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Mechanisms of the pro-inflammatory action of the thyroid stimulating hormone on human abdominal subcutaneous differentiated adipocytes

by

Melanie L Langille

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the M.Sc. degree in Biochemistry

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ABSTRACT

Subclinical hypothyroidism, characterized by elevated thyroid stimulating hormone (TSH) levels with normal thyroid hormone levels, is associated with an increased risk for cardiovascular disease. Human adipocytes express the TSH receptor and may be a bystander target of TSH action. Treatment of human differentiated adipocytes with TSH stimulates interleukin (IL)-6 release through activation of the nuclear factor-kappa B (NF-κB) pathway. I identified intermediates implicated in TSH-induced activation of NF-κB and its upstream regulator, inhibitor of kappa B (IκB) kinase (IKK)β. My results also suggest that TSH-induced NF-κB activation and IL-6 production are not dependent on protein kinase A (PKA) activity. Studies demonstrate that protein kinase Cδ and reactive oxygen species are upstream of IKKβ/NF-κB activation. Furthermore, TSH induces monocyte chemoattractant protein-1 production in an IKKβ- and PKA-dependent manner. Further analysis of the TSH-induced inflammatory response in human adipocytes will broaden our understanding of TSH action in subclinical hypothyroidism.

DEDICATION

To dad and mom and the fam

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ABBREVIATIONS

Asn: asparagines

AP-1: activator protein-1

AP-2β: activating protein-2β

Bcl10: B cell chronic lymphocytic leukemia 10

BMI: body mass index

cAMP: cyclic adenosine monophosphate

CARD10: caspase recruiting domain 10; CARMA3

CBP: CREB binding protein

C/EBP: CCAAT/enhancer binding protein

CKGF: cystine knot growth factor

CREB: cAMP response element binding protein

CRP: C-reactive protein

CVD: cardiovascular disease

DPI: diphenyleneiodonium chloride

FSHR: follicle stimulating hormone receptor

GPCR: G-protein coupled receptor

hTSH: human thyroid stimulating hormone

IκB: inhibitor of κB

ICAM-1: intercellular adhesion molecule 1

IKKβ: inhibitor of κB kinase β

IL-6: interleukin-6

Ins(1,4,5)P_3: inositol 1,4,5-triphosphate

LHR: luteinizing hormone receptor

LPA: lysophosphatidic acid

LPS: lipopolysaccharide

L-T4: levo-thyroxine

MALT: mucosa-associated lymphoid tissue

MALT1: MALT lymphoma translocation gene 1

MCP-1: monocyte chemoattractant protein-1

NF-κB: nuclear factor κB

PI3K: phosphoinositide 3-kinase

PKA: cAMP-dependent protein kinase

PKC: protein kinase C

PLC: phospholipase C

PVN: paraventricular nucleus

RBP4: retinol binding protein 4

rhTSH: recombinant human thyroid stimulating hormone

ROS: reactive oxygen species

T3: triiodothyronine

T4: thyroxine

TNF-α: tumor necrosis factor-α

TRH: thyrotropin releasing hormone

TSH: thyroid stimulating hormone; thyrotropin

TSHR: thyroid stimulating hormone receptor

VCAM-1: vascular cell adhesion molecule 1

VEGF: vascular endothelial growth factor

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INTRODUCTION

My Masters project has looked at the extrathyroidal signaling of thyroid stimulating hormone (TSH) in human differentiated adipocytes as it pertains to inflammatory responses. My thesis examines whether the extrathyroidal role of TSH on adipocytes might induce inflammatory responses that may explain the increased risk for cardiovascular disease (CVD) observed in patients with subclinical hypothyroidism.

This introduction will cover characteristics of TSH and its receptor in addition to TSH action in the body and its signaling in thyrocytes. Furthermore, subclinical hypothyroidism will be discussed along with its link to CVD. Finally, interleukin (IL)-6 and other adipokines will be examined as they relate to inflammatory responses.

THYROID STIMULATING HORMONE (THYROTROPIN)

TSH is a 28- to 30-kDa glycoprotein hormone produced and secreted by thyrotrophs located in the anterior pituitary gland. It belongs to the family of glycoprotein hormones including follicle stimulating hormone, luteinizing hormone and the human chorionic gonadotropin. The hormones of this family are heterodimeric cystine-knot glycoproteins containing a common α-subunit and a unique β-subunit. The distinct β-subunit is what gives the hormones their biological specificity (Pierce 1981; Lapthorn 1994).

TSH GENES

The common α–subunit and the TSH β-subunit of human TSH (hTSH) are encoded by genes found on chromosomes 6 and 1, respectively (Dracopoli 1986). The α-subunit is

composed of four exons and three introns and the β-subunit contains three exons and two introns, resulting in the α-subunit gene being approximately two times larger (9.4 kB) than the β-subunit gene (4.9 kB). The first exon of both genes is short, untranslated and separated from the coding region by a large intron. A TATA box that binds RNA polymerase II is found upstream of the only transcription start site in each gene. Whereas the α-subunit gene demonstrates a more general expression, β-subunit gene expression is restricted to thyrotrophs (Szkudlinski 2002).

TSH PROTEIN

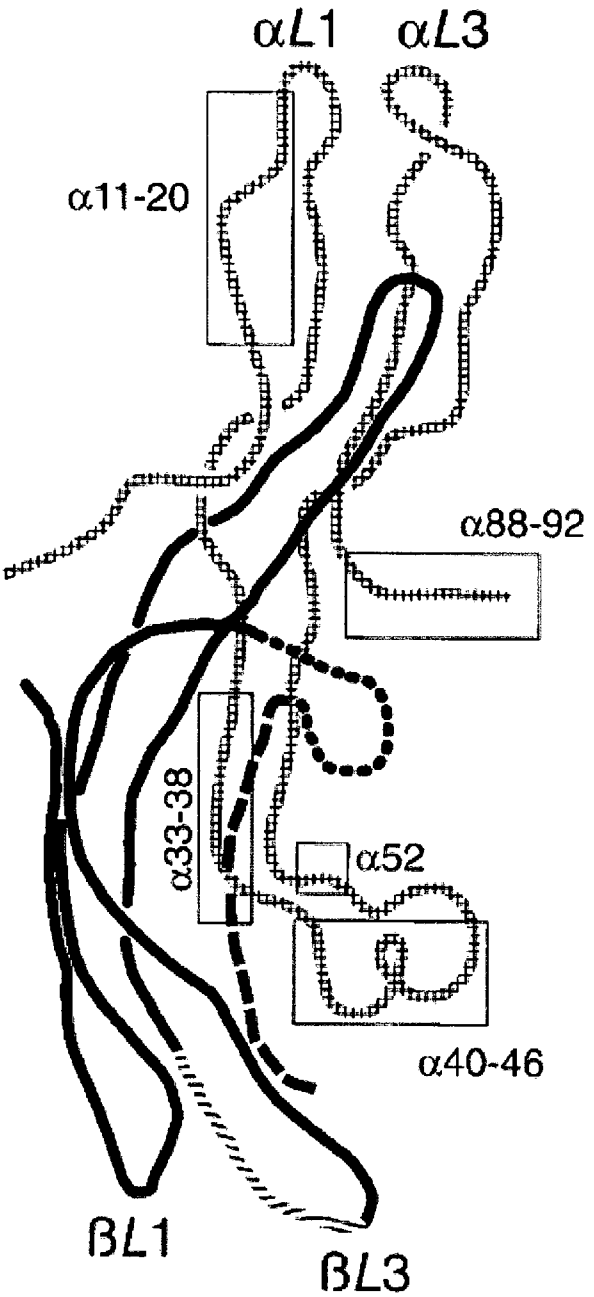
hTSH is comprised of two noncovalently linked subunits; the α-subunit which is 92 amino acids in length and shared with other human glycoprotein hormones, and the TSH β-subunit. The coding sequence of the TSH β-subunit predicts a 118 amino acid subunit (Wondisford 1988) but when isolated from cadaver pituitary, it is found to be 112 amino acids in length (Pierce 1981), a discrepancy that can most likely be explained by proteolytic cleavage during the purification process. However, it has been noted that deletion of amino acids 113-118 of the β-subunit does not affect the bioactivity of recombinant hTSH (rhTSH) which suggests that these C-terminal amino acids are not important for hormone activity (Takata 1989).

TSH is classified as a member of the glycoprotein hormone family, which in turn is structurally classified as part of the cystine knot growth factor (CKGF) superfamily of related proteins (Lapthorn 1994; Wu 1994). Structurally related proteins have been characterized and their crystal structure revealed that each of the two subunits contains a cystine-knot and three loops, two β-hairpin loops on one side of the cystine-knot and a long

loop on the other side; the long loop of the α-subunit having a two-turn α-helix. This structure has also been found in various growth factors including platelet-derived growth factor and vascular endothelial growth factor (VEGF). The two subunits of glycoprotein hormones are stabilized by a "seatbelt" segment of the β-subunit which wraps around the α-subunit long loop. This extra stabilization, seen in glycoprotein hormones compared with other members of the CKGF superfamily, might be necessary due to the large amounts of glycosylation in glycoprotein hormones, accounting for up to one-third of the weight of these hormones.

The α–subunit has two carbohydrate chains linked to asparagine- (Asn) 52 and Asn-78 whereas the β-subunit has only one carbohydrate chain at Asn-23. Several studies have been conducted to elucidate the role of each glycan. The glycan at Asn-78 seems to be required for the stability of the TSH protein (Van Zuylen 1998) by stabilizing the hydrophobic packing of the β-sheets within the subunit and therefore affecting the interaction of the two β-hairpin loops of the α-subunit with the extracellular domain of the TSH receptor (TSHR) (Leitolf 2000; Szkudlinski 1996). However, the glycan at Asn-52 in the α-subunit long loop is highly mobile in the free subunit but this mobility is restricted in the heterodimer form due to the seatbelt (Heikoop 1998). It is however known that the α33-57 region is highly important for signal transduction. The importance of the Asn-23 glycan is less well understood. Overall, the structural backbone of the heterodimer is flexible and can undergo a variety of conformational changes in order to acquire various active and inactive states (Figure 1A).

Figure 1A. Human TSH ribbon model highlighting important domains for hTSH activity. Taken from Grossmann et al. *Endocrine Reviews* 2007.



Grossmann et. al. *Endocrine Reviews* 1997

THYROID STIMULATING HORMONE RECEPTOR

TSHR is a related member of the rhodopsin/β-adrenergic receptor family in the G-protein coupled receptor (GPCR) superfamily characterized by seven transmembrane domains. The TSHR, along with other glycoprotein hormone receptors, contains a large extracellular domain 300- to 400- amino acids in length and containing at least 8 highly conserved cysteine residues, which are involved in the tertiary structure of this domain, along with ligand binding and the inactive receptor conformation (Nagayama 1992). The structure of GPCRs, in addition to the seven transmembrane domains, contains the amino-terminus in the extracellular space, three extracellular and three cytoplasmic loops in addition to a cytoplasmic carboxy terminus (Vassart 1995).

The human TSHR gene is located on chromosome 14q31 (Rousseau-Merck 1990). The extracellular domain, accounting for approximately one-half the molecular size of the receptor (Kohn 1989; Kohn 1995; Rees Smith 1988; Segaloff 1993), is encoded by the first nine exons and a part of the tenth whereas the rest of the receptor is encoded solely by the tenth exon. TSHR, along with the luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR), is classified more specifically in the leucine-rich repeats-containing GPCR proteins. These leucine rich repeats are encoded by separate exons and are thought to be important in the binding of the hormone to its receptor. There are two structural characteristics unique to the TSHR compared to both LHR and FSHR. Firstly, there is an 8-amino acid insertion near the amino terminus of TSHR that is important for TSH binding (Rapoport 1998; Wadsworth 1990) and secondly, there is a 50-amino acid insertion (residues 317-366), which seems to have no effect on TSHR function.

Some functional differences between TSHR and other glycoprotein hormone receptors include: 1) TSHR is more often affected by gain-of-function mutations which increases signaling without a change in ligand binding affinity (Van Sande 1995; Vassart 1995), 2) TSHR transduces a signal via adenylate cyclase in the absence of a ligand; both proteolytic degradation and minor deletions in its extracellular domain can lead to TSHR activation (Van Sande 1996; Zhang 1995), 3) there is no distinction between recognition and activation sites in the TSHR (Grossmann 1997; Leitolf 2000; Szkudlinski 1996), 4) TSH binding to TSHR is not altered in the presence of a nonhydrolyzable GTP analog as it is with many other GPCRs (Akamizu 1994) and 5) TSHR is unique in that mature receptors on the cell surface are capable of being cleaved into two subunits (A and B) that remain linked by disulfide bonds (Rapoport 1998; Rees Smith 1988; Davies 2002). The exact implications of the cleavage with regards to receptor activity have not been established but it has been suggested that the cleavage increases the constitutive activity of the B subunit in the absence of a ligand. Various multimers have been reported due to subunit associations during intracellular processing and subunit shedding of the mature receptor, the physiological significance of which is not yet understood (Davies 2005; Graves 2000; Rapoport 1998). However, the receptor associations and the intact receptor have been shown to be competent with respect to TSH signaling (Chen 2006; Bell 2000a; Bell 2002). Posttranslational modifications shown to be important for TSHR function include N-linked glycosylation of Asn-77 and Asn-113, which is important in the expression of biologically active TSHR at the cell surface.

TSHR mRNA transcripts and/or protein have been found in lymphocytes, adipocytes, retroocular fibroblasts, neuronal cells and astrocytes (Crisanti 2001; Graves 2000; Mengistu

1994; Paschke 1995) in addition to thyrocytes, the main target of TSH. Furthermore, there are more than 10 different inactivating mutations that have been described in the TSHR, usually causing resistance to TSH ultimately leading to primary hypothyroidism. On the other hand, there are more than 30 different activating mutations leading to nonautoimmune hyperthyroidism (Golos 1991; Vassart 1995) due to the constitutive activity of the receptor.

TSH ACTION

In the 1990s, thyrotropin-releasing hormone (TRH), the first hypothalamic hormone to be isolated and structurally characterized (Boler 1969; Burgus 1969), was found to be present mainly in the neurons of the paraventricular nucleus (PVN) of the hypothalamus, with smaller amounts found in the suprachiasmatic nucleus and the sexually dimorphic nucleus of the hypothalamus (Fliers 1994). While thyroid hormone receptor isoforms $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ are found in various regions of the brain, prominent expression has been found in the found in the TRH expressing neurons in the PVN (Bradley 1989; Cook 1992; Lechan 1994; Fliers 2006). When low blood levels of thyroid hormone are detected by the hypothalamus, TRH is released into portal capillaries and transported to the anterior pituitary where it stimulates thyrotrophs to produce and release TSH into circulation. The pituitary is also capable of responding to low thyroid hormone blood levels and to increase TSH secretion. TSH then travels to its primary target, the thyroid gland where it acts on thyrocytes via its receptor, TSHR, to increase the uptake of iodine by the thyroid, increase production and secretion of thyroid hormones, as well as induce cell proliferation and growth.

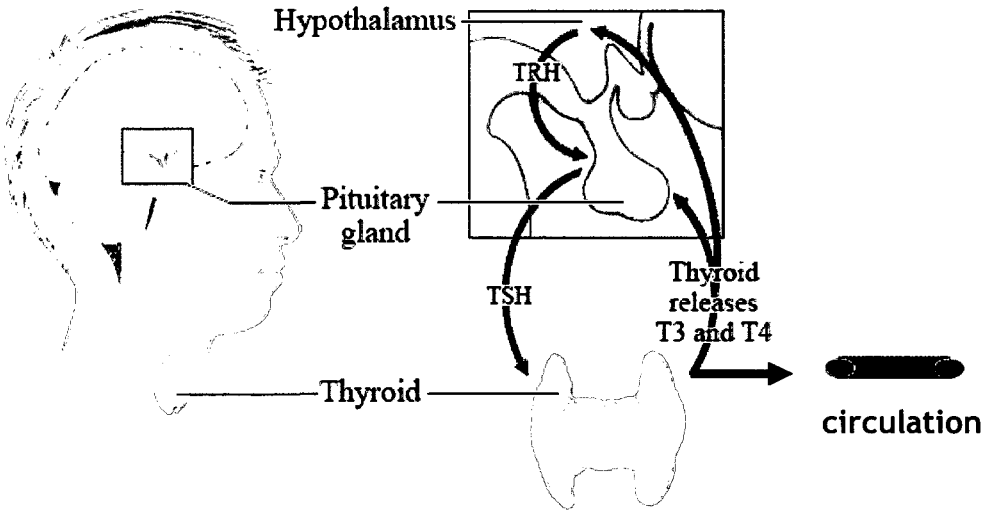
The thyroid gland produces two thyroid hormones, triiodothyronine (T3) and thyroxine (T4). Once the levels of circulating thyroid hormone are restored within the normal range by the thyroid gland, thyroid hormones act on the hypothalamus and pituitary to inhibit TRH and TSH production, respectively. Thyroid hormones also enter circulation where they perform their metabolic functions. The three glands, namely the hypothalamus, pituitary and thyroid, along with the hormones they produce, comprise what is termed the Hypothalamic-Pituitary-Thyroid axis (Fliers 2006) (Figure 1B).

TSH SIGNALING IN THYROCYTES

TSH is able to activate several regulatory cascades by binding TSHR. The classical pathway, activated in all species studied thus far, is the cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) pathway. TSHR in thyrocytes have been shown to be coupled via $G\alpha_s$ proteins to adenylate cyclase, increasing levels of cAMP. cAMP acts through its main target, PKA, to regulate proliferation and differentiation of thyrocytes, in addition to regulating thyroid hormone secretion (Vassart 1992; Dremier 2002). Secondly, it has recently been demonstrated that TSH activates phospholipase C (PLC) via $G\alpha_q$ proteins in human thyroid cells (Van Sande 2006). Upon activation, PLC hydrolyzes phosphatidylinositol 4,5-phosphate into inositol 1,4,5-triphosphate ($Ins(1,4,5)P_3$) and diacylglycerol which can then activate downstream cascades. The $Ins(1,4,5)P_3$ Ca^{+2} pathway has been found to be involved in thyroid hormone synthesis (Vassart 1992; Van Sande 2006). Furthermore, TSHR has been shown to couple to $G\alpha_i$, $G\alpha_{12}$ and $G\alpha_{13}$ in human thyroid cells, but much less is known about signaling through these mechanisms (Laugwitz 1996). Studies showed that the TSHR ability to raise cAMP levels in response to TSH were

increased when $G\alpha_i$ was inhibited. These results indicated that TSHR couples to two opposing pathways, $G\alpha_i$ in addition to $G\alpha_s$, to modulate cAMP levels (Laugwitz 1996). In human follicular thyroid carcinoma cells and nonneoplastic human thyrocytes, TSH stimulation activated p44/42 mitogen-activated protein kinase via TSHR coupling to $G\alpha_{13}$ (Buch 2008).

Figure 1B. Model of TSH regulation and action in the body.



Adapted from: health.com

SUBCLINICAL HYPOTHYROIDISM

Subclinical hypothyroidism is characterized by TSH levels above the reference range (0.45 to 4.5 mU/L) with serum thyroid hormone levels within the normal reference range (Wilson 2005). Subclinical hypothyroidism is usually diagnosed through routine screening or by the evaluation of nonspecific symptoms including but not limited to fatigue, cold intolerance, and weight gain. Subclinical hypothyroidism is caused by mild thyroid gland failure, usually the result of chronic autoimmune destruction, which then causes thyroid hormone production to weaken. However, as the pituitary increases TSH output, thyroid hormone levels are restored to the normal range. This compensatory state may remain for many years, or may eventually lead to overt hypothyroidism characterized by inadequate amounts of circulating thyroid hormone levels.

PREVALENCE OF SUBCLINICAL HYPOTHYROIDISM

The prevalence of subclinical hypothyroidism world wide ranges from 1 to 10 percent, with the highest age- and sex-specific rates being in women older than 60 years of age (Tunbridge 1977; Canaris 2000). There was a 12-15% prevalence of subclinical hypothyroidism reported for a healthy US population over the age of 65 (Karlin 2004). Up to 75% of patients with subclinical hypothyroidism have only mildly elevated TSH levels (5-10 mU/L) and 50-80% have positive tests for antibodies against thyroperoxidase (Cooper 2001). There is also evidence for an increased prevalence of subclinical hypothyroidism during pregnancy. Fetal development, especially of the brain, is dependent on the mother's thyroid function. It has been shown that mothers with subclinical hypothyroidism who are not adequately treated with thyroid hormone replacement therapy give birth to babies with

lower intelligence quotients or impaired psychomotor development (Pop 1999). Some data suggest that subclinical hypothyroidism is associated with ovulatory dysfunction and infertility (Cooper 2001). Since there are few symptoms associated with subclinical hypothyroidism, routine screenings have been put in place by some physicians (Danese 1996). Although, since benefits of thyroxine therapy for patients with subclinical hypothyroidism have yet to be established long-term, population screening is not accepted unanimously. However, screening of pregnant women in addition to the elderly female population seems to be widely accepted.

TREATMENT OF SUBCLINICAL HYPOTHYROIDISM

Whether or not subclinical hypothyroidism should be treated is still under much debate. To begin, the TSH reference range used to diagnose subclinical hypothyroidism itself is under discussion, complicating the decision of whether or not to treat these individuals. The threshold TSH concentration, above which an individual is diagnosed as having subclinical hypothyroidism, is population-based. Some clinicians feel the threshold should be lowered ($\geq$ 2.5 mU/L) (Wartofsky 2005) while others believe it should remain unchanged ($\geq$ 4.0 mU/L) (Surks 2004). There are no randomized clinical trials that assess the long-term benefits or adverse effects of early treatment for subclinical thyroid dysfunction and therefore, some physicians are hesitant to treat these patients. Another concern has to do with the risk of overtreatment i.e. iatrogenic hyperthyroidism which could lead to more dangerous outcomes such as osteopenia and atrial fibrillation (Marqusee 1998). Another drawback of treating all patients might be the cost, however medication is fairly affordable. Follow up monitoring would be needed for both treated and untreated cases,

however, further cost-benefit analyses need to be conducted (Feldt-Rasmussen 2009). On the other hand, the rationale for treatment is based on the adverse cardiovascular profile in patients with subclinical hypothyroidism compared with controls, and the improved lipid profile, endothelial function and cardiac performance seen in subclinical hypothyroid patients treated with thyroxine. Another argument for treatment is that subclinical hypothyroidism is a part of the disease spectrum of hypothyroidism. The benefits of thyroxine therapy rely on three main points, each of which is still being debated: progression of overt hypothyroidism may be prevented (Tunbridge 1977), improved serum lipid profiles and decreased risk of death from CVD may result (Elder 1990; Danese 2000) and symptoms, if any, of subclinical hypothyroidism may be reversed (Cooper 2001). The one matter in which clinicians do seem to agree however, is that fertile women with subclinical hypothyroidism should be treated before conception and monitored closely during pregnancy for reasons mentioned above.

SUBCLINICAL HYPOTHYROIDISM AND THE RISK OF CARDIOVASCULAR DISEASE

Although the association of overt hypothyroidism and increased CVD risk is well studied and accepted, the association of subclinical hypothyroidism and CVD is much less understood. The Whickham Study showed that baseline measurements revealed a weak association between subclinical hypothyroidism and electrocardiographic changes (Tunbridge 1977). However, the 20-year follow-up on these patients did not show an increase in CVD in these patients, perhaps because they received thyroid hormone therapy throughout the years (Vanderpump 1996). The issue of whether patients with subclinical

hypothyroidism were at an increased risk for developing CVD resurfaced in 2000 with The Rotterdam Study. A cross-sectional study was performed on post-menopausal women. Subclinical hypothyroidism was present in approximately 11% of the population studied and was associated with a larger prevalence of aortic atherosclerosis (odds ratio 1.7) and myocardial infarction (odds ratio 2.3). These results were independent of body mass index (BMI), total and high-density lipoprotein cholesterol level, smoking status and blood pressure (Hak 2000). Furthermore, the attributable risk was found to be in the range of that for known major risk factors of CVD.

Most recently, Ochs *et al.* performed a meta-analysis on studies conducted between 1950 and January 2008 and found that there was a small link between subclinical hypothyroidism and CVD that did not quite reach significance. However, when analyzing only those studies involving subjects less than 65 years of age, it was determined that these subclinical hypothyroid patients are at an increased risk for CVD (RR 1.51) (Ochs 2008). Finally, another group performed a meta-analysis in 2008 to determine the effect of age on the association of subclinical hypothyroidism and CVD. Similar to the Ochs group, they found that subclinical hypothyroid patients under the age of 65 years were at an increased risk for ischemic heart disease whereas those above 65 years of age showed no increased risk for ischemic heart disease (Razvi 2008). Age is therefore an important factor when examining the risk for CVD in subclinical hypothyroid patients. The reason for this is not yet understood but it has been suggested that perhaps the elderly are less responsive to TSH (Gussekloo 2004). Further research is needed in the elderly population before conclusions can be drawn.

MEDIATORS POTENTIATING CVD RISK IN SUBCLINICAL HYPOTHYROIDISM

The mediators that are involved in elevating the risk for CVD in patients with subclinical hypothyroidism are not well understood. In a 2006 study, it was revealed that patients with subclinical hypothyroidism had increased levels of circulating C-reactive protein (CRP) and IL-6. This low grade inflammation was correlated with endothelial dysfunction in these patients, possibly contributing to their increased risk of heart disease (Taddei 2006). IL-6 is a main regulator of hepatic CRP production which has been accepted as an indicator for CVD risk (Blake 2003). In addition to being a marker of a pro-inflammatory, pro-atherogenic state, both IL-6 and CRP have also been implicated in the actual progression of atherosclerosis (Li 2004; Pepys 2003; Pepys 2006). However, there is still some debate on this topic with regards to CRP. Although most scientists agree that CRP can have toxic effects on cells, reviews are questioning whether or not CRP has a causal effect on the progression of atherosclerosis (Schunkert 2008; Tall 2004).

Recently, it was also found that patients with subclinical hypothyroidism have higher levels of retinol binding protein 4 (RBP4) independent of obesity (Choi 2008). RBP4 delivers retinol from liver stores to peripheral tissues and has recently been identified as an adipokine that contributes to insulin resistance (von Eynatten 2008), which may also link subclinical hypothyroidism to CVD.

IL-6 AND CVD

Human IL-6 begins as a 212 amino acid protein but is secreted as a 184 amino acid protein following cleavage of the signal peptide. Depending on the glycosylation state of the protein, the final protein has a molecular weight of approximately 23-32 kD (Wong 2003).

IL-6 is a pro-inflammatory adipokine, found to be elevated in patients with CVD (Biasucci 1996; Miyao 1993). A recent review confirmed that serum IL-6 concentrations predict the onset of CVD (Lobbes 2006) and subsequent studies have also demonstrated IL-6 as a predictive marker of the disease (Lee 2006; Stork 2006; Fisman 2006). As such, IL-6 has been deemed an independent risk factor for CVD (Danesh 2008; Ridker 2000).

As mentioned above, since IL-6 is the main regulator for CRP, higher levels of IL-6 is likely what leads to the high levels of CRP in patients with CVD (Blake 2001). CRP has been shown to increase LDL uptake by macrophages (Zwaka 2001), upregulate the expression of adhesion molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (Pasceri 2000; Pasceri 2001) and to suppress nitric oxide production by endothelial cells, all of which are involved in foam cell formation and the development of atherosclerosis (Blake 2003). However, as mentioned above, recent data has started to cast doubt on the causal effects of CRP levels on the actual progression of atherosclerosis (Schunkert 2008; Tall 2004). In addition, IL-6 also increases the risk for CVD independently of CRP levels. It can do so via actions on the endothelium of vessel walls. In studies done with endothelial cells, IL-6 was shown to have a positive role in local inflammatory responses increasing leukocyte recruitment. IL-6 also induced endothelial-dependent expression of the monocyte chemoattractant protein-1 (MCP-1) and IL-8 (Romano 1997). Furthermore, mice treated with recombinant IL-6 showed increased circulating levels of IL-1β and tumor necrosis factor (TNF)-α, both involved in systemic inflammation. In addition, IL-6 administration in mice potentiated the development of lesions characteristic of atherosclerosis (Huber 1999). Finally, IL-6 exposure reduced

glucose uptake in 3T3-L1 adipocytes (Rotter 2003) and was found to reduce insulin-dependent hepatic glycogen synthesis also in mice (Klover 2003; Senn 2002).

ADIPOSE TISSUE FUNCTION

In addition to being involved in energy storage and release, adipose tissue has been shown to release a variety of cytokines (termed adipokines) that have a wide range of physiological effects on pathways that regulate appetite, energy expenditure and insulin sensitivity. Deregulation of adipokine production leads to a pro-inflammatory, pro-atherogenic state increasing the risk for CVD and type 2 diabetes. Both inflammation and insulin resistance in the adipose tissue have been shown to contribute to the progression of atherosclerosis (Libby 2006; Shoelson 2006).

ADIPOSE TISSUE CELL MODELS

Most of our knowledge with regards to adipogenesis and adipose tissue studies have come from studies using 3T3-L1 and 3T3-F442A immortalized preadipocyte cell lines derived from mouse embryo fibroblasts (MacDougald 2002). However, some major differences between these cell lines and human primary adipose cells need to be considered when interpreting results. The cell lines are aneuploid and immortalized compared to human primary adipose cells which are diploid and have a limited life span (Ailhaud 1982). Furthermore, the cell lines and human primary adipose cells represent different stages of development; preadipose cell lines are obtained during the prenatal period whereas human primary preadipocytes are obtained postnatally (Gregoire 1998). Mitotic clonal expansion is a process that the 3T3 cell lines need to undergo prior to differentiation whereas it is thought

that human primary preadipocytes have already undergone this process *in vivo* and are therefore a later stage of differentiation than the cell lines even though they are all committed to the adipocyte lineage (Entenmann 1996). Finally, adipose cell lines do not allow for the study of differences of anatomical depot with regard to hormone effects or cytokine secretion (Rosen 2000).

Human primary preadipocytes are therefore used in order to strengthen the validity of results obtained from preadipocyte cell lines and in order to obtain information that is more physiologically relevant. Human primary adipocytes are obtained from adipose tissue through collagenase digestion, centrifugation, filtration and erythrocyte lysis procedures (Hauner 1989). Upon reaching confluence, human primary preadipocytes are differentiated in culture when placed in a differentiation cocktail for 14 days. The differentiation medium is supplemented with fetal bovine serum, insulin, dexamethasone, isobutylmethylxanthine and indomethacin (Adams 1997). Insulin serves to activate pro-adipogenic transcription factors such as cyclic AMP response element-binding protein (CREB) and to repress anti-adipogenic transcription factors such as those from the forkhead and GATA family, via growth factor-1 receptor or insulin receptor signaling (Rosen 2006). Dexamethasone is a synthetic member of the glucocorticoid family of steroid hormones and it activates CCAAT/enhancer binding protein (C/EBP)δ synthesis, required for differentiation to occur, and represses the anti-adipogenic protein pref-1 (Smas 1999). Isobutylmethylxanthine is a non-specific inhibitor of phosphodiesterases which in turn leads to an increase in cAMP levels required for CREB activation and consequently C/EBPβ expression (Zhang 2004). Finally, indomethacin is a PPARγ agonist, required for differentiation.

Human primary adipose cells are not without their own disadvantages and setbacks. Firstly, obtaining these cells requires informed consent and access to patients and a surgical team in order to obtain the adipose tissue for preadipocyte isolation. Secondly, these preadipocytes are not immortalized and thus can only be sustained for about 3 passages (after which they start to lose their capacity to differentiate). Thirdly, the differentiation process is quite timely taking between 14 and 18 days compared to between 6 and 8 for preadipocyte cell lines. Finally, due to variation between donor characteristics such as age and BMI, the percentage of differentiation obtained with human primary preadipocytes is variable (Avram 2007; Cartwright 2007). Nevertheless, human primary preadipocytes are invaluable in the field of research of adipose tissue as they represent a more physiological cell model compared to the 3T3-L1 cell model.

TSH SIGNALING IN ADIPOSE TISSUE

The study of TSH action on adipose tissue began in the mid 1960s by 1994 Nobel Prize in Physiology or Medicine winner Rodbell who showed that TSH induces lipolysis in fat cells (Rodbell 1964). Furthermore, Marcus *et al* showed that in human newborns, TSH is the major lipolytic hormone and that this effect decreases with age (Marcus 1988). Janson *et al.* conducted studies that concluded that the lipolytic effect of TSH can be reproduced with stimulatory TSHR autoantibodies and that this effect is blocked with inhibitory TSHR autoantibodies. Furthermore, although TSHR stimulation in adipocytes increases cAMP levels, indicating that TSHR is most likely to be coupled to adenylate cyclase, it is still uncertain whether or not this is the pathway required for lipolysis (Janson 1995). This paper

also concluded that in infants, TSH is the major lipolytic inducer rather than catecholamines. (Janson 1995).

Our lab has investigated the effects of TSH on 3T3-L1 and human differentiated adipocytes. TSH stimulated an increase in IL-6 release from 3T3-L1 adipocytes requiring PKA activity (Antunes 2005). When analyzing stage of differentiation and fat depot origin of human adipocytes, it was found that basal IL-6 release was higher in preadipocytes than differentiated adipocytes, regardless of fat depot. Also, preadipocytes from the omental depot had higher basal IL-6 release compared to those from the subcutaneous depot, a feature that did not hold true for differentiated adipocytes. TSH-stimulated IL-6 release was only observed in subcutaneous differentiated adipocytes (Bell 2003; Antunes 2005). Furthermore, TSH treatment of human abdominal subcutaneous differentiated adipocytes was found to activate the inhibitor of kappa B (IκB) kinase (IKK)β/nuclear factor κB (NF-κB) pathway (Antunes 2008).

NF-κB refers to a group of transcription factors encompassing 5 factors which are: p65 (RelA), c-Rel, RelB, p50 (NF-κB1) and p52 (NF-κB2). Various homo or heterodimers can form between these 5 factors, however only p65, RelB and c-Rel are capable of transcriptional activation and thus in order to be transcriptionally active, one of these three factors must be part of the dimer. The most common dimer is the p65/p50 dimer which is often simply referred to as NF-κB (de Winther 2005).

NF-κB is found in the cytoplasm in an inactive state because it is bound to a family of inhibitor proteins IκB α, β or ε. The binding of IκB to NF-κB hides the nuclear localization signal of the transcription factor. When the cell is stimulated, IκB is phosphorylated by an upstream kinase complex, IKK (α, β, γ). This phosphorylation is a

signal for ubiquitination of IκB which then leads to its degradation by the proteosome. NF-κB is then liberated and can enter the nucleus and transcribe its target genes, one of which is IL-6 (Schmid 2008).

TSH stimulation increased levels of phosphorylated IKKβ (required for IKK activity) and decreased IκBα (most common IκB) levels, indicating its degradation. TSH also increased NF-κB translocation to the nucleus as well as DNA-binding. In addition, it was found that TSH-induced IL-6 release is dependent on IKKβ activity and the translocation of NF-κB to the nucleus. Finally, TSH was demonstrated to stimulate IKKβ phosphorylation and IL-6 mRNA levels in a TSHR-dependent manner. TSH was only capable of inducing IKKβ phosphorylation and IL-6 mRNA expression in CHO cells expressing human TSHR and not in control cells (Antunes 2008).

It has been proposed that GPCRs can activate the NF-κB pathway through upstream regulators including the generation of ROS via NADPH oxidase (Hirotani 2002; Park 2006; Wegener 2007). Adipocytes are known to express these enzymes, specifically NOX1, NOX2, and NOX4 isoforms (Sautin 2006). Little is known about how TSH may regulate NADPH oxidase activity in adipocytes.

TSH has also been shown to regulate leptin release from adipocytes but with conflicting results. TSH and leptin are found to have coordinated pulsatility in plasma and seem to regulate each other (Mantzoros 2001). In rat epididymal adipocytes, TSH decreased the concentration of leptin found in the medium, in a dose-dependent manner (Shintani 1999) whereas TSH-stimulated human omental adipose tissue resulted in increased leptin secretion *in vitro* (Menendez 2003). Furthermore, Cecoli *et al* recently showed that rhTSH administration to thyroidectimized patients did not affect circulating leptin levels (Cecoli

2008). However, this result could be due to the acute exposure that the patients had to rhTSH; perhaps it was not long enough to see a response in leptin levels. It could also be due to the concentration of rhTSH used because these patients were undergoing thyroxine therapy and therefore endogenous TSH levels were suppressed which may have resulted in the adipose tissue no longer being as responsive to TSH. Furthermore, perhaps rhTSH is in fact upregulating leptin expression locally but it is not reaching circulation. On the other hand, perhaps rhTSH administration in fact has no effect on leptin regulation *in vivo*.

IL-6 AND ADIPOSE TISSUE

Adipose tissue is a major source of circulating IL-6, accounting for up to 30% in a normal state and up to 50% in conditions of increased adiposity, insulin resistance and diabetes (Mohamed-Ali 1997, Kern 2001; Yudkin 1999). Fried showed that abdominal omental adipose tissue secretes more IL-6 basally than does abdominal subcutaneous adipose tissue (Fried 1998). More specifically, there are numerous cell types within the adipose tissue itself that are capable of producing and secreting IL-6 which include preadipocytes, adipocytes, endothelial cells and macrophages. Adipocytes were found to account for only about 10% of the total IL-6 production from adipose tissue, suggesting that the other cells within the tissue must also be important contributors to IL-6 release. Elaborating on this finding, adipose tissue macrophages in the obese state were shown to secrete the same amount of IL-6 as do adipocytes (Weisberg 2003; Curat 2006).

Importantly, our lab has shown that adipocytes, and not preadipocytes, are the cells responsive to TSH with regards to IL-6 secretion (Antunes 2006).

IL-6 released from adipose tissue has autocrine and paracrine functions within this tissue (Sopasakis 2004). IL-6 treatment of adipose tissue increases its own secretion (Lagathu 2003) in addition to the secretion of pro-inflammatory factors including MCP-1 (Fasshauer 2004) and plasminogen activator inhibitor-1 (Kralisch 2006a). Furthermore, IL-6 treatment decreases the secretion of adiponectin from adipose tissue (Sopasakis 2004; Fasshauer 2003a), a protein known to improve insulin sensitivity. Additionally, IL-6 released from adipose tissue can exert effects in an endocrine fashion as a significant amount reaches circulation (Mohamed-Ali 1997). Since circulating IL-6 levels have been associated with various states of inflammation, a possible endocrine target of IL-6 is the liver. This is supported by the fact that IL-6 was found to inhibit insulin-stimulated glycogen metabolism in rat hepatocytes *in vitro* (Kanemaki 1998). Furthermore, mice treated with IL-6 developed insulin resistance (Klover 2003) and fasting blood glucose levels were found to be elevated in human patients receiving recombinant IL-6 treatment (Tsigos 1997).

REGULATION OF IL-6

IL-6 is regulated by four different transcription factors: NF-κB, C/EBP, activator protein-1 (AP-1) and CREB. Which transcription factors and to what extent each are needed for IL-6 transcriptional regulation is both cell type specific and ligand-specific (Hershko 2002). For instance, in Caco-2 cells, a human intestinal epithelial cell line, all four transcription factors are needed in order to activate the IL-6 gene (Hershko 2002).

Regulation in preadipocytes and adipocytes

IL-6 secretion has been reported to be stimulated by β-adrenergic receptor stimulation

in response to catecholamines, such as isoproterenol, and inhibited by glucocorticoids in human breast adipocytes (Path 2001). Furthermore, pro-inflammatory proteins such as IL-1β, TNF-α (Flower 2003), lipopolysaccharide (LPS) (Harkins 2004), angiotensin II (Skurk 2004), and IL-6 itself (Fasshauer 2003b; Hoene 2008) have been shown to induce IL-6 production from adipose tissue. Insulin and growth hormone stimulated IL-6 mRNA expression in 3T3-L1 adipocytes (Fasshauer 2003b) while adiponectin was found to suppress LPS-induced IL-6 expression in pig adipocytes (Ajuwon 2005).

In preadipocytes and adipocytes, the transcription factor of particular importance to IL-6 production is NF-κB. The differentiation of 3T3-L1 preadipocytes leads to an increase in NF-κB expression and thus it has been suggested that NF-κB is a key regulator of adipokine release in the later stages of differentiation (Berg 2004). Furthermore, TNF-α and LPS have been shown to activate IL-6 release via NF-κB activation in 3T3-L1 adipocytes (Berg 2004).

In addition to classical NF-κB activators, GPCR have been shown to a activate NF-κB in order to induce inflammatory responses and release various cytokines (Ye 2001). This is beginning to be explored in adipose tissue. In 2004 Skurk *et al.* demonstrated that angiotensin II stimulates the release of IL-6 from human adipocytes *in vitro* by activating NF-κB (Skurk 2004). However, our understanding of regulators of IL-6 production from preadipocytes and adipocytes is far from being complete.

EXTRA-THYROIDAL EFFECTS OF TSH: RECOMBINANT HUMAN TSH STUDIES

The use of rhTSH administration for thyroid cancer monitoring presents an interesting model for studying the *in vivo* effect of TSH. Following total thyroidectomy and

radioactive iodine ablation of any residual thyroid gland tissue, thyroid cancer patients are placed on lifelong levo-thyroxine (L-T4) therapy. To monitor for recurrence, these patients, while continuing L-T4 treatment, will often undergo a protocol involving administration of rhTSH. The response with respect to serum thyroglobulin levels and body scan results are used to determine if there is any indication of recurrent thyroid cancer. (Schlumberger 2007)

Since TSHR mRNA transcripts and/or protein have been found in lymphocytes, adipocytes, retroocular fibroblasts, neuronal cells and astrocytes (Crisanti 2001; Graves 2000; Mengistu 1994; Paschke 1995), in addition to thyrocytes, studies involving the treatment of thyroidectomized patients with rhTSH became of interest in order to gain insight into some of the extra-thyroidal functions of TSH *in vivo*.

When rhTSH was administered to thyroidectimized patients, VEGF levels decreased significantly (Sorvillo 2003) and the endothelium-dependent vasodilation was potentiated by rhTSH levels (Napoli 2008; Napoli 2009). However, another study showed that rhTSH administration decreased endothelium-dependent vasodilation (Dardano 2006). This latter study also showed that rhTSH treatment significantly elevated blood IL-6 and TNFα levels. Finally, our lab also showed that rhTSH administration increased serum IL-6 levels in thyroidectimized patients (Antunes 2006). As these results have been found in thyroidectimized patients, this indicates an extra-thyroidal function of TSH.

OTHER ADIPOKINES

Monocyte chemoattractant protein-1

MCP-1 is a small cytokine of molecular weight 13 kDa. MCP-1 recruits monocytes, memory T cells and dendritic cells to sites of tissue injury and infection and into sites of

early atherosclerotic lesions (Carr 1994; Xu 1996). MCP-1 is secreted from adipose tissue (Guzik 2006; Fain 2006) and its secretion increases in states of obesity. MCP-1 also plays a key role in the recruitment of macrophages into the adipose tissue (Gustafson 2007) and is a pro-inflammatory cytokine involved in the progression of diabetes as it has been shown to be able to induce insulin resistance (Sell 2007). A study using 3T3-L1 adipocytes and an *in vivo* insulin resistant mouse (ob/ob) model revealed that MCP-1 regulation remains insulin sensitive even in states of insulin resistance; insulin increased MCP-1 expression in both these models (Sartipy 2003). Since hyperinsulinemia often results from obesity and insulin resistance, this increase in circulating insulin may account at least in part for the increase in MCP-1 levels observed in obese mice. In turn, increased MCP-1 levels can affect adipocyte function as MCP-1 stimulation of adipocytes *in vitro* decreased insulin-stimulated glucose uptake (Sartipy 2003). Furthermore, MCP-1 expression was found to be stimulated by growth hormone, IL-6 (Fasshauer 2004) and isoproterenol treatment (Kralisch 2006b) in 3T3-L1 adipocytes while thrombin increased MCP-1 production in human adipose cells *in vitro* (Strande 2009).

MCP-1 overexpression in the adipose tissue of mice led to an increase in macrophage recruitment into the tissue and also led to insulin resistance (Kamei 2006; Kanda 2006). Furthermore, human differentiated omental adipocytes treated with macrophage-conditioned medium resulted in an increase in IL-6 mRNA expression and protein levels (Bassols 2009). Monocyte-derived macrophage infiltration to the adipose tissue has been associated with both increased tissue and systemic inflammation. This chronic low grade inflammation can then result in deregulation of adipokine production, including increased IL-6 release, which can in turn lead to cardiovascular complications, as discussed above.

Vascular Endothelial Growth Factor A

VEGF is a sub-family of the platelet-derived growth factor family and is involved in both vasculogenesis and angiogenesis and is mainly produced by endothelial cells. VEGF is also produced by adipose tissue. VEGFA (subsequently referred to as VEGF) is viewed as the most important positive regulator of both vascular permeability and angiogenesis. VEGF has also been implicated in wound repair and tumorigenesis (Chodorowska 2004). VEGF levels are increased with increased adiposity; levels which have been shown to play a role in hypertension and atherogenesis. Whereas it is not surprising that VEGF levels would increase with increasing adiposity, as it allows for an increase in the vascular bed which is needed to meet the needs of the expanding adipose tissue (Berg 2005), these increased levels play a role in increasing aortic subendothelial macrophage accumulation and intimal thickening (Mick 2002). Still to be determined is the importance of the obesity-induced changes in VEGF levels in the context of cardiovascular risks.

Retinol binding protein 4

RBP4 is a 21-kDa protein produced and secreted by the liver and adipose tissue and has been implicated in insulin resistance. RBP4 expression is increased in the adipose tissue of insulin-resistant adipose GLUT4(-/-) and RBP4 injection induced insulin resistance in mice (Yang 2005; von Eynatten 2008). Overexpression of RBP4 or recombinant RBP4 treatment resulted in insulin resistance in mice via increased expression of the gluconeogenic enzyme phosphoenolpyruvate in mouse liver and via impaired skeletal muscle insulin signaling (Yang 2005; Rabe 2008). In human studies, RBP4 levels correlated with cases of obesity and insulin resistance and an intervention of exercise training and lifestyle changes

that led to improved insulin sensitivity, also led to decreased RBP4 levels (Graham 2006; Balagopal 2007; Haider 2007; Lim 2008).

Adiponectin

Adiponectin is a 30 kDa adipokine solely secreted by adipose tissue. After secretion, adiponectin forms trimers (low molecular weight), hexamers (mid-molecular weight) and dodecamers (high molecular weight) in circulation. Circulating adiponectin levels are inversely proportional to adiposity and therefore obese subjects have lower levels of circulating adiponectin than do lean individuals (Scherer 1995). Adiponectin is an anti-inflammatory adipokine and has a role in liver insulin sensitivity and metabolism. Furthermore, adiponectin has been implicated in cardiovascular health as a serum marker for the prediction of the onset of cardiovascular complications. The mechanism by which adiponectin is implicated in cardiovascular health is still not well understood but studies have suggested that adiponectin elicits its anti-atherosclerotic effect directly on vasculature rather than indirectly through insulin sensitivity (Goldstein 2004). In addition to adiponectin's potential anti-inflammatory and anti-atherosclerotic properties, adiponectin has also been shown to protect against cardiac hypertrophy (Ouchi 2004).

HYPOTHESIS

TSH acts on human adipocytes to release pro-inflammatory cytokines via activation of the IKKβ/NF-κB pathway.

RATIONALE

TSH-induced inflammatory responses from adipocytes may explain the increased risk for CVD in patients with subclinical hypothyroidism.

OBJECTIVES

Objective 1: To identify the intermediates implicated in TSH-induced activation of NF-κB and its upstream regulator IKKβ, leading to the stimulation of IL-6 production.

Objective 2: To explore whether other adipokines are regulated by TSH treatment and to begin to elucidate the mechanism by which TSH regulates their expression and release.

MATERIALS AND METHODS

Isolation, culture and differentiation of human stromal preadipocytes

Human abdominal subcutaneous adipose tissue was obtained from 21 patients (18 female, 3 male), age 49 ± 9 years (mean ± SD), with a 28 ± 7 body mass index (BMI; mean ± SD) undergoing elective abdominal surgery. Written consent was obtained for each patient and the study was approved by The Ottawa Hospital Research Ethics Board (1995023-01H). The stromal preadipocytes were isolated as described (Antunes 2006). Briefly, blood vessels and fibrous tissue were carefully removed. Collagenase digestion followed (CLS type I; 600 U/g of tissue) for 1 hour at 37°C. Following digestion, collagenase activity was inhibited by adding Dulbecco's Modified Eagle's Medium (DMEM):F12 (17 μM pantothenate, 33 μM biotin and antibiotics [100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin]). The sample was then passed through a 200 μm filter followed by centrifugation at 200 x g for 20 minutes at room temperature. The lipid layer found at the top of the tube was then removed. Fetal bovine serum (FBS; 10%) was then added to the remainder of the sample in the tube and the pellet was resuspended. The sample was then filtered though sterile 100 μm, 50 μm and 25 μm filters followed by centrifugation at 200 x g at room temperature for 20 minutes. The supernatant was then removed without disturbing the pellet and 10 mL of red cell lysis buffer (155 mM NH_4Cl, 5.7 mM K_2HPO_4, 0.1 mM EDTA; pH 7.4) was added for 5 minutes followed by the addition of 10 ml DMEM/10% FBS/antibiotics. The sample was then spun for 5 minutes at 200 x g at room temperature. Finally, the supernatant was removed without disturbing the pellet and the pellet was resuspended in 500 μL DMEM/F12 medium. Cells were then grown in DMEM supplemented with 10% FBS and antibiotics.

Upon reaching 80-90% confluence, some of these samples were either subsequently seeded for a maximum of three passages or cryopreserved until needed. Preadipocytes were thawed and grown in DMEM supplemented with 10% FBS in the presence of antibiotics.

Preadipocytes were seeded overnight at a density of $3x10^4$ cells/cm^2 in DMEM supplemented with 10% FBS and antibiotics, and induced to differentiate the next day. Differentiation was induced by addition of DMEM supplemented with 10% FBS, 0.85 μM insulin, 100 μM indomethacin, 0.5 μM dexamethasone, 0.25 mM isobutylmethylxanthine and antibiotics for 14 days. On day 14, differentiation medium was removed, cells were washed once in DMEM supplemented with 10% FBS and antibiotics, maintained in this same medium for 2 more days, and cell stimulation studies occurred on day 17. The extent of differentiation averaged 70%.

Immunoblot analysis

Human abdominal subcutaneous differentiated adipocytes were placed in Krebs Ringer HEPES (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, 5.6 ml glucose; pH 7.4) supplemented with 1% calf serum and treated with 50 mU/ml TSH (Calbiochem) or vehicle (H_2O) for 30 min. Where indicated, cells were treated with 20 μM H89, 10 μM LY294002, 100 nM wortmannin, 10 μM DPI, 10 μM rottlerin, or vehicle (0.1% dimethylsulfoxide) for the indicated times before the addition of TSH. After treatment, cell monolayers were lysed in Laemmli buffer (Laemmli 1970) supplemented with 5 mM EGTA, 5 mM sodium pyrophosphate, 50 mM NaF and 1 mM Na_3VO_4. Lysate protein concentrations were determined with the modified Lowry method (Bio-Rad; Mississauga, Ontario, Canada), using bovine serum albumin as a standard. Equal

amounts of solubilized protein (10–75 μg, depending on the experiment) were resolved by SDS-PAGE, followed by electrophoretic transfer to a nitrocellulose membrane. Nonspecific binding sites were blocked, and the membrane was probed with either antiphospho-IKKα ser180/IKKβ ser181 (1:500); anti-IκBα (1:500), antiphospho-CREB Ser 133 (1:1000; all from Cell Signaling Technology, Beverly, MA), or anti-IKKβ (clone 10AG2; 2 μg/ml; Upstate Biotechnology, Lake Placid, NY) followed by the appropriate horseradish peroxidase-conjugated antibody (Cell Signaling). Immunoreactivity was detected by chemiluminescence (Immobilon Western Chemiluminescence HRP Substrate; Millipore). Relative intensity of the bands was determined with AlphaEaseFC software (Alpha Innotech, San Leandro, CA) and data expressed as integrated optical density (IOD) units.

Real time PCR

Human abdominal subcutaneous differentiated adipocytes were placed in DMEM supplemented with 1% calf serum and antibiotics. Cells were treated with 50 mU/ml TSH (Calbiochem) or vehicle (H_2O) for 2 hours. Where specified, adipocytes were pretreated with 100 μM sc-514, 20 μM H89, 10 μM rottlerin, or vehicle (0.1% dimethyl sulfoxide) for the times indicated prior to TSH stimulation. Following stimulation, RNA was extracted with TRI Reagent, and treated with DNAse I, according to manufacturer's instructions (Ambion). Total RNA (1 μg; heat denatured) was reversed transcribed in a reaction volume of 20 μl, containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 6 μg of random primers, 0.5 mM of each dNTP, 10 U of RNase inhibitor and 100 U of reverse transcriptase (all reagents were from Invitrogen). Control assays, without reverse transcriptase, were performed for all reactions. Real time PCR was performed using the

QuantiTect SYBR Green RT-PCR kit (Qiagen) with 900 nM target primers (Table 1), or QuantiTect Probe RT-PCR kit with 18S rRNA primers (Qiagen), according to the manufacturer's protocol. Amplification consisted of 1 cycle at 95°C for 15 minutes and 45 cycles at 95°C for 15 seconds (denaturation) followed by 30 seconds at 60°C (annealing) and then 30 seconds at 72°C (extension) performed in a Roche Light Cycler Real-Time PCR System using Light Cycler Software 4.05. mRNA expression data are expressed as relative quantification (RQ).

Table 1. Target primers used for quantification of mRNA using real time PCR

Target		
IL-6	Forward	5'CCACACAGACAGCCACTCACCTC3'
	Reverse	5'CTGGCTTGTTCCTCACTACTCTC3'
MCP-1	Forward	5'CAGCCAGATGCAATCAATGC3'
	Reverse	5'GTGGTCCATGGAATCCTGAA3'
VEGF	Forward	5'CCTTGCTGCTCTACCTCCAC3'
	Reverse	5'CCATGAACTTCACCACTTCG3'
RBP4	Forward	5'GCCTCTTTCTGCAGGACAAC3'
	Reverse	5'GCACACGTCCCAGTTATTCA3'
Adiponectin	Forward	5'GCAGAGATGGCACCCCTG3'
	Reverse	5'GGTTCACCGATGTCTCCCTTA3'
TSHR	Forward	5'GATATTCAACGCATCCCCAG3'
	Reverse	5'GGCAGATTAGAAAATGCATGA3'

Quantification of IL-6 and MCP-1 release

Human abdominal subcutaneous differentiated adipocytes were placed in DMEM supplemented with 1% calf serum and antibiotics. Cells were treated with 50 mU/ml TSH (Calbiochem) or vehicle (H_2O) for 4 hours. Where specified, adipocytes were pretreated with 100 μM sc-514, 20 μM H89, 10 μM rottlerin, or vehicle (0.1% dimethyl sulfoxide) for the times indicated prior to TSH stimulation. After treatment, medium was collected and centrifuged (500 x g for 5 min at 4°C) to remove cell debris. The supernatants were collected

and IL-6 and MCP-1 protein in the medium was measured by enzyme immunometric assay (Assay Designs, Ann Arbor, MI; R&D Systems, Minneapolis, MN, respectively), following the manufacturer's instructions.

Cell monolayers were lysed, and protein concentration was measured by the modified Lowry method (Bio-Rad; Mississauga, ON, Canada), using bovine serum albumin as a standard. IL-6 and MCP-1 released into the medium was normalized to total cell lysate protein.

Immunoprecipitation and Kinase Assay

Human abdominal subcutaneous differentiated adipocytes were placed in KRH buffer supplemented with 1% calf serum and treated with 50 mU/ml TSH (Calbiochem) or vehicle (H_2O) for 15 minutes. KRH was then aspirated and the cells were subsequently lysed and scraped using 500 µl lysis buffer containing 1X PBS, 1% Nonidet P-40 (NP-40), 50 mM NaF, 4 mg/ml benzamidine, 1 mM β-glycerophosphate, 200 µM Na_3VO_4, 0.1 mg/ml phenylmethyl-sulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin and 5 mM NaPPi. Cell lysates were centrifuged at 4°C at 14000 x g for 10 minutes. Supernatants were transferred to new tubes and assayed for protein content using the BCA™ Protein Assay Reagent Kit (Pierce). Equal amounts of protein from cell lysates were pre-cleared with protein A-sepharose, then incubated for 90 minutes at 4°C with 1 µg rabbit anti-protein kinase C (PKC)δ antibody (Santa Cruz Biotechnology) adsorbed to the sepharose beads. Immunoprecipitated proteins were recovered by centrifugation (14000 x g; 1 min) and washed 3 times with ice-cold lysis buffer. Samples were then washed twice with kinase assay buffer containing 25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.5 mM EGTA, 1mM

dithiothreitol, 20 μg phosphatidylserine, 20 μM ATP and were resuspended in 30 μl kinase buffer containing 5 μg histone H1 as a substrate and 5 μCi of [γ-^{32} ATP] was added. The reaction incubated for 5, 15 or 30 minutes at room temperature. The reaction was stopped by adding 30 μl of 2 X Laemmli buffer with 10% β-mercaptoethanol. Samples were boiled then analyzed by 15% SDS-PAGE gel. Phospho-histone H1 was detected by autoradiography (Kodak BioMax MS Film).

Statistical analysis

Statistical analysis was performed with Student's t test for paired values or ANOVA with Newman-Keuls post-hoc test, as appropriate, using GraphPad Instat version 3.05 (GraphPad Software Inc., San Diego, CA), with $p<0.05$ considered significant.

RESULTS

TSH-DEPENDENT REGULATION OF IL-6 PRODUCTION IN HUMAN DIFFERENTIATED ADIPOCYTES:

To identify the intermediates implicated in TSH-induced activation of NF-κB and its upstream regulator IKKβ, as it relates to IL-6 regulation, various pathways were studied.

TSH-induced IL-6 production from human differentiated adipocytes is dependent on IKKβ activity

Because our laboratory had previously shown that TSH induces IL-6 protein release from human differentiated adipocytes, I firstly wanted to confirm these findings at the mRNA level. Indeed, upon TSH stimulation, IL-6 mRNA expression was increased by more than 100-fold over basal levels (Figure 2). Furthermore, our laboratory demonstrated that this TSH-dependent induction of IL-6 protein release was dependent on IKKβ activity (Antunes 2008). To determine whether IL-6 mRNA regulation in response to TSH stimulation requires IKKβ activity, human abdominal subcutaneous differentiated adipocytes were pretreated with 100 μM sc-514, a selective IKKβ inhibitor, followed by TSH stimulation. The TSH-induced IL-6 mRNA expression levels were inhibited by 48% with sc-514 pretreatment ($p<0.01$; Figure 3).

Figure 2. TSH stimulates the expression of IL-6 mRNA. Human abdominal subcutaneous differentiated adipocytes were stimulated with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. IL-6 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 5 separate patient samples. RQ: relative quantification. *denotes $p<0.05$ compared to control.

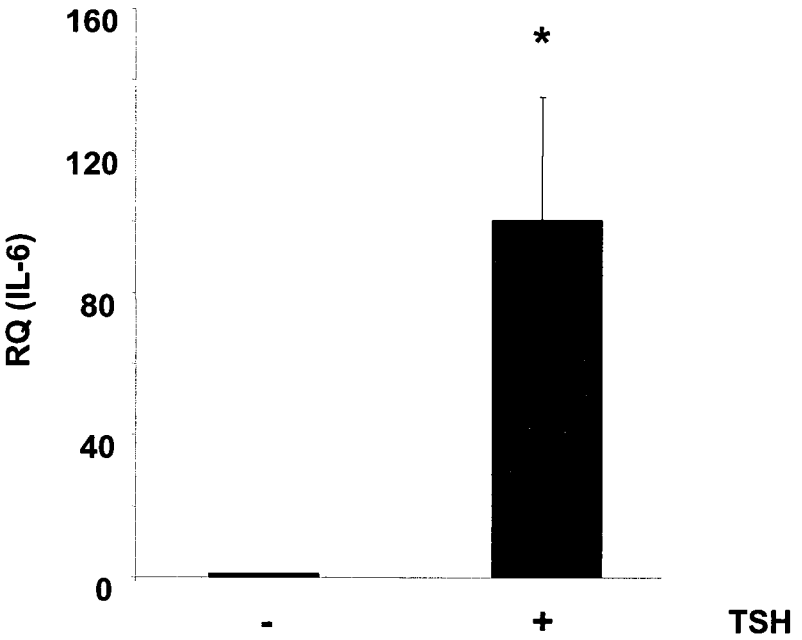
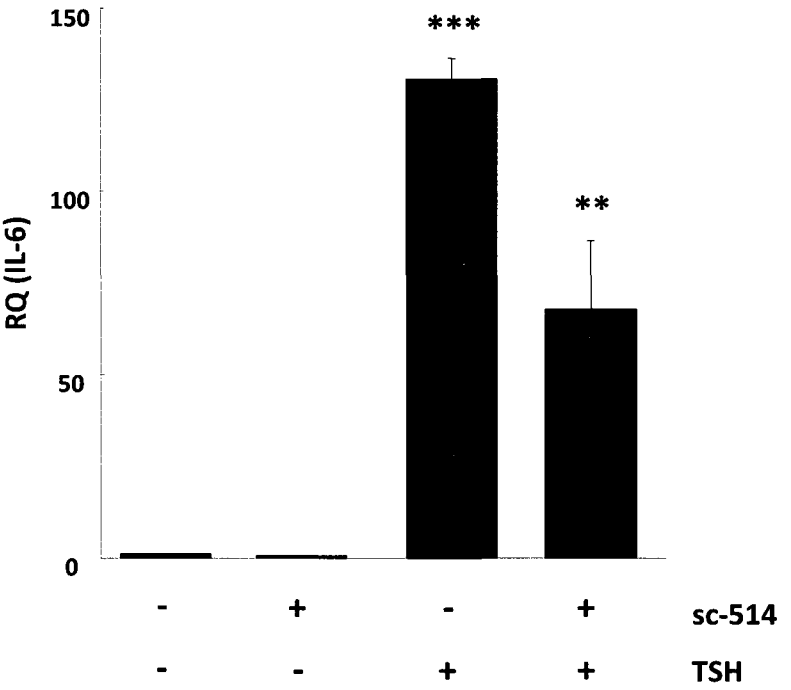

160
120
80
40
0
RQ (IL-6)
*
-
+
TSH

Figure 3. TSH-stimulated expression of IL-6 mRNA depends on IKKβ activity. Human abdominal subcutaneous differentiated adipocytes were pre-treated with 100 μM sc-514 or vehicle for 1 hour prior to stimulation with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. IL-6 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 4 separate patient samples. RQ: relative quantification. *** denotes $p<0.001$ compared to control; ** denotes $p<0.01$ compared to TSH.


150
100
50
0
RQ (IL-6)
***
**
-
+
-
+
sc-514
-
-
+
+
TSH

TSH-induced IKKβ/NF-κB activation and IL-6 production from human differentiated adipocytes is not dependent on PKA activity

We have previously shown that TSH-stimulated IL-6 release from differentiated 3T3-L1 adipocytes requires PKA activity (Antunes 2005), and that this same response requires the IKKβ/NF-κB pathway in human abdominal subcutaneous differentiated adipocytes (Antunes 2008). Therefore, I investigated whether PKA is upstream of the TSH-dependent activation of the IKKβ/NF-κB pathway in my cell model. Human abdominal subcutaneous differentiated adipocytes were pretreated with 20 μM H89, a selective PKA inhibitor, followed by TSH stimulation. H89 pretreatment did not alter either TSH-stimulated IKKβ phosphorylation or IκBα degradation although it did successfully inhibit TSH-dependent phosphorylation of CREB, a known PKA-dependent event (Figure 4).

Although H89 did not affect TSH-induced IKKβ/NF-κB activation, I determined whether PKA is important for IL-6 production in these cells, as it could act through a pathway distinct from IKKβ/NF-κB. However, H89 pretreatment did not affect either the TSH-dependent increase in IL-6 mRNA expression (Figure 5) or protein release (Figure 6).

TSH-induced IKKβ/NF-κB activation in human differentiated adipocytes is not dependent on PI3K activity

HeLa cells expressing the B2-type bradykinin GPCR receptor have been shown to require phosphoinositide 3-kinase (PI3K) activity for the activation of the NF-κB pathway in response to bradykinin stimulation (Xie 2000). I therefore tested whether PI3K was upstream of IKKβ/NF-κB activation in my cell model. Human abdominal subcutaneous differentiated adipocytes were pretreated with 10 μM LY294002 or 100 nM wortmannin,

Figure 4. TSH-stimulated activation of the IKKβ/NF-κB pathway does not depend on PKA activity. Human abdominal subcutaneous differentiated adipocytes were pre-treated with 20 μM H89 or vehicle for 1 hour prior to stimulation with 50 mU/ml TSH or vehicle for 30 min. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibody against phospho-IKK (pIKK), phospho-CREB (pCREB) and IκBα. Blots were stripped and reprobed with antibody against IKKβ. Representative immunoblots from a single experiment are shown. Densitometric analysis from 3 different patient samples are expressed as mean +/- SE. IOD: integrated optical density. * denotes $p<0.05$ compared to TSH; ** denotes $p<0.01$ compared to control and H89.
Note: All data in this figure were obtained from my experiments.

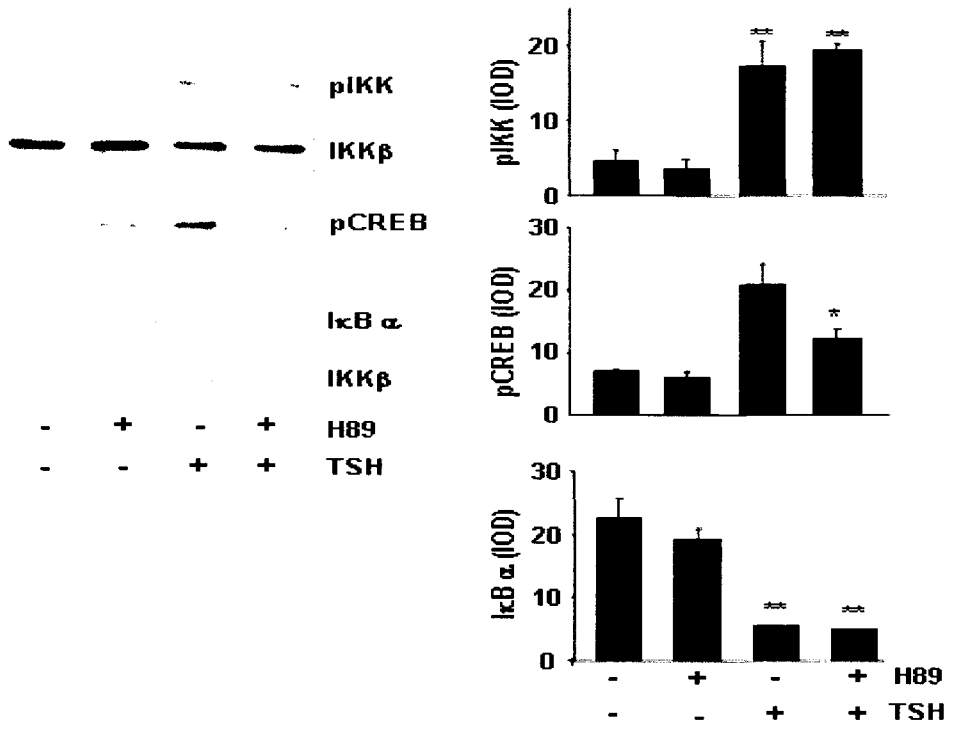


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Figure 5. TSH-stimulated expression of IL-6 mRNA does not depend on PKA activity. Human abdominal subcutaneous differentiated adipocytes were pre-treated with 20 μM H89 or vehicle for 1 hour prior to stimulation with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. IL-6 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 3 separate patient samples. RQ: relative quantification. ** denotes $p<0.01$ compared to control and H89.

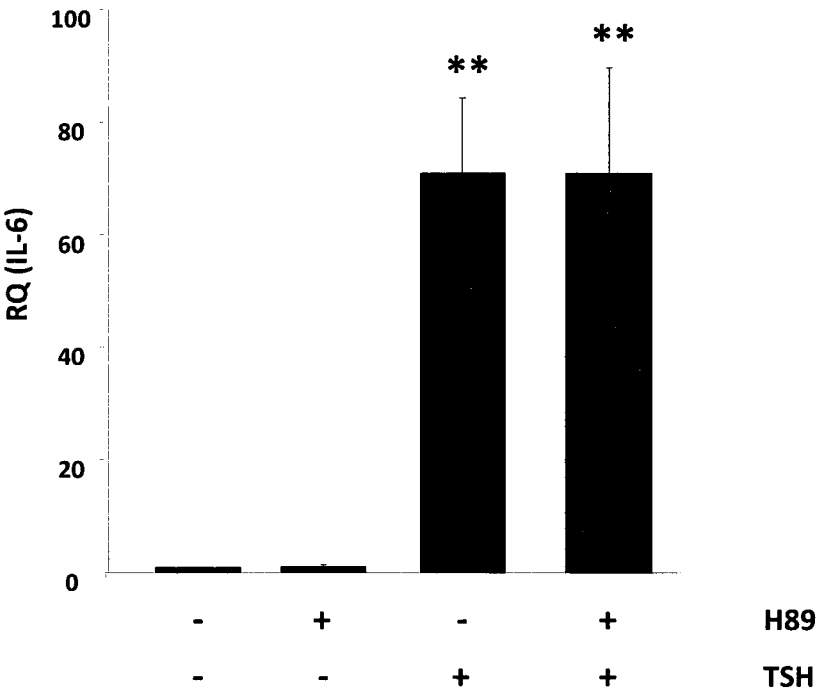
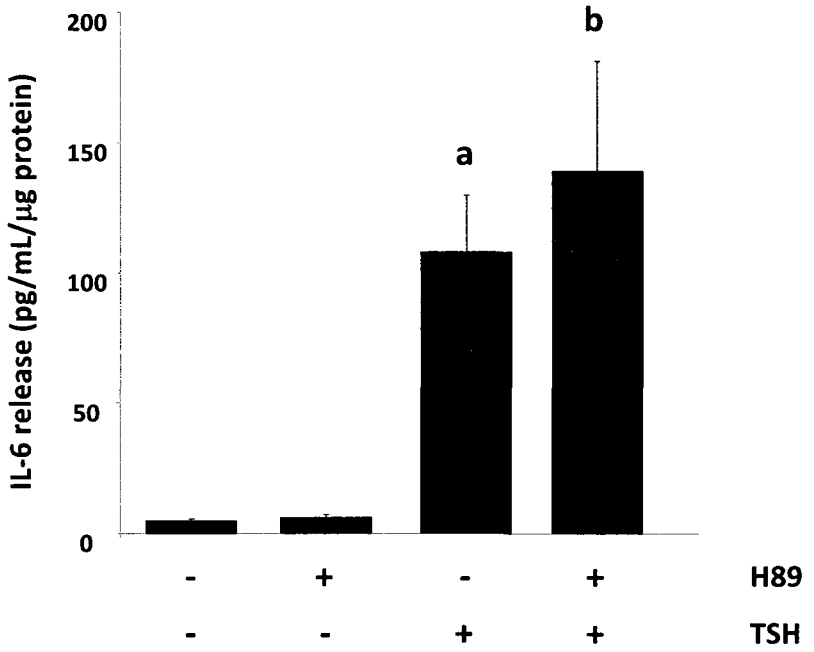

100
80
60
40
20
0
RQ (IL-6)
**
**
-
+
-
+
H89
-
-
+
+
TSH

Figure 6. TSH-stimulated IL-6 protein release does not depend on PKA activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 20 μM H89 or vehicle for 1 hour prior to simulation with 50 mU/ml TSH or vehicle for 4 hours. IL-6 protein in the medium was measured as described. Data are expressed as mean ± SE from 3 separate patient samples, each performed in duplicate. a denotes $p<0.05$ compared to control ; b denotes $p<0.01$ compared to H89 and $p<0.05$ compared to control.


IL-6 release (pg/mL/µg protein)
200
150
100
50
0
a
b
-
+
-
+
H89
-
-
+
+
TSH

both PI3K inhibitors, followed by stimulation with TSH. TSH-dependent IKKβ phosphorylation and IκBα degradation were unaltered in the presence of either PI3K inhibitor (Figure 7).

TSH-induced IKKβ/NF-κB activation in human differentiated adipocytes is dependent on NADPH oxidase activity

Angiotensin II and thrombin have been show to increase levels of reactive oxygen species (ROS) following stimulation of their respective GPCRs in vascular smooth muscle cells (Griendling 1994; Patterson 1999). Furthermore, the production of ROS has been suggested as a mediator of NF-κB activation by GPCRs including that for angiotensin II (Hirotani 2002; Wegener 2007). I therefore investigated whether NADPH oxidase activity, a complex responsible for the production of ROS, is necessary for IKKβ/NF-κB activation. Human abdominal subcutaneous differentiated adipocytes were pretreated with10 μM diphenyleneiodonium chloride (DPI), an NADPH oxidase inhibitor, followed by TSH stimulation. The phosphorylation of IKKβ was reduced by 43% with the inhibition of NADPH oxidase activity ($p<0.01$; Figure 8). Furthermore, the degradation of IκBα was significantly reversed by more than 46% with DPI pretreatment ($p<0.01$; Figure 8). TSH-induced phosphorylation of CREB was also inhibited with DPI pretreatment (Figure 8A).

Figure 7. TSH-stimulated activation of the IKKβ/NF-κB pathway does not dependent on PI3K activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 10 μM LY294002, 100 nM wortmannin, or vehicle for 15 minutes prior to stimulation with TSH or vehicle for 30 minutes. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibody against pIKK and IκBα. Blots were stripped and reprobed with antibody against IKKβ. A: Representative immunoblots from a single experiment are shown. B: Densitometric analysis from 3 different patient samples are expressed as mean +/- SE. IOD: integrated optical density. Top panel: a denotes $p<0.01$ compared to control and LY294002; b denotes $p<0.01$ compared to control and wortmannin. Bottom panel: a denotes $p<0.05$ compared to control; b denotes $p<0.05$ compared to LY294002; c denotes $p<0.05$ compared to wortmannin.

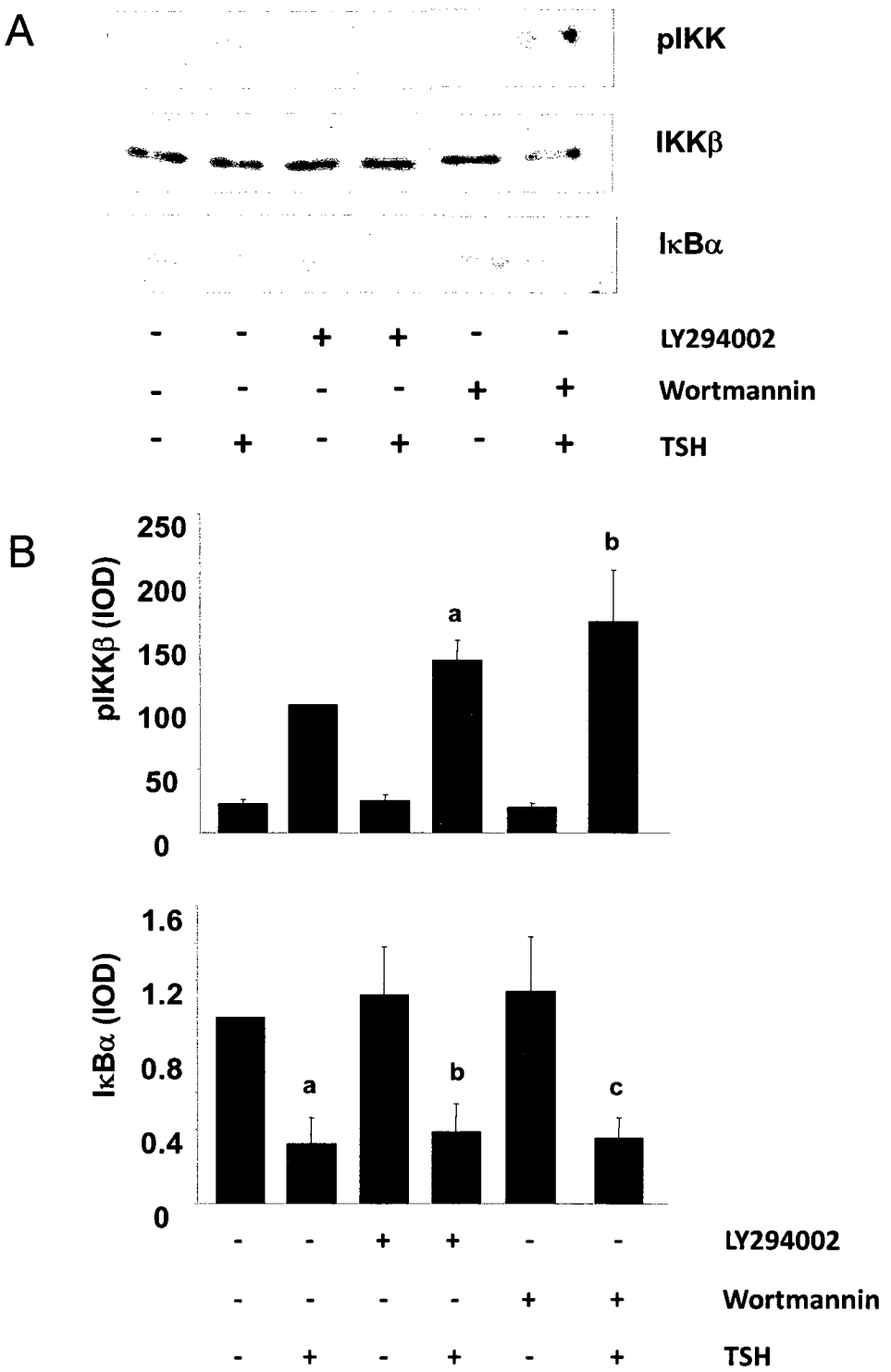
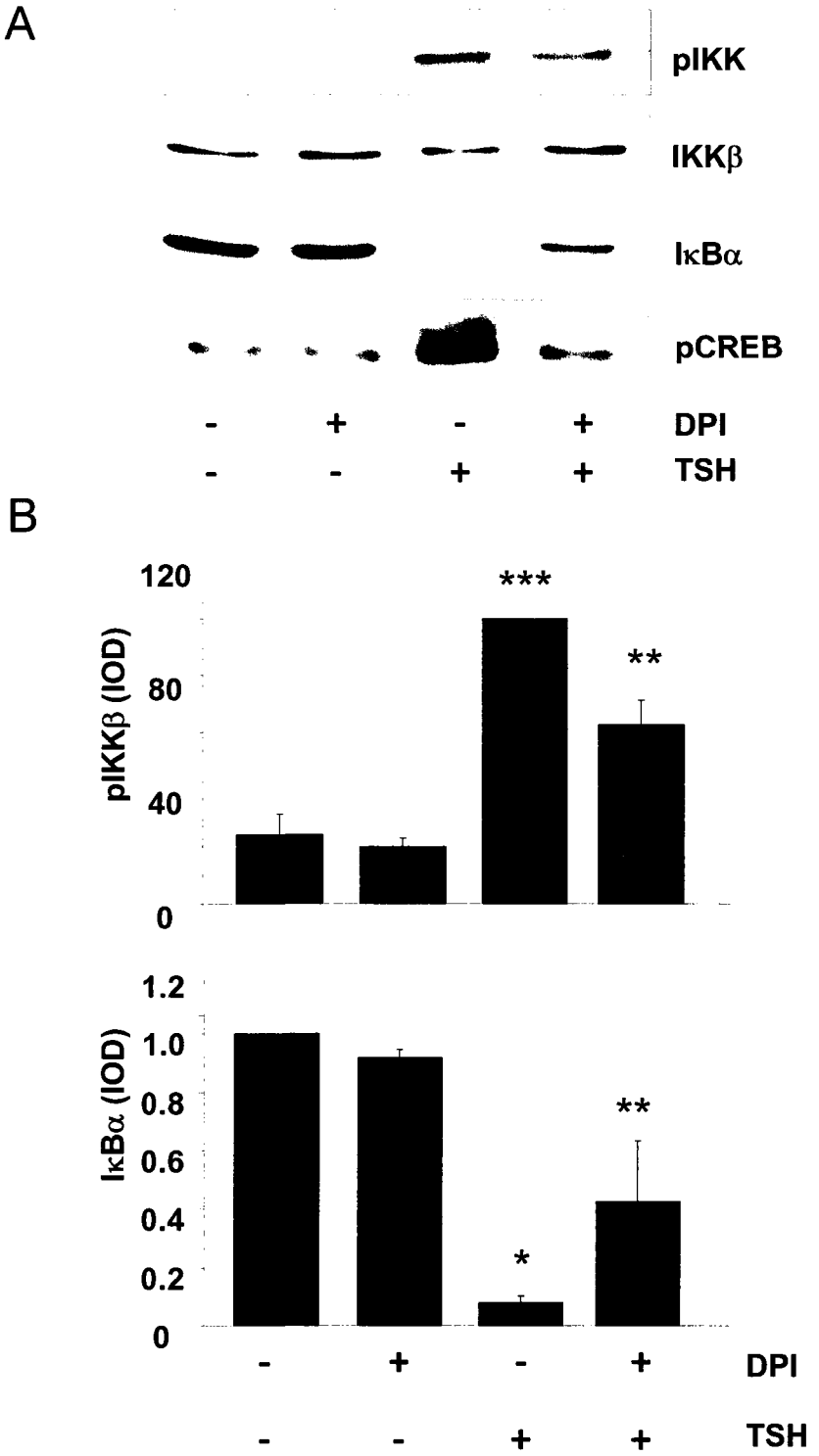

A
pIKK
IKKβ
IκBα
- - + + - - LY294002
- - - - + + Wortmannin
- + - + - + TSH
B
pIKKβ (IOD)
250
200
150
100
50
0
a
b
IκBα (IOD)
1.6
1.2
0.8
0.4
0
a
b
c
- - + + - - LY294002
- - - - + + Wortmannin
- + - + - + TSH

Figure 8. TSH-stimulated activation of the IKKβ/NF-κB pathway depends on NADPH oxidase activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 10 μM DPI or vehicle for 30 minutes prior to stimulation with 50 mU/ml TSH or vehicle for 30 minutes. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibody against pIKK, IκBα and pCREB. Blots were stripped and reprobed with antibody against IKKβ. A: Representative immunoblots from a single experiment are shown. B: Densitometric analysis from 3 different patient samples are expressed as mean +/- SE. IOD: integrated optical density. *** denotes $p<0.001$ compared to control; ** denotes $p<0.01$ compared to TSH; * denotes $p<0.05$ compared to control.


A
pIKK
IKKβ
IκBα
pCREB
- + - + DPI
- - + + TSH
B
120
80
40
0
pIKKβ (IOD)
***
**
1.2
1.0
0.8
0.6
0.4
0.2
0
IκBα (IOD)
**
*
- + - + DPI
- - + + TSH

TSH-induced IKKβ/NF-κB activation and IL-6 production from human differentiated adipocytes is dependent on PKCδ activity

Because NADPH oxidase activity is important for TSH-induced IKKβ/NF-κB activation, I investigated potential upstream regulatory factors. PKCδ has been shown to activate $p47^{phox}$, a subunit in the NADPH oxidase complex responsible for ROS production (Reeves 1999; Fontayne 2002). I therefore determined whether PKCδ activity is also important for IKKβ/NF-κB activation in my cell model, indicating the possibility that it could be positioned upstream of ROS production. To investigate the role of PKCδ in the activation of the IKKβ/NF-κB pathway by TSH, human abdominal subcutaneous differentiated adipocytes were pretreated with rottlerin, a PKCδ inhibitor, followed by TSH stimulation. The TSH-induced increase in IKKβ phosphorylation was inhibited by 55% with rottlerin pretreatment ($p<0.05$; Figure 9). Furthermore, IκBα degradation following TSH stimulation was significantly blocked by ~35% ($p<0.01$) and TSH-induced CREB phosphorylation was inhibited with rottlerin pretreatment Figure 9).

Taking it one step further, I examined whether rottlerin pretreatment inhibited TSH-induced IL-6 release. Indeed, rottlerin pretreatment did significantly blunt the TSH-dependent increase in IL-6 release by 60% ($p<0.05$ Figure 10).

To determine whether PKCδ is activated in my cell model upon TSH stimulation, PKCδ was immunoprecipitated from human abdominal subcutaneous differentiated adipocytes and an *in vitro* kinase assay was performed using histone H1 as a substrate. Preliminary data shows an increase in PKCδ activity with TSH stimulation (Figure 11).

Figure 9. TSH-stimulated activation of the IKKβ/NF-κB pathway depends on PKCδ activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 10 μM rottlerin or vehicle for 30 minutes prior to stimulation with 50 mU/ml TSH or vehicle for 30 minutes. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibody against pIKK, IκBα and pCREB. Blots were stripped and reprobed with antibody against IKKβ. A: Representative immunoblots from a single experiment are shown. B: Densitometric analysis from 3 different patient samples are expressed as mean +/- SE. IOD: integrated optical density. a denotes $p<0.001$ compared to control and $p<0.05$ compared to rottlerin-TSH; b denotes $p<0.001$ compared to control and $p<0.01$ compared to rottlerin-TSH.

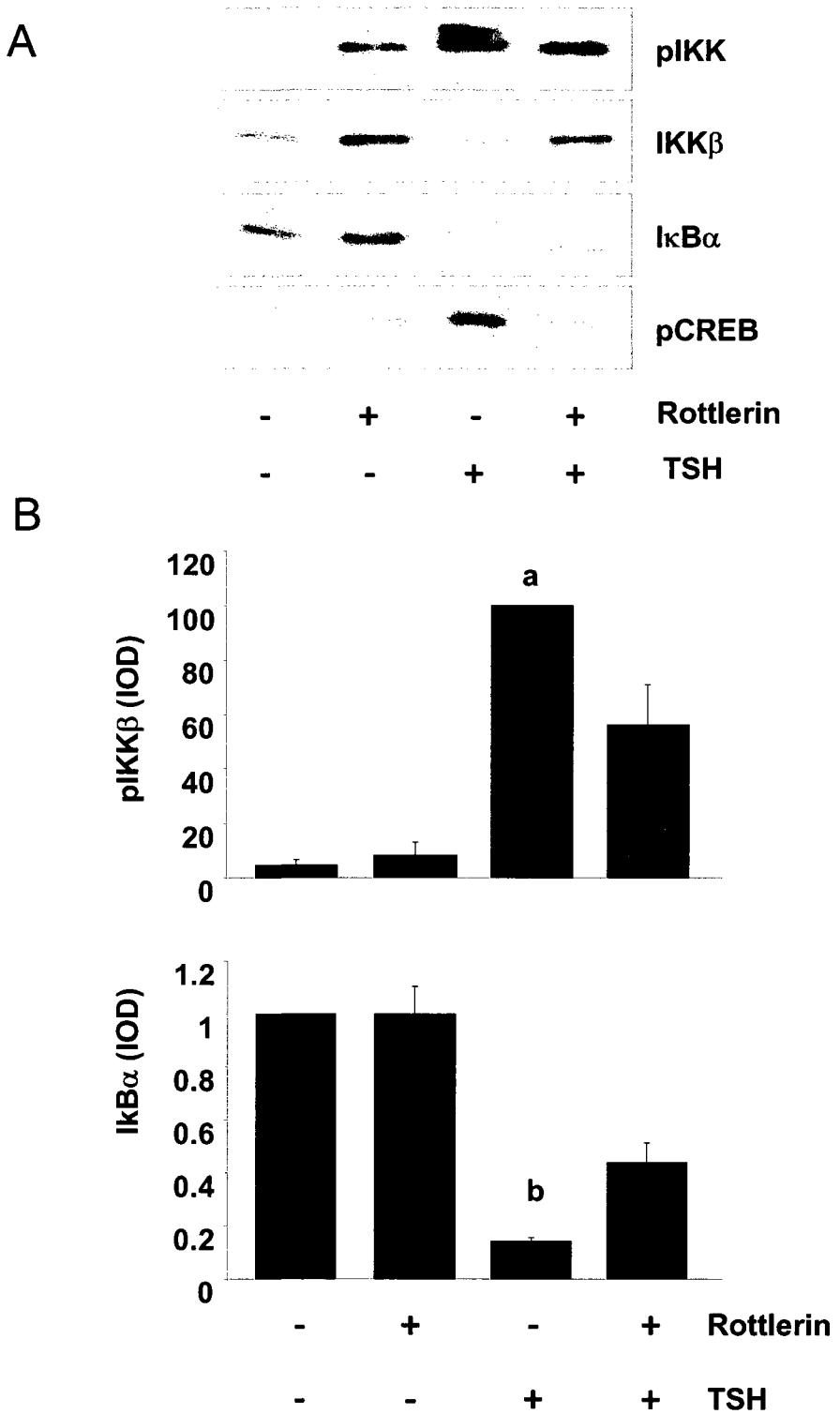

A
pIKK
IKKβ
IκBα
pCREB
- + - + Rottlerin
- - + + TSH
B
pIKKβ (IOD)
120
100
80
60
40
20
0
a
IkBα (IOD)
1.2
1
0.8
0.6
0.4
0.2
0
b
- + - + Rottlerin
- - + + TSH

Figure 10. TSH-stimulated IL-6 protein release depends on PKCδ activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 10 μM rottlerin or vehicle for 30 minutes prior to stimulation with 50 mU/ml TSH or vehicle for 4 hours. IL-6 protein in the medium was measured as described. Data are expressed as mean ± SE from 3 separate patient samples * denotes $p<0.05$ compared to control and rottlerin-TSH.

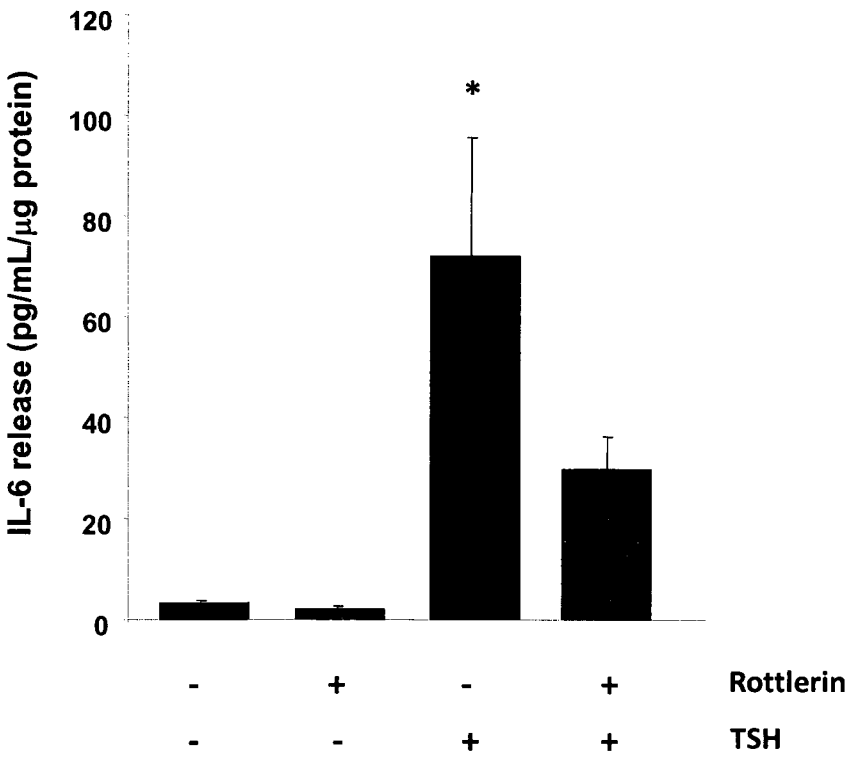
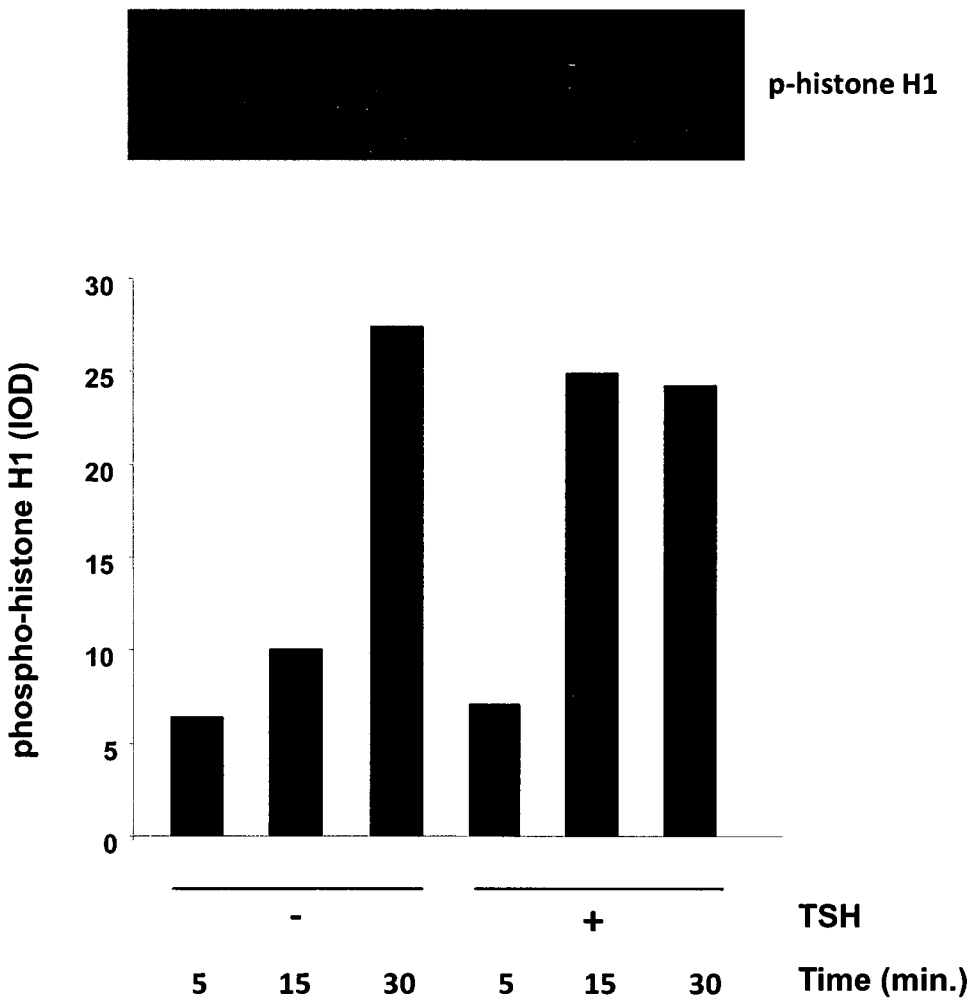

IL-6 release (pg/mL/µg protein)
120
100
80
60
40
20
0
*
-
+
-
+
Rottlerin
-
-
+
+
TSH

Figure 11. TSH stimulates PKCδ activity. Human abdominal subcutaneous differentiated adipocytes were stimulated with 50 mU/ml TSH or vehicle for 15 minutes. Cells were lysed, PKCδ was immunoprecipitated and its activity was determined by *in vitro* kinase assay using histone H1 as substrate as described. Reactions were incubated for the indicated times. A single exposure of phospho-histone H1 (p-histone H1) is shown, and densitometric analysis was performed for this one patient sample.


p-histone H1
phospho-histone H1 (IOD)
30
25
20
15
10
5
0
-
+
TSH
5
15
30
5
15
30
Time (min.)

TSH-DEPENDENT INDUCTION OF ADIPOKINE mRNA EXPRESSION:

To investigate whether other candidate adipokines are regulated by TSH, human abdominal subcutaneous differentiated adipocytes were stimulated with TSH. MCP-1 mRNA levels were increased by more than 12-fold ($p<0.05$ Figure 12) and VEGF levels by ~3-fold ($p<0.05$, Figure 13) in response to TSH. However, no change in RBP4 (Figure 14) or adiponectin (Figure 15) mRNA levels were noted upon TSH stimulation.

TSH-DEPENDENT REGULATION OF MCP-1 PRODUCTION IN HUMAN DIFFERENTIATED ADIPOCYTES:

Since MCP-1 had the greatest increase in mRNA expression in response to TSH, I decided to further study its regulation by TSH. In order to start elucidating the mechanisms important for TSH-dependent MCP-1 regulation, two pathways were studied.

TSH-induced MCP-1 production from human differentiated adipocytes is dependent on IKKβ activity

First of all, I wanted to determine whether MCP-1 regulation in response to TSH requires IKKβ activity, as did IL-6. To do so, human abdominal subcutaneous differentiated adipocytes were pretreated with 100 μM sc-514, followed by TSH stimulation. Data revealed that the TSH-induced MCP-1 mRNA expression was inhibited by almost 59% with sc-514 pretreatment ($p<0.001$; Figure 16). Furthermore, I observed a significant increase in

Figure 12. TSH stimulates the expression of MCP-1 mRNA. Human abdominal subcutaneous differentiated adipocytes were stimulated with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. MCP-1 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 5 separate patient samples. RQ: relative quantification. *denotes $p<0.05$ compared to control.

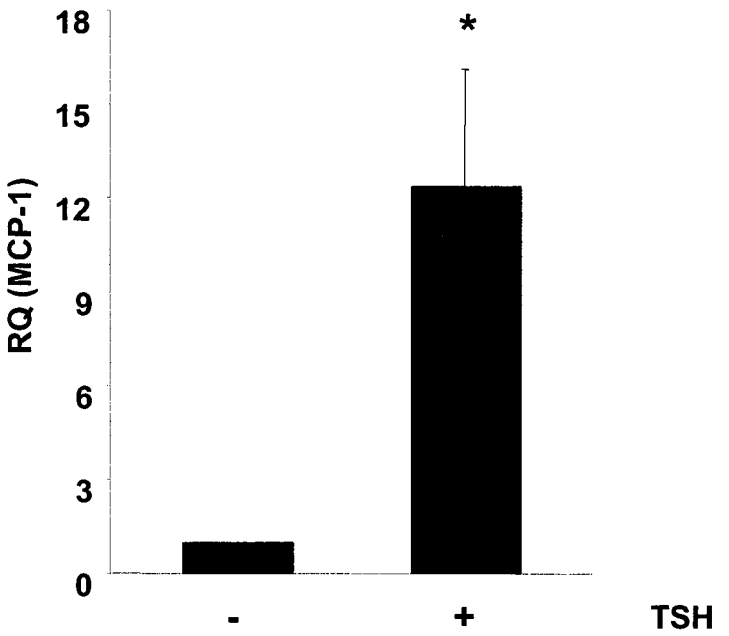

18
15
12
9
6
3
0
RQ (MCP-1)
*
-
+
TSH

Figure 13. TSH stimulates the expression of VEGF mRNA. Human abdominal subcutaneous differentiated adipocytes were stimulated with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. VEGF mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 5 separate patient samples. RQ: relative quantification. *denotes $p<0.05$ compared to control.

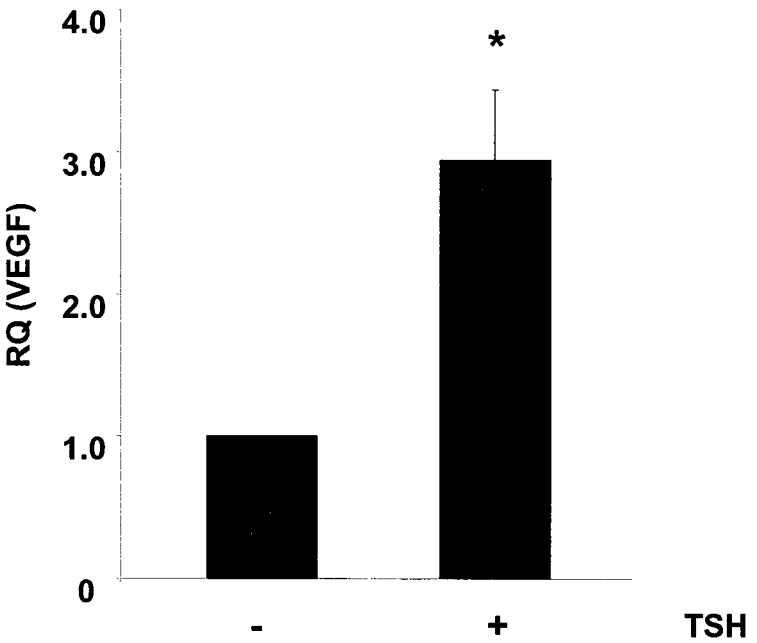

4.0
3.0
2.0
1.0
0
RQ (VEGF)
*
-
+
TSH

Figure 14. TSH stimulation does not affect the expression of RBP4 mRNA. Human abdominal subcutaneous differentiated adipocytes were stimulated with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. RBP4 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 5 separate patient samples. RQ: relative quantification.

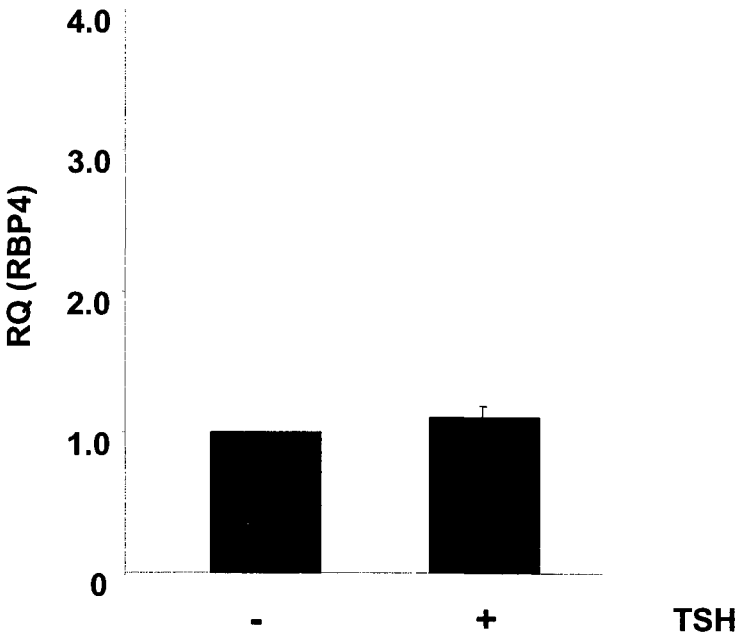

4.0
3.0
2.0
1.0
0
RQ (RBP4)
-
+
TSH

Figure 15. TSH stimulation does not affect the expression of adiponectin mRNA. Human abdominal subcutaneous differentiated adipocytes were stimulated with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. Adiponectin mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 5 separate patient samples. RQ: relative quantification.

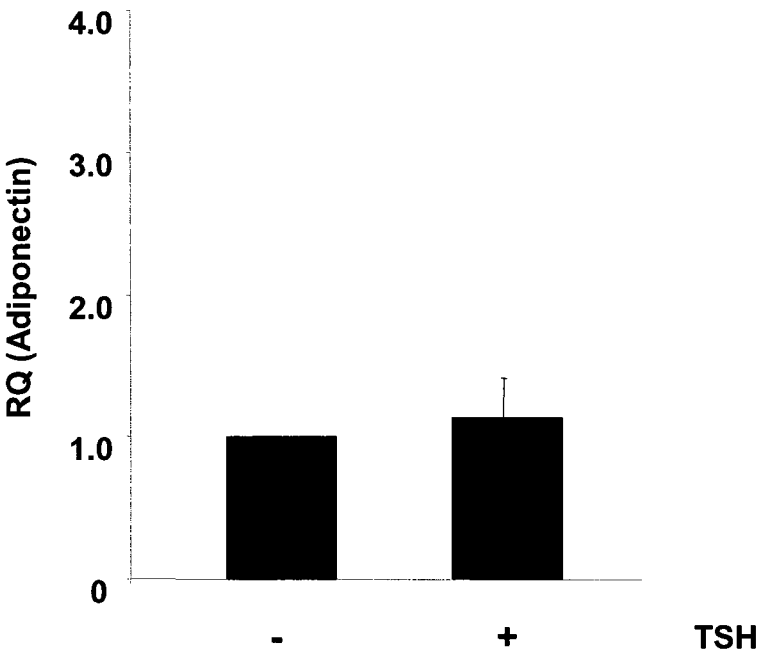
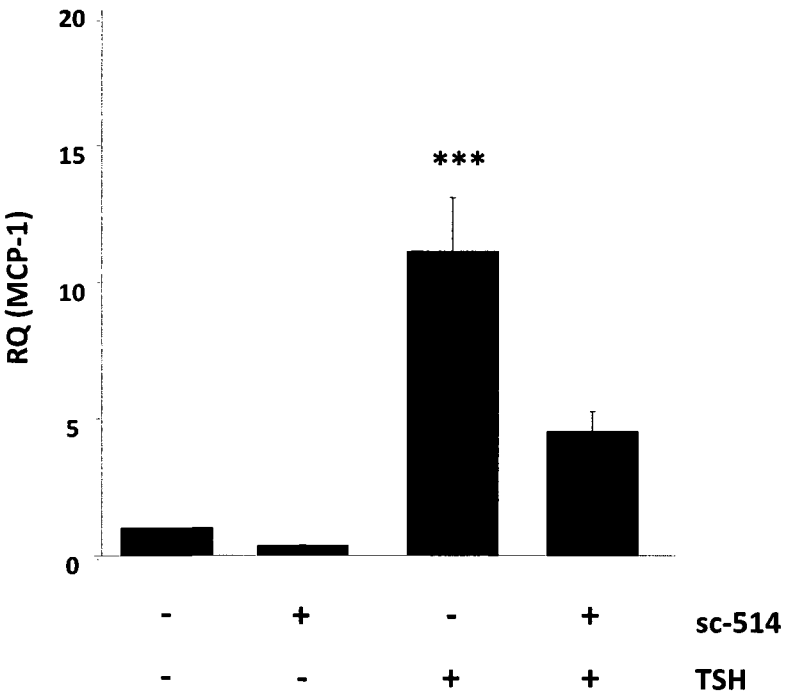

4.0
3.0
2.0
1.0
0
RQ (Adiponectin)
-
+
TSH

Figure 16 . TSH-stimulated expression of MCP-1 mRNA depends on IKKβ activity. Human abdominal subcutaneous differentiated adipocytes were pre-treated with 100 μmol/l sc-514 or vehicle for 1 hour prior to stimulation with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. MCP-1 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 4 separate patient samples. RQ: relative quantification. *** denotes $p<0.001$ compared to control and sc-514-TSH.


20
15
10
5
0
RQ (MCP-1)
***
-
+
-
+
sc-514
-
-
+
+
TSH

MCP-1 protein release following TSH treatment, a response that was inhibited by more than 70% with sc-514 pretreatment ($p<0.05$ Figure 17).

TSH-induced MCP-1 production from human differentiated adipocytes is dependent on PKA activity

To evaluate the role of the PKA pathway in the TSH-dependent regulation of MCP-1, human abdominal subcutaneous differentiated adipocytes were pretreated with 20 μM H89, followed by TSH stimulation. My results demonstrated that TSH-induced MCP-1 mRNA expression was inhibited by more than 55% with H89 pretreatment ($p<0.05$; Figure 18). This was also observed at the protein level, with TSH-induced MCP-1 protein release into the medium being inhibited by almost 55% with H89 pretreatment ($p<0.01$; Figure 19).

Figure 17. TSH-stimulated MCP-1 protein release from human adipocytes depends on IKKβ activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 100 μM sc-514 or vehicle for 1 hour prior to simulation with 50 mU/ml TSH or vehicle for 4 hours. MCP-1 protein in the medium was measured as described. Data are expressed as mean ± SE from 3 separate patient samples. ** denotes $p<0.01$ compared to control; * denotes $p<0.05$ compared to TSH.

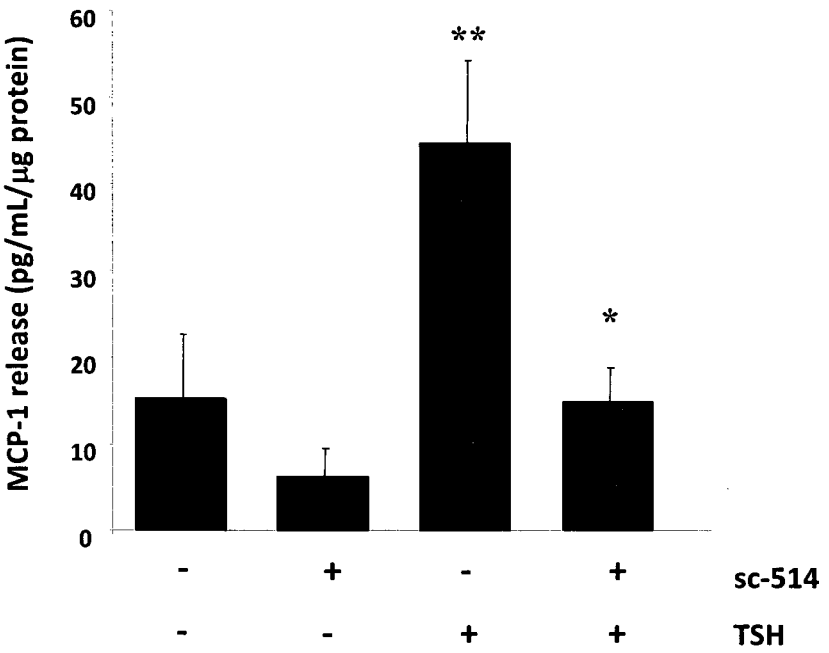

MCP-1 release (pg/mL/μg protein)
60
50
40
30
20
10
0
**
*
-
+
-
+
sc-514
-
-
+
+
TSH

Figure 18. TSH-stimulated expression of MCP-1 mRNA depends on PKA activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 20 μM H89 or vehicle for 1 hour prior to stimulation with 50 mU/ml TSH or vehicle for 2 hours. RNA was extracted and quantified by real time PCR as described. MCP-1 mRNA levels were normalized to endogenous 18S mRNA levels (reference) and relative to a control (calibrator). Data are expressed as mean ± SE from 4 separate patient samples. RQ: relative quantification. a denotes $p<0.01$ compared to control and $p<0.05$ compared to H89-TSH.

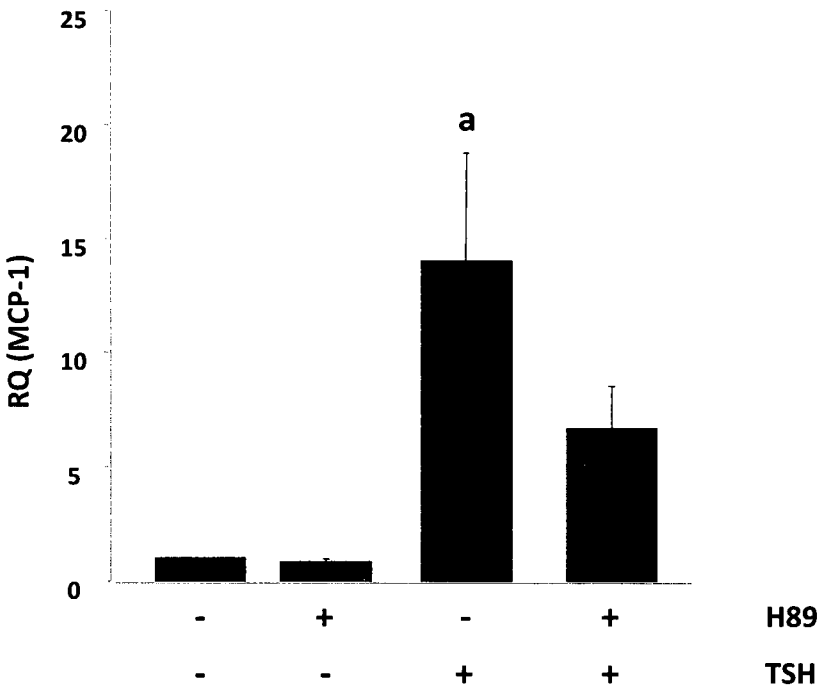
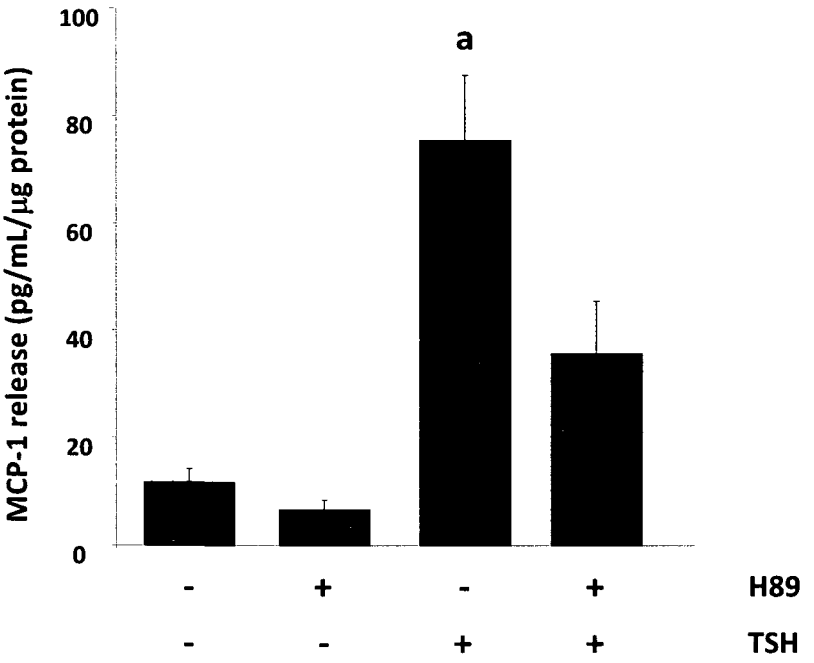

25
20
15
10
5
0
RQ (MCP-1)
a
-
+
-
+
H89
-
-
+
+
TSH

Figure 19. TSH-stimulated MCP-1 protein release from human adipocytes depends on PKA activity. Human abdominal subcutaneous differentiated adipocytes were pretreated with 20 μM H89 or vehicle for 1 hour prior to simulation with 50 mU/ml TSH or vehicle for 4 hours. MCP-1 protein in the medium was measured as described. Data are expressed as mean ± SE from 3 separate patient samples, each performed in duplicate. a denotes $p<0.001$ compared to control; $p<0.01$ compared to H89-TSH.


MCP-1 release (pg/mL/μg protein)
100
80
60
40
20
0
a
-
+
-
+
H89
-
-
+
+
TSH

DISCUSSION

Subclinical hypothyroidism is characterized by elevated levels of TSH and normal thyroid hormone levels. Subclinical hypothyroid patients are at an increased risk for CVD independent of traditional risk factors. TSHR are found in human adipocytes and TSH stimulation of human adipocytes leads to an increase in IL-6 release, a cytokine that has been independently linked to CVD. Therefore, TSH-induced inflammatory responses in adipocytes may explain the increased risk for CVD in patients with subclinical hypothyroidism.

Our laboratory has shown that the TSH-induced IL-6 protein release from human abdominal subcutaneous differentiated adipocytes is an IKKβ/NF-κB-dependent event (Antunes 2008). My research aimed firstly to identify the intermediates implicated in TSH-induced activation of NF-κB and its upstream regulator IKKβ, to better understand how this leads to the stimulation of IL-6 expression and secretion. Secondly I explored whether other adipokines are regulated by TSH treatment and began to elucidate the mechanism by which TSH regulates their expression and release.

REGULATION OF IL-6 PRODUCTION BY TSH IN HUMAN DIFFERENTIATED ADIPOCYTES:

Effect of IKKβ inhibition on the regulation of IL-6 production by TSH in human differentiated adipocytes

My data demonstrating that TSH stimulates IL-6 mRNA expression are in agreement with previous data published by our laboratory showing that TSH induces IL-6 protein release from human abdominal subcutaneous differentiated adipocytes (Bell 2003; Antunes 2008). My results using the inhibitor sc-514 demonstrating that the TSH-induced increase in IL-6 mRNA expression is dependent upon IKKβ activity also confirm similar results found at the protein release level (Antunes 2008). TSH has not been reported to activate the IKKβ/NF-κB pathway in either adipocytes or thyrocytes previous to the publication by our laboratory (Antunes 2008).

Although there was a significant reduction in IL-6 mRNA expression with inhibition of IKKβ activity, it was not complete. This might be due to incomplete pharmacological inhibition as it is sometimes difficult for the inhibitor to reach its target through the lipid droplets in adipocytes. Furthermore, the concentration and pretreatment time for sc-514 were taken from past experiments done in our laboratory; dose-dependent experiments or time courses were not performed by me for my studies, and therefore the concentration of sc-514 used may not be optimal for my cell model. Another possibility is that other pathways that do not involve IKKβ are involved in TSH-induced IL-6 mRNA expression. An ideal option would be to use an inducible system (in order that it may be induced in the mature cells so as not to affect the differentiation process) to knockdown IKKβ in my cell model

using siRNA. Our laboratory is currently attempting to optimize this procedure in human primary adipose cells. However, primary cells are difficult to work with in regards to producing knockdowns and this is proving to be a setback for us. Another option would be to use multiple inhibitors simultaneously to see if blocking multiple pathways would lead to a complete inhibition. More studies would have to be performed in order to determine other candidate pathways in this cell model.

Effect of PKA inhibition on the regulation of IKKβ/NF-κB activation and IL-6 production by TSH in human differentiated adipocytes

TSH activates two main regulatory cascades in thyrocytes via activation of its receptor. The classical pathway, activated in all species studied thus far, is the PKA pathway. The second pathway, involving PKC signaling, will be discussed below. When coupled to $G\alpha_s$, TSHR stimulation leads to the activation of adenylate cyclase, which increases levels of cAMP. cAMP then acts through its main target, PKA, to regulate proliferation and differentiation of thyrocytes, in addition to regulating thyroid hormone secretion (Vassart 1992; Dremier 2002). More specific to my project studying adipocytes, PKA was also shown to be important for TSH-stimulated IL-6 protein release from differentiated 3T3-L1 adipocytes (Antunes 2005). On another note, our laboratory has also previously shown that TSH activates the IKKβ/NF-κB pathway in human differentiated adipocytes. We demonstrated that TSH induces IKKβ phosphorylation, IκBα degradation and NF-κB activation (Antunes 2008). Based on these findings, I decided to see if PKA was involved upstream of IKKβ/NF-κB activation in response to TSH. I therefore determined whether or not PKA activity was involved in the first two steps of the activation of the NF-

κB pathway, namely IKKβ phosphorylation and IκBα degradation. I noted the expected increase in IKKβ phosphorylation and IκBα degradation in response to TSH stimulation; however, the PKA inhibitor H89 did not affect either of these responses. In case PKA activity might be important for TSH-induced IL-6 regulation independent of the NF-κB pathway, I further examined whether the inhibition of PKA affected TSH-induced IL-6 mRNA expression or protein release. TSH treatment led to the expected increase in both IL-6 mRNA expression and protein release but PKA inhibition had no effect on these responses.

My data in differentiated human adipocytes differ with data previously reported by our laboratory demonstrating that TSH-induced IL-6 release from differentiated 3T3-L1 adipocytes is PKA dependent (Antunes 2005). It is not known if TSH induces IKKβ phosphorylation, IκBα degradation or IL-6 mRNA expression in 3T3-L1 differentiated adipocytes. The only comparison that can be made between the two cell models is the TSH-dependent IL-6 protein release. Although cAMP levels are similarly increased in response to TSH stimulation in both cell models (Bell 2002; Antunes 2005; Bell 2000b), PKA activity, a main downstream target of cAMP, does not appear to be important for IL-6 release in human differentiated adipocytes as it was in 3T3-L1 differentiated adipocytes. Although TSH treated cells, in the presence of H89, seemed to display higher amounts of phospho-CREB than did control cells (Figure 4), this difference is not statistically significant, indicating that H89 was effective in blocking PKA activity. A possible reason for the differences seen between 3T3-L1 and human differentiated adipocytes could be the fact that the 3T3-L1 cells are immortal and are aneuploid and thus may not behave as would either primary mouse or human differentiated adipocytes. The abnormal number of chromosomes found in the 3T3-L1 cells might affect the signaling mechanisms employed by

the cell by affecting the actual proteins involved in the activated signaling cascade. It could also affect the transcriptional or translational regulation or even the secretory pathways of the cell depending on which proteins might be affected by the abnormal chromosomes therefore helping to explain the differences seen in PKA dependency for TSH-induced IL-6 release in the two cell models. Furthermore, although murine cells are often used as an alternative to studying human cells, they are not identical. This is not the first time differences have been noted in signaling. Differences were observed between 3T3-L1 and human differentiated adipocytes in the susceptibility to apoptosis as a factor of the stage of differentiation relating to neuronal apoptosis inhibitor protein expression (Magun 1998a; Magun 1998b; Papineau 2003). These studies have clearly shown a difference in the signaling mechanisms employed by 3T3-L1 versus human adipocytes.

Effect of PI3K inhibition on the regulation of IKKβ/NF-κB activation by TSH in human differentiated adipocytes

Another pathway found downstream of GPCR activation that has been implicated upstream of the NF-κB pathway is the PI3K pathway. One group found that HeLa cells transfected with the B2-type bradykinin GPCR have been shown to require PI3K and its downstream target protein kinase B (also known as Akt) for the activation of the NF-κB pathway in response to bradykinin stimulation (Xie 2000). Another study implicating PI3K downstream of GPCR activation was published recently studying the melanocortin 5 receptor in HEK 293 cells transfected with the receptor (Rodrigues 2009). Since PKA was not required for the TSH-induced NF-κB activation or IL-6 regulation, I decided to see if PI3K might be important for IKKβ/NF-κB activation in response to TSH.

My data do not indicate a role for PI3K in TSH-dependent activation of classical NF-κB signaling. Inhibition of PI3K using either LY294002 or the more potent covalent inhibitor wortmannin, did not alter the TSH-induced IKKβ phosphorylation levels nor did it alter the TSH-induced IκBα degradation. However, PI3K-dependent IκBα degradation and NF-κB activation have been shown to occur independent of IKKβ activity (Tergaonkar 2003). In another study, NF-κB was shown to be directly phosphorylated, not requiring IKKβ activity or IκBα degradation (Bohuslav 2004). My studies did not examine whether PI3K might play a role in the direct activation of NF-κB or IL-6 mRNA expression or protein release.

Effect of NADPH oxidase inhibition on the regulation of IKKβ/NF-κB activation by TSH in human differentiated adipocytes

Angiotensin II and thrombin both signal through their respective GPCR to increase ROS levels in vascular smooth muscle cells (Griendling 1994; Patterson 1999). In addition, ROS production has been suggested as a mediator of NF-κB activation by GPCRs, including the angiotensin II receptor (Hirotani 2002). I therefore examined whether the production of ROS is necessary for IKKβ/NF-κB activation in response to TSH. I used the NADPH oxidase inhibitor DPI to treat human abdominal subcutaneous differentiated adipocytes prior to TSH stimulation. Although DPI is used by many investigators to study NADPH oxidase, it should be noted that, in addition to NADPH oxidase, DPI can also inhibit other flavin-containing oxidases.

The expected TSH-induced phosphorylation of IKKβ and IκBα degradation were noted. Both of these processes were significantly inhibited with DPI pretreatment indicating

that NADPH oxidase activity and therefore ROS production is important for TSH-induced IKKβ/NF-κB activation. This is in accordance with another study that demonstrated that TSH induces oxidative stress in hTSHR-CHO cells and FRTL5 cells (rat thyroid cell line) (Pomerance 2000). However, it must be noted that it has yet to be shown that TSH actually activates NADPH oxidase and increases ROS production in my model system. These studies will need to be done in order to validate the interpretation of the DPI experiments.

Since DPI inhibits TSH-induced IKKβ phosphorylation and IκBα degradation, and since IKKβ activity is required for TSH-dependent IL-6 regulation, I expect that DPI will inhibit IL-6 mRNA expression and protein release. However, I did not check this during my studies due to time limitations, but these experiments will be performed in the future by other members of the laboratory. It should be noted that TSH-induced CREB phosphorylation was also decreased with DPI pre-treatment. This might suggest that CREB could be another route by which DPI would inhibit IL-6 expression. However, since I have shown that TSH-induced CREB phosphorylation is inhibited by the PKA inhibitor H89, and since this did not affect IL-6 mRNA or protein levels, it is unlikely that IL-6 regulation by TSH is dependent on CREB phosphorylation.

Effect of PKCδ inhibition on the regulation of IKKβ/NF-κB activation and IL-6 regulation by TSH in human differentiated adipocytes

Because I had shown that DPI, an NADPH oxidase inhibitor, reduced TSH-induced IKKβ/NF-κB activation, I investigated potential upstream regulatory factors. In 2000, Volk *et al.* demonstrated that the secretion of IL-6 from endothelial cells in response to TNF-α and IL-1 was dependent on ROS production (Volk 2000). Furthermore, PKCδ has been

shown to have a direct interaction with $p47^{phox}$ (Reeves 1999) and has been shown to directly phosphorylate $p47^{phox}$, induce its translocation to the cell membrane and subsequently activate NADPH oxidase. p47phox is a subunit in the NADPH oxidase complex responsible for ROS production (Fontayne 2002). In addition, lysophosphatidic acid (LPA) is a bioactive lipid that elicits many of its biological functions through its GPCR and LPA-induced NF-κB activation has been shown to be mediated by PKCδ in human bronchial epithelial cells (Cummings 2004). I therefore determined if PKCδ might be positioned upstream of ROS production in my experimental model.

My results suggest a role for PKCδ in both the activation of IKKβ/NF-κB and in the release of IL-6 in response to TSH stimulation. Pretreatment with the PKCδ inhibitor rottlerin decreased TSH-induced IKKβ phosphorylation as well as IκBα degradation. In addition, when examining IL-6 protein release, rottlerin pretreatment blunted the TSH-dependent increase in IL-6 release. I also noted that TSH-induced CREB phosphorylation was inhibited with rottlerin pretreatment but as mentioned above, when we inhibited CREB phosphorylation with H89, it did not affect TSH-induced IL-6 mRNA or protein secretion. Therefore I presume that IL-6 regulation by TSH will prove to be reliant on a PKCδ-dependent effect on IKKβ phosphorylation rather than its effect on CREB phosphorylation.

Recent data have implicated caspase recruiting domain 10 (CARD10), B cell chronic lymphocytic leukemia 10 (Bcl10) and mucosa-associated lymphoid tissue (MALT) lymphoma translocation gene 1 (Malt1) adapter proteins downstream of PKCδ in GPCR-stimulated cells activating the NF-κB pathway (Klemm 2007; McAllister-Lucas 2007; Wang 2007; Wegener 2007). The Bcl10 gene was discovered by its translocation in a case of MALT lymphoma. The Malt gene was also found to be rearranged in chromosomal

translocation in cases of MALT lymphomas. CARD10 (also known as CARMA 3) has been found to be involved in apoptosis signaling and to often be associated with Bcl10 and Malt1. Blocking Bcl10 (Klemm 2007) as well as Malt1 deficiency (Wang 2007) in mouse embryonic fibroblasts were found to inhibit NF-κB activation via LPA-dependent activation of its GPCR. Furthermore, CARD10 was found to link GPCR activation to the Bcl-Malt-NF-κB pathway; deficiency in CARD10 blocked angiotensin II-mediated activation of NF-κB via its GPCR (McAllister-Lucas 2007). Therefore, the CARD10-Bcl10-Malt1-NF-κB pathway could account for one of the ways in which PKCδ could affect IKKβ/NF-κB activation and subsequently IL-6 regulation in human differentiated adipocytes. Further studies are needed however to determine whether the components of this complex are expressed in human differentiated adipocytes.

On the other hand, as mentioned above, since NADPH oxidase activity was shown to be important for TSH-dependent IKKβ/NF-κB activation and IL-6 secretion in my cell model, another pathway needs to be examined. PKCδ has been shown to activate $p47^{phox}$, a subunit in the NADPH oxidase complex responsible for ROS production (Reeves 1999; Fontayne 2002). ROS in turn has been found upstream of NF-κB activation in many cell types. It is thought that ROS mediates NF-κB activation by inhibiting phosphatases, which in turn leads to the activation of downstream kinases, such as IKKβ (Gloire 2006). Increased ROS production in response to PKCδ activation may therefore be important in the activation of the IKKβ/NF-κB pathway in human differentiated adipocytes. Again, further studies will need to be performed to establish whether this pathway is important in this cell model.

The inhibition with either DPI or rottlerin was not complete with regards to IKKβ phosphorylation, IκBα degradation or IL-6 regulation and this again could be due to

incomplete inhibition using pharmacological inhibitors, sub-optimal concentration and pretreatment times for the inhibitors, or the implication of more than one pathway in these responses. It would therefore be interesting to use both inhibitors (DPI and rottlerin) simultaneously and determine whether that would lead to complete inhibition of TSH-induced IKKβ/NF-κB activation and IL-6 regulation.

I wanted to determine firstly whether PKCδ is expressed in my cell model and if so, whether its activity is increased in response to TSH treatment. I determined that PKCδ is in fact expressed in my cell model and preliminary data revealed an increase in PKCδ activity upon TSH stimulation with a 15 minute kinase assay. Results indicated no difference between the control sample and the TSH-stimulated sample after a 30 minute kinase assay. However, this may be due to an inadequate amount of substrate being present in the assay. More experiments will need to be performed in order to confirm these results and to determine optimal rottlerin concentration and pretreatment times, as well as TSH stimulation times, for our studies.

REGULATION OF ADIPOKINES BY TSH IN HUMAN DIFFERENTIATED ADIPOCYTES:

Adipokines are released from adipose tissue in response to various stimuli. I examined whether or not TSH is capable of regulating other adipokines in human abdominal subcutaneous differentiated adipocytes.

TSH-induced MCP-1 mRNA expression in human differentiated adipocytes

Upon TSH stimulation, I noted a significant increase in MCP-1 mRNA expression which was paralleled by an increase in MCP-1 protein release. As its name suggests, MCP-1 serves a major role in the recruitment of macrophages to sites of injury and infection and perpetuates inflammation. MCP-1 levels are elevated in states of obesity, one possible explanation being that obese individuals usually have a characteristic hyperinsulinemic phenotype and insulin is a regulator of MCP-1 (Sartipy 2003). MCP-1 is recognized as a pro-inflammatory cytokine and has been shown to have an effect on adipocyte function. MCP-1 stimulation of adipocytes decreased insulin-stimulated glucose uptake (Sartipy 2003) and eventually induced insulin resistance (Sell 2007). High levels of TSH may act on adipocytes to increase MCP-1 levels *in vivo*, and this may be a mechanism by which patients with subclinical hypothyroidism experience increased risk for CVD.

MCP-1 released by adipocytes can act locally and lead to the recruitment of macrophages into the adipose tissue itself. Adipose tissue-infiltrating macrophages in turn lead to an increased inflammatory state, as they display a pro-inflammatory phenotype. A greater state of inflammation then leads to more deregulation of adipokines which can cause cardiovascular complications (Bastard, J.P.; Heilbronn 2008; Bourlier 2009). For instance, MCP-1 can upregulate IL-6 production which can promote atherosclerosis by acting on the endothelium of vessel walls and having a positive role in leukocyte recruitment.

TSH-induced elevation of MCP-1 could also directly promote atherosclerosis. Development of atherosclerosis requires the recruitment of monocytes and T lymphocytes into the innermost layer of blood vessels. MCP-1 is one such cytokine that has been shown to recruit monocytes, memory T cells and dendritic cells to sites of early atherosclerotic

lesions (Carr 1994; Xu 1996; Linton 2003; Charo 2004). In addition, low-density lipoprotein receptor-deficient mice, a model of atherosclerosis, lacking MCP-1 had fewer macrophages in their aortic wall and were less prone to atherosclerosis (Gu 1998). Also, when the MCP-1 receptor was knocked out in apoE-deficient mice, another well studied model of atherosclerosis, aortic lesion areas decreased in size and were less advanced, indicating a role of MCP-1 signaling in the progression of atherosclerosis (Dawson 1999). MCP-1 also plays a role in plaque instability and blocking MCP-1 in apoE-deficient mice resulted in decreased progression of preexisting aortic lesions and alteration of the composition of these lesions; namely, they contained less macrophages and lympocytes compared to those mice with MCP-1 (Inoue 2002). Finally, in a study of patients with acute coronary syndromes, elevated MCP-1 levels were associated with traditional atherosclerotic risk factors as well as increased risk for myocardial infarction (de Lemos 2003).

My data demonstrating that TSH induces MCP-1 mRNA expression and protein release from human adipocytes suggests a possible mechanism underlying the increased risk for CVD seen in patients with subclinical hypothyroidism.

TSH-induced VEGF mRNA expression in human differentiated adipocytes

TSH induced a significant increase in VEGF mRNA expression in human differentiated adipocytes. While VEGF is mainly produced by endothelial cells, it is also produced by adipocytes (Mick 2002; Rega 2007; Gealekman 2008; Trayhurn 2008). VEGF is thought to be the most important positive regulator of vascular permeability and angiogenesis. Similar to MCP-1, VEGF levels increase with increasing adiposity and these levels have been shown to play a role in the development of hypertension and atherogenesis.

It is intuitive that VEGF levels would increase in response to increasing adiposity, as it enables the expansion of the vascular bed into newly formed adipose tissue (Berg 2005). VEGF mRNA expression and protein release are responsive to insulin in adipocytes, much like MCP-1 (Mick 2002). Therefore in states of hyperinsulinemia, increasing VEGF levels might promote the development of vascular complications seen in states of inflammation. For example, VEGF levels correlate with increased aortic subendothelial macrophage accumulation and intimal thickening (Mick 2002), important steps in the development of CVD. Elevated levels of TSH characteristic of subclinical hypothyroidism may therefore induce VEGF mRNA expression and protein release from human adipose tissue and thus add to the risk for CVD observed in these patients.

VEGF however has some beneficial effects in addition to its detrimental effects mentioned above. A recent review discusses the detrimental effect of VEGF in the involvement in the neoangiogenetic process in atherosclerotic plaque, an early step in the development of CVD. On the other hand, a beneficial outcome of VEGF signaling is the formation of collateral blood vessels which could potentially bypass obstructed arteries, an idea underlying current therapeutic approaches using VEGF (Testa 2008). It is therefore difficult to draw any conclusions regarding the implications of increased VEGF mRNA levels in my cell model until further studies are performed.

TSH-induced RBP4 mRNA expression in human differentiated adipocytes

RBP4 is a protein secreted by the liver and adipose tissue that has been implicated in insulin resistance. In human studies, RBP4 levels correlate with cases of obesity and insulin resistance. An intervention of exercise training and lifestyle changes that led to improved

insulin sensitivity also decreased RBP4 levels (Graham 2006; Balagopal 2007; Haider 2007; Lim 2008). Elevated RBP4 levels are positively correlated with risks for CVD (Graham 2006). One study demonstrated that RBP4 levels were elevated in patients with subclinical hypothyroidism compared to euthyroid patients (Choi 2008) and that RBP4 levels correlated with TSH levels in these patients.

On this basis, I had thought that TSH would increase RBP4 expression. In contrast, my data show no effect of TSH on RBP4 mRNA expression in human differentiated adipocytes. However, a time course was not performed to thoroughly investigate the possibility of an RBP4 response (if any) to TSH, nor was a dose-dependent study performed with regards to RBP4. Therefore, perhaps a higher concentration and/or longer stimulation time is required for TSH to affect RBP4 mRNA levels. Another possibility is that TSH may not affect mRNA expression of RBP4 in human differentiated adipocytes, but may in fact affect the secretion of the protein itself. These are possibilities that will need to be explored in the future before drawing any conclusions with respect to TSH-dependent regulation of RBP4 in my cell model.

TSH-induced adiponectin mRNA expression in human differentiated adipocytes

Adiponectin is secreted solely by adipose tissue and its circulating concentration is inversely proportional to adiposity (Scherer 1995). In states of chronic inflammation, such as obesity, adiponectