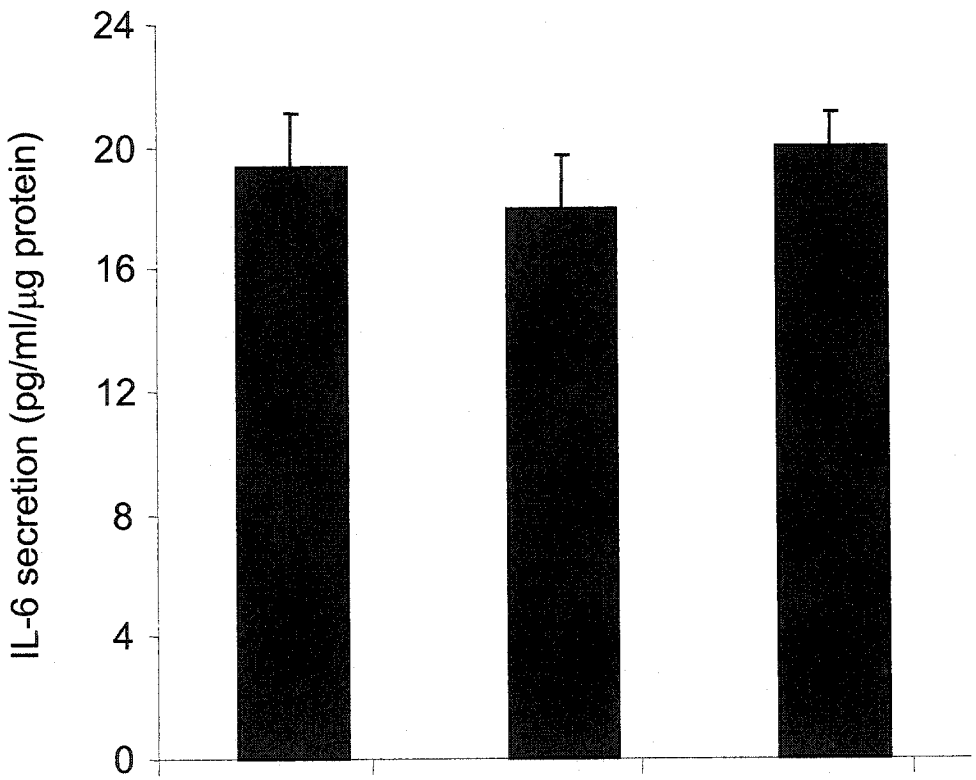

24
20
16
12
8
4
0
IL-6 secretion (pg/ml/μg protein)
TSH (5 μM)
Isoproterenol (1 μM)
-
-
+
-
-
+

Figure 31. TSH does not effect IL-6 secretion from human abdominal subcutaneous preadipocytes. Confluent human preadipocytes were stimulated under serum-free conditions with TSH (1 μM) for 4 h and IL-6 secretion was measured, as described. Data are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. $p>0.05$, versus control (ANOVA).

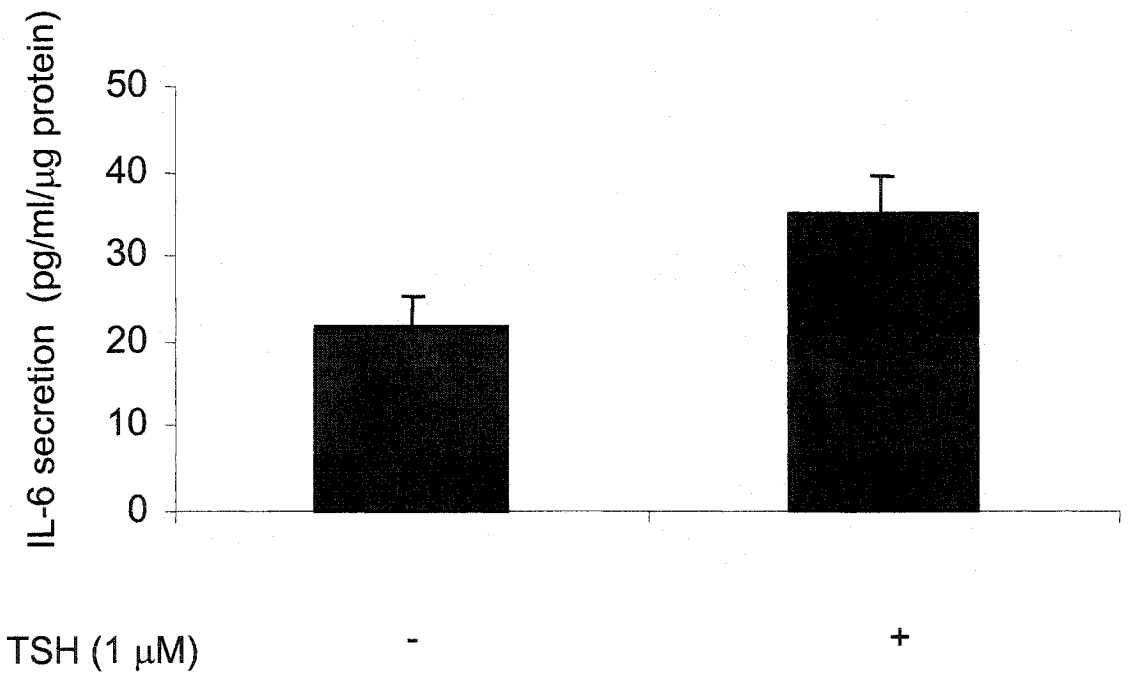
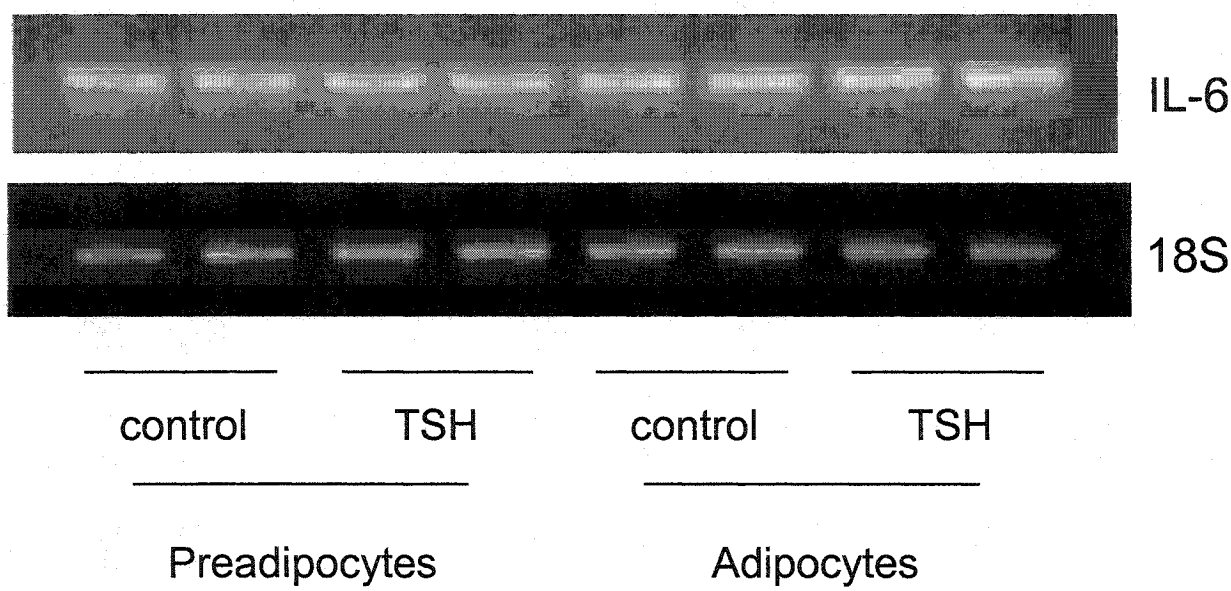
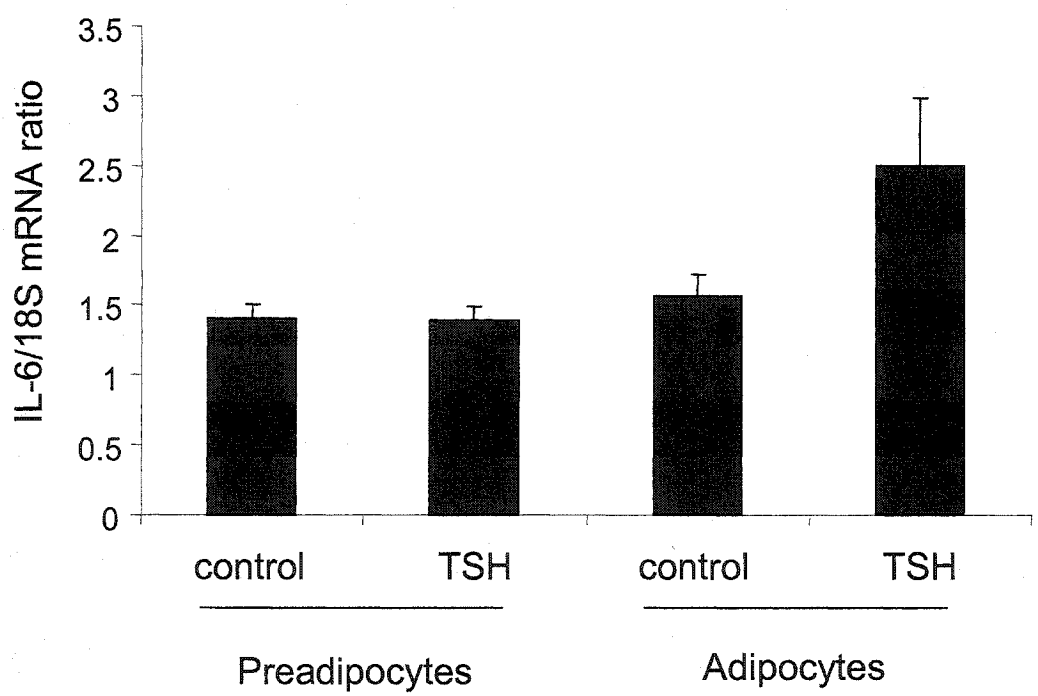

IL-6 secretion (pg/ml/μg protein)
50
40
30
20
10
0
TSH (1 μM)
-
+

Figure 32. IL-6 mRNA is present in human abdominal subcutaneous preadipocytes and differentiated adipocytes. TSH does not enhance IL-6 secretion from either of these cell types. RNA was isolated from human preadipocytes and differentiated adipocytes in culture and subjected to RT-PCR analysis, as described. Band intensity was measured using molecular analyst. (A) A single representative ethidium bromide staining of cDNA fragments of IL-6 and 18S amplified by PCR, as described. (B) Densitometric analysis was performed and IL-6/18S mRNA ratios were calculated. The values are expressed as means±S.E.M. of 3 separate experiments each performed in duplicate. $p=0.057$ (ANOVA).

A


IL-6
18S
control
TSH
control
TSH
Preadipocytes
Adipocytes

B


IL-6/18S mRNA ratio
3.5
3
2.5
2
1.5
1
0.5
0
control
TSH
control
TSH
Preadipocytes
Adipocytes

since responsiveness varies due to the donor heterogeneity observed across human samples.

IV. DISCUSSION

We have shown TSH activates the PI3K-PKB/Akt-p70 S6K pathway in 3T3-L1 preadipocytes, and promotes survival of serum-starved 3T3-L1 preadipocytes. TSH increases IL-6 secretion from 3T3-L1, 3T3-F442A, and human adipocytes differentiated in culture. In order to discuss the distinct findings regarding TSH signaling and cellular function in preadipocytes versus adipocytes, this section of the thesis has been divided into two major sections. First, we will address the effect of TSH on 3T3-L1 preadipocyte signaling and survival, as described in Chapter 1. Second, we will examine the effect of TSH on adipocyte signaling and IL-6 secretion, as described in Chapter 2.

Our previous studies in human preadipocytes indicate TSHR is present, and TSH signal transduction is linked to p70 S6K (Bell et al., 2000). Separate reports examining dog and rat thyrocyte cell models also implicate p70 S6K as a novel target of TSH signal transduction (Cass and Meinkoth, 1998; Coulonval et al., 2000). Meinkoth's group demonstrated that TSH activates p70 S6K in a cAMP-dependent manner, and leads to DNA synthesis in a rat thyrocyte cell model. In a separate study, they showed that the PI3K-PKB/Akt pathway is involved in proliferation, though it acts independently of the PKA signal transduction pathway that activates p70 S6K (Cass et al., 1999). Coulonval et al. demonstrated that TSH activates p70 S6K in a cAMP-dependent fashion, and leads to proliferation of dog thyrocytes. However, they found that TSH was incapable of inducing activation of the PI3K-PKB/Akt pathway.

Our findings demonstrate that TSH activates p70 S6K as well as PI3K-PKB/Akt in 3T3-L1 preadipocytes. We proceeded to explore the potential involvement of the cAMP pathway. Classic TSH signaling involves TSHR activation of G_s, coupling to adenylyl cyclase, and upregulation of cAMP production. We examined the possibility of TSHR coupling to cAMP production in our model system. However, TSH did not raise cAMP levels in 3T3-L1 preadipocytes. To confirm that cAMP alone was incapable of activating PKB/Akt, we added forskolin to raise cAMP levels. Forskolin-elevated cAMP levels were insufficient for activation of PKB/Akt. Our results indicating TSH activates p70 S6K and PKB/Akt in a cAMP-independent manner are inconsistent with the findings in the thyrocyte cell models. Reasons for these differences could include cell type or culture conditions.

We continued to explore potential TSHR-coupled signals targeting p70 S6K and PKB/Akt activation. Given that PI3K is located upstream of PKB/Akt in many cell signal transduction pathways, we decided to test whether TSH activates PI3K. A number of reports in the literature have implicated G-proteins in the activation of class I_A and class I_B PI3K (Brock et al., 2003; Daulhac et al., 1999; Leopoldt et al., 1998; Murga et al., 2000). Since phosphotyrosine-containing proteins associate with and activate PI3K, we initially tested whether TSH induces phosphorylation of tyrosine-containing proteins. Importantly, a role for TSH in tyrosine phosphorylation has been reported in thyrocyte cell models. TSH stimulation of thyroid epithelial cell lines leads to activation of nonreceptor tyrosine kinases (Ariga et al., 2000; Nedachi et al., 2000). We demonstrate

TSH induces a prominent increase in protein tyrosine phosphorylation in the 60 and 125 kDa region. In our search for candidate proteins, we examined whether TSH might activate the ~125 kDa JAK1. TSHR has been shown to activate JAK1 in rat thyroid cells and CHO-hTSHR transfected cells (Park et al., 2000). Our results indicate TSH does not activate JAK1 in 3T3-L1 preadipocytes. The ~120 kDa proline-rich tyrosine kinase 2 (PYK2) has been positioned downstream of another GPCR, endothelin-1, in 3T3-L1 adipocytes (Park et al., 2001). We thought PYK2 could potentially be activated by TSH in preadipocytes, but we were unable to detect PYK2 using western analysis of 3T3-L1 preadipocyte lysate treated with or without TSH (data not shown). We did not attempt to identify the 60 kDa protein but it is conceivable that TSH activates a member of the Src family of tyrosine kinases. A separate GPCR gastrin/CCK-B receptor activates Src-family tyrosine kinases (~60 kDa) that phosphorylate IRS-1, and subsequently lead to PI3K activation (Daulhac et al., 1999). It is also possible that the 60 kDa tyrosine phosphorylated protein is PKB/Akt. One report indicates that PKB/Akt can be phosphorylated at Tyr 474 (Conus et al., 2002). Further studies will be necessary to identify tyrosine-containing proteins that are phosphorylated upon TSH stimulation.

We proceeded to show that PI3K is associated with phosphotyrosine-containing proteins when 3T3-L1 preadipocytes are stimulated with TSH. Our studies examining TSH action in the presence of the PI3K inhibitors wortmannin and LY294002 reveal that PI3K inhibition prevents TSH activation of p70 S6K and PKB/Akt. Wortmannin inhibits PI3K activity by irreversible binding to the p110 catalytic subunit PI3K, but it may also

bind to other PI3K-like proteins (Nakanishi et al., 1995b; Vanhaesebroeck et al., 2001). We also used low concentrations of wortmannin, since higher concentrations have been shown to inhibit myosin light chain kinase and PI4K (Nakanishi et al., 1995a; Nakanishi et al., 1992; Okada et al., 1994). Use of the LY294002 compound in addition to wortmannin is important since it acts through a separate mechanism involving competitive binding with ATP. A given finding can be attributed to PI3K inhibition with more confidence if the same result is observed with both inhibitors. Results from the PI3K *in vitro* kinase assay and PI3K inhibitor studies together suggest a role for PI3K in p70 S6K and PKB/Akt activation. The identity of the TSH-activated PI3K in 3T3-L1 preadipocytes is currently unknown. Given our results demonstrating TSH-stimulated tyrosine-containing proteins associate with and activate PI3K, it seems possible that TSH activates class I_A PI3K since GPCR has been linked to PI3K activation via phosphotyrosine-containing proteins. Alternatively, TSH may lead to $G\beta\gamma$ subunit association with and activation of class I_A PI3K, as this linkage has been described in the literature (Daulhac et al., 1999; Hu et al., 1996b; Murga et al., 2000). $G\beta\gamma$ subunit activation of class I_B p110γ is not likely operating in 3T3-L1 preadipocytes since p110γ expression appears to be restricted to white blood cells (Vanhaesebroeck et al., 2001). Further studies will be necessary to delineate which phosphotyrosine proteins are activated by TSH, and how TSHR affects their phosphorylation.

The cellular function of TSH signaling in preadipocytes was unknown when we began our studies. Given that TSH activates PI3K-PKB/Akt signaling, it seemed

plausible that TSH could promote survival of 3T3-L1 preadipocytes. Earlier studies by Dudek et al. implicated another hormone, IGF-1, in neuronal survival mechanisms acting through the PI3K-PKB/Akt pathway (Dudek et al., 1997). Numerous studies since then have confirmed PI3K-PKB/Akt involvement in anti-apoptotic signaling (Vanhaesebroeck et al., 2001). Recent studies have implicated TSH in FRTL-5 thyroid cell survival (Li et al., 1999; Sato et al., 1999). TSH-mediated survival of serum-deprived FRTL-5 cells is governed by cAMP-dependent events. The possibility of PI3K involvement in TSH-induced thyrocyte survival was not addressed in those studies. Our laboratory has previously demonstrated that IGF-1 survival pathways in preadipocytes are PI3K-PKB/Akt-dependent (Gagnon et al., 2001).

We tested the effect of TSH on preadipocyte survival using two different assessments of cell death that complement each other. Cell enumeration showed that cell death is decreased when serum-starved preadipocytes are treated with TSH. Second, we used the TUNEL assay to confirm that TSH prevented cell death through anti-apoptotic mechanisms. Cell counts enable one to conveniently determine cell death over an extended period of time, but a decrease in cell number cannot be directly attributed to apoptosis. The apoptosis-specific detection assay TUNEL identifies the number of apoptotic cells at a given moment in time. Our data indicating 4% overall apoptosis in 3T3-L1 preadipocytes is comparable to apoptotic rates observed in the literature for human preadipocytes (Niesler et al., 1998). We have not directly demonstrated that PI3K-PKB/Akt is involved in TSH-mediated survival of serum-starved 3T3-L1 preadipocytes.

However, further studies testing the effect of PI3K inhibitors on TSH-stimulated preadipocyte survival using cell counts and TUNEL assay will shed light on this matter.

As another assessment of apoptosis, we measured the levels of cleaved activated caspase 3 in the absence and presence of TSH. The majority of apoptotic signaling occurs through caspase activation. Since PKB/Akt has been shown to inhibit apoptosis by preventing caspase 3 activation, we tested whether TSH could also regulate caspase 3 cleavage and activation. We show that TSH induces cell survival by reducing the levels of active cleaved caspase 3. Our results suggest TSH may play a role in adipose tissue cellular turnover. Another report in the literature has implicated TNFα activation of caspase 3 in the regulation of adipose tissue cell number (Qian et al., 2001).

Lastly, we examined whether T_3 can modulate 3T3-L1 preadipocyte cell death since TSH and T_3 act in concert within the pituitary-thyroid axis. In addition, T_3 has previously been shown to augment lymphocyte apoptosis (Mihara et al., 1999). T_3 did not significantly alter TSH-induced survival of serum-starved preadipocytes.

In contrast to our findings, one report employing a rat model of preadipocytes has indicated that TSH stimulates proliferation, inhibits differentiation (lipoprotein lipase mRNA), and has no effect on apoptosis (DNA laddering) (Haraguchi et al., 1999). The partly differentiated nature of their cells, and the culture conditions they applied, might account for the differences compared with our data.

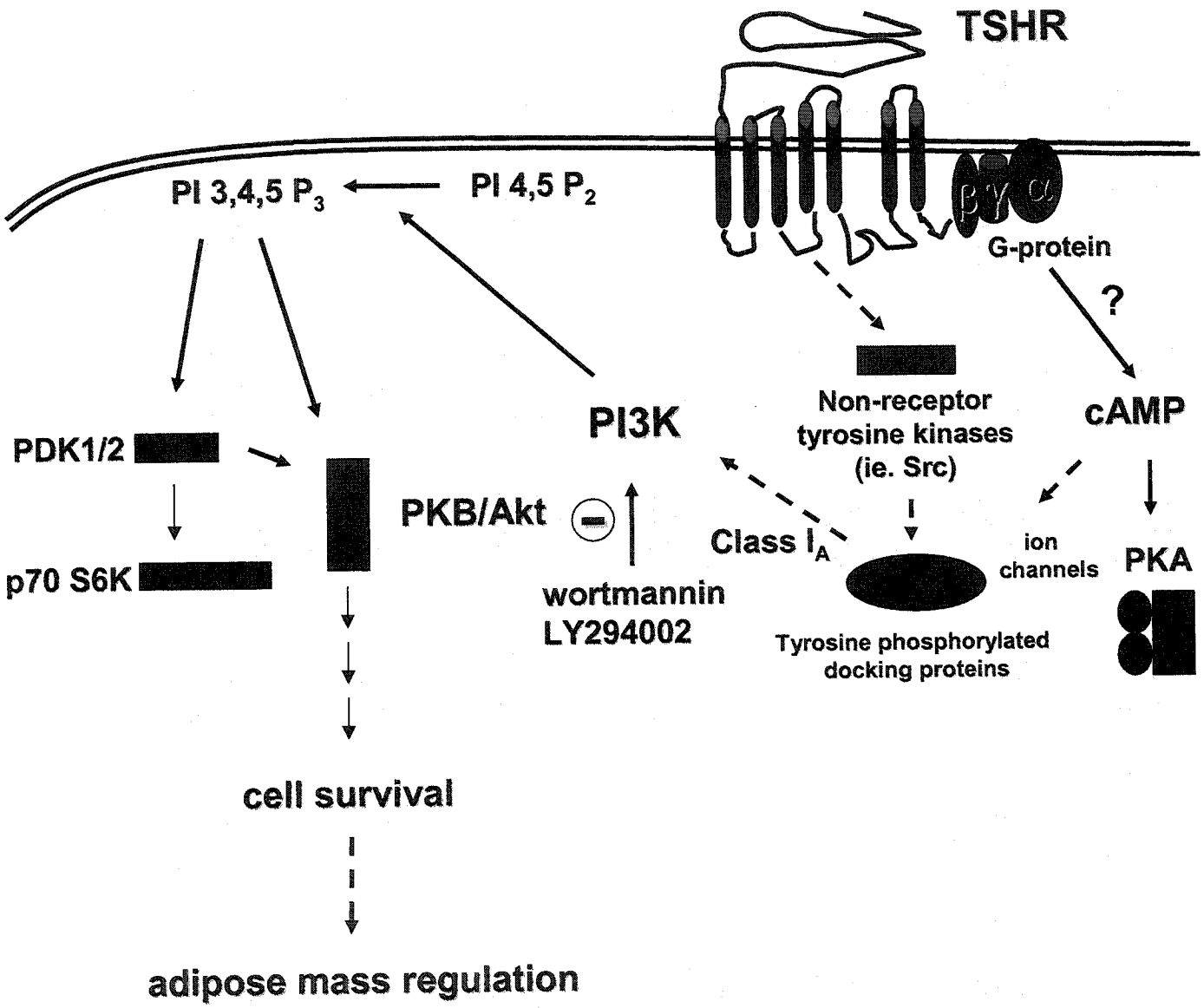
Therefore, to conclude this section, we know that adipose tissue is influenced by the processes of proliferation, differentiation and apoptosis. Our results indicate that TSH

promotes survival of 3T3-L1 preadipocytes. A potential model is developing for TSH signaling in 3T3-L1 preadipocytes as shown in Figure 33. TSH activates PI3K-PKB/Akt signaling that appears responsible for inducing cell survival in serum-starved preadipocytes. We speculate that apoptosis at the cellular level may influence *in vivo* physiology of normal and hypothyroid individuals. Our results raise the possibility that TSH could potentially influence the weight gain observed in hypothyroid individuals though this has not been proven.

In Chapter 2, we turned to the *in vitro* model of TSH signaling in differentiated adipocytes. TSH plays a role in the regulation of adipocytokine secretion. TSH was recently shown to stimulate leptin secretion from human omental adipose tissue (Menendez et al., 2003). In contrast, TSH decreases leptin secretion from rat adipocytes (Shintani et al., 1999). The differences observed in TSH-modulated leptin secretion may be due to the variations between the cell models.

We postulated that TSH may regulate secretion of the pro-atherogenic IL-6, and thus provide a link between TSH and CVD. As described in the introduction, in the population-based cross-sectional Rotterdam study of over 1000 women, TSH was identified as an independent risk factor for CVD comparable to that of other major CVD risk factors (Hak et al., 2000). An additional study of over 2500 subjects revealed that subclinical hypothyroidism is associated with atherosclerosis in men (Imaizumi et al., 2004).

Figure 33. Model of TSH-induced survival signaling in 3T3-L1 preadipocytes. TSHR protein is expressed in 3T3-L1 preadipocytes. TSH does not appear to elevate cAMP levels though increased levels of phosphotyrosine-containing proteins are observed. TSH promotes the association of PI3K with phosphotyrosine containing proteins. Addition of TSH leads to phosphorylation and activation of PKB/Akt and p70 S6K. TSH promotes survival of serum-starved 3T3-L1 preadipocytes in culture.


TSHR
PI 3,4,5 P3
PI 4,5 P2
G-protein
?
PDK1/2
PI3K
Non-receptor
tyrosine kinases
(ie. Src)
cAMP
PKB/Akt
p70 S6K
Class IA
ion
channels
PKA
wortmannin
LY294002
Tyrosine phosphorylated
docking proteins
cell survival
adipose mass regulation

IL-6 release from subcutaneous and omental adipocytes has been well documented (Fried et al., 1998; Mohamed-Ali et al., 2001). Stimulation of adipocytes with insulin and catecholamines leads to a rise in IL-6 secretion (Vicennati et al., 2002). We demonstrate that TSH elevates IL-6 secretion from 3T3-L1, 3T3-F442A, and human abdominal subcutaneous adipocytes. The TSH signal transduction pathway leading to IL-6 secretion in differentiated adipocytes is largely unexplored.

We undertook studies to examine the mechanism of IL-6 protein secretion from human adipocytes. In contrast to the human preadipocyte model, we show TSH elevates cAMP levels in human differentiated adipocytes. Therefore, upon adipocyte differentiation, a change in TSHR coupling to cAMP is observed. Given the cAMP involvement in adipocyte signaling, we tested whether PKA, a well-known effector of cAMP, plays a role in TSH signaling in these cells. Surprisingly, PKA appears to play only a minor role in TSH-induced IL-6 secretion from human adipocytes. However, our results are based solely on the use of the cell permeable H89 inhibitor. H89 has been used extensively in studies of PKA action. It inhibits PKA through competitive binding with ATP; however, it is important to note that a number of limitations exist with regard to its use (Penn et al., 1999). For example, studies employing high concentrations of H89 (28-38 μM) have indicated that H89 is also capable of inhibiting myosin light chain kinase, Ca2+/calmodulin-dependent protein kinase II, protein kinase C, and casein kinase I (Chijiwa et al., 1990). In our studies, we used a low dose of H89 (10 μM) that appears to be specific for PKA (Tsygankova et al., 2001).

It has been recently recognized that there are alternate cAMP effectors other than PKA. Novel cAMP targets include ion channels and GEFs for small GTPases Rap1a, Rap2 and Ras (Sprang, 2001). GEFs may be involved in TSH-induced IL-6 secretion from human adipocytes. Epac is a particular example of a GEF whose main effectors include the small GTPases Rap1 and Rap2 (Bos, 2003). Three papers have raised the possibility that Epac may play a role in TSH signaling in the thyroid (Dremier et al., 2000; Iacovelli et al., 2001; Vanvooren et al., 2001). One study suggests Epac can lead to activation of p42/44 MAPK in TSH-induced proliferation of FRTL-5 cells (Iacovelli et al., 2001).

We explored other potential downstream targets of cAMP signaling in human adipocytes. As described previously, the PI3K-PKB/Akt pathway has been implicated in TSH signaling in thyrocyte cell models. Our preliminary studies, using the PI3K inhibitors wortmannin and LY294002, indicate that PI3K does not appear to be involved in TSH-induced IL-6 secretion from human adipocytes. Additional studies employing the PI3K *in vitro* kinase assay should be performed to determine whether TSH activates PI3K.

Some studies have implicated the MAPK pathways in the control of IL-6 secretion from T cells. For example, p42/44 MAPK has been implicated in signal transduction leading to IL-6 secretion from T cells (Dumont et al., 1998). Our studies employing the synthetic MEK inhibitor PD98059 revealed that p42/44 MAPK plays a significant role in TSH-induced IL-6 secretion from human adipocytes. PD98059 binds

inactive MEK and prevents its phosphorylation and activation (Alessi et al., 1995). GPCR activation of MAPK signaling pathways has been described for numerous stimuli operating in many different cell types (Pierce et al., 2002). Activation of p42/44 MAPK in particular has been shown to occur through Gβγ activation of Ras or Src family members (Lopez-Ilasaca, 1998). The precise mechanisms involved in these signaling pathways are still under investigation.

Future experiments should be carried out to confirm that TSH activates p42/44 MAPK in human adipocytes. Human differentiated adipocytes can be stimulated with TSH and MAPK activation can be examined using western analysis with phospho-MAPK antibodies. In addition, human adipocytes can be stimulated with TSH and MAPK can be immunoprecipitated and subjected to *in vitro* kinase assay. Future studies will be necessary to confirm the possible control points of human adipocyte secretion of IL-6.

A new concept is developing in the field of adipose tissue biology. A number of non-fat cells including macrophages, appear to be a new source of factors that influence signaling in adipose tissue. The non-digestible stromal fraction of adipose tissue also appears to release cytokines in culture. Traditionally, macrophages are associated with immune system processes. In the last few years, investigators have observed a macrophage infiltration in the adipose tissue of obese individuals (Savage et al., 2001; Steppan et al., 2001a; Weisberg et al., 2003; Xu et al., 2003). Macrophages share similarities with adipocytes, including lipid storage and cytokine release (Lehrke and

Lazar, 2004). Additional studies will be necessary to tease out the function of macrophages and other non-fat cells within the highly dynamic adipose tissue setting.

The regulation of IL-6 secretion has not been well characterized at the transcriptional or posttranscriptional level. The nuclear transcription factor NF-κB appears to play a role in transcriptional regulation of IL-6 secretion from numerous cell types but this remains to be determined in adipocytes. A study by Hauner's group hints at NFκB involvement in IL-6 secretion, but direct activation has yet to be demonstrated in adipocytes (van Harmelen et al., 2003). Little is known about posttranscriptional regulation of IL-6 release. Insulin has been implicated in the regulation of vesicle transport pathways for GLUT-4 and leptin in adipocytes. The mechanism of insulin-induced leptin secretion occurs independently of transcription (Bradley et al., 2001). Our data suggest IL-6 secretion is regulated at the posttranscriptional level. Future studies will be necessary to elucidate posttranscriptional events that result in IL-6 secretion.

The cellular source of IL-6 expression within adipose tissue is currently debated. There has been some controversy in the literature over whether IL-6 is released from preadipocytes, adipocytes, or macrophages. A number of studies have demonstrated IL-6 is released from adipocytes (Fried et al., 1998; Mohamed-Ali et al., 2001; Vicennati et al., 2002). However, in the case of preadipocytes, some groups but not others have indicated that IL-6 is expressed or released from these cells. One study over a decade ago revealed IL-6 mRNA expression early in 3T3-L1 preadipocyte differentiation (Stephens et al., 1993). IL-6 mRNA has also been detected in adipose stromal cells, a fraction that

includes preadipocytes (Crichton et al., 1996). Another report suggests that IL-6 protein is released from human preadipocytes (Vicennati et al., 2002). Yudkin and Mohamed-Ali stated that basal IL-6 expression at the mRNA and protein levels were highest in preadipocytes, and lower in mature adipocytes (Clarke and Mohamed-Ali, 2000; Yudkin et al., 2000). In contrast, Path et al. stated that IL-6 protein expression was not detected in human preadipocytes using immunohistochemistry (Path et al., 2001). It is not clear why the IL-6 protein levels differ in preadipocytes located in the breast region versus abdominal preadipocytes (Path et al., 2001).

Our data from preadipocytes in three different cell models indicate basal secretion of IL-6. However, we did not detect an increase in IL-6 protein secretion from human abdominal subcutaneous preadipocytes treated with TSH (1 μM). To determine whether TSH elevates IL-6 protein secretion via transcriptional regulation, we tested IL-6 mRNA levels in preadipocytes versus adipocytes using RT-PCR. Our results demonstrate IL-6 mRNA is present in both human preadipocytes and differentiated adipocytes. An upward trend was observed in TSH-stimulated mRNA levels in differentiated adipocytes though our results did not reach statistical significance. This may result from donor heterogeneity and the data might prove significant given an increased number of patient samples.

Some studies have raised concerns about the purity of commercially available TSH preparations, suggesting that contaminants in the TSH preparations could possibly induce signal transduction pathways independent of TSHR (Correze et al., 2000; Vandeput et al., 2003). To confirm that TSH activation of PKB/Akt did not result from

potential contaminants in the commercial TSH preparation, we examined the effect of TSH on CHO-hTSHR cells. TSH activation of p(PKB/Akt) in CHO-hTSHR cells was very prominent in comparison to TSH activation of p(PKB/Akt) in non-hTSHR expressing cells. Our results demonstrate that the TSH-stimulated rise in p(PKB/Akt) levels in CHO-hTSHR cells is dependent on the presence of TSHR, and argue against the possibility of contaminants in the TSH preparation leading to activation of PKB/Akt. To further alleviate concerns that the responses we observed may be due to contaminants in the commercial TSH fraction, we show that the addition of recombinant human TSH or highly purified bovine TSH induce PKB/Akt activation in preadipocytes and IL-6 secretion in adipocytes.

In these studies, we have used TSH at a dose as low as 0.01 μM. This concentration is comparable to the TSH dose applied in studies of TSH-stimulated thyrocyte cell models studied *in vivo* and *in vitro* (1 mIU/ml or 20 nM). Since TSH concentrations applied in our cell models and in the thyrocyte cell models in the literature are supraphysiological, we postulated that other hormones might act in concert with TSH to augment cellular responsiveness, as they do in thyrocyte cell models. Other groups have demonstrated that IGF-1 enhances TSH signaling in thyrocytes (Ariga et al., 2000; Dremier et al., 2002; Kimura et al., 2001; Takahashi et al., 1991). Pretreatment of FRTL-5 cells with TSH prior to IGF-1 stimulation enhances the tyrosine phosphorylation of IRS-2 and activation of PI3K, and upregulates Shc leading to activation of p42/44 MAPK. TSH pretreatment of FRTL-5 cells has been shown to complement IGF-1-

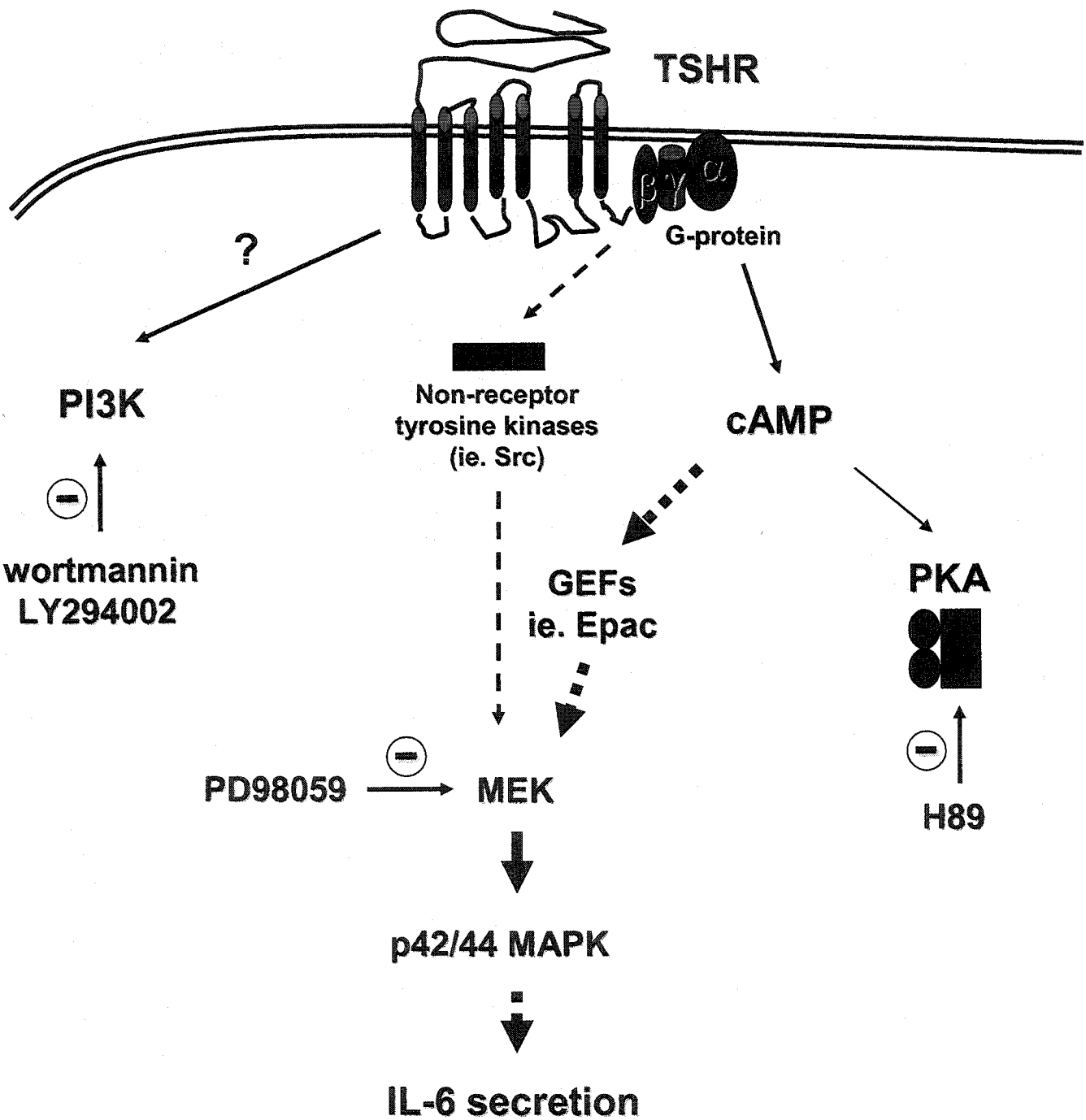
induced DNA synthesis by increasing key regulators of cell cycle, cyclin D1 and cyclin E and synergistically expediting DNA synthesis (Yamamoto et al., 1996). In line with the above findings, we have demonstrated that IGF-1 enhances TSH-responsiveness in human adipocytes by elevating IL-6 secretion levels. We have yet to determine how IGF-1 acts synergistically with TSH in our system.

A potential model for TSH signaling in human adipocytes is developing as shown in Figure 34. Our findings suggest adipocytes not preadipocytes respond to TSH by augmenting IL-6 secretion levels. In our model system, TSHR appears to couple to adenylyl cyclase since TSH stimulation of human adipocytes leads to elevated cAMP production. Inhibitor studies reveal a potentially small role for PKA and a more significant role for p42/44 MAPK. The mechanisms of IL-6 secretion are still largely unknown and warrant further investigation.

Further studies will be necessary to determine whether TSH regulates IL-6 secretion from adipose tissue. *In vivo* studies are also important to illuminate whether TSH alters IL-6 secretion from adipose tissue. Furthermore, it will be interesting to know whether circulating IL-6 levels are elevated in subclinical hypothyroid patients. Elevated levels of IL-6 will help explain our suggested mechanism providing the link between TSH and CVD.

In summary, TSH promotes cell survival in 3T3-L1 preadipocytes. Our data suggest a role for TSH may exist in adipose tissue development and remodeling. TSH increases IL-6 secretion from human, 3T3-L1 and 3T3-F442A adipocytes. TSH

Figure 34. Model of TSH-induced IL-6 secretion in human adipocytes. TSH elevates cAMP levels in human abdominal subcutaneous adipocytes. TSH does not appear to act through activation of PKA or PI3K. A role for p42/44 MAPK in TSH signal transduction was revealed by the use of the MEK inhibitor PD98059. TSH promotes IL-6 release from human abdominal subcutaneous adipocytes differentiated in primary culture.


TSHR
G-protein
?
PI3K
wortmannin
LY294002
Non-receptor
tyrosine kinases
(ie. Src)
cAMP
GEFs
ie. Epac
PKA
H89
PD98059
MEK
p42/44 MAPK
IL-6 secretion

modulation of IL-6 release suggests a novel molecular mechanism by which subclinical hypothyroidism may predispose to CVD.

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VI. Contributions of Collaborators

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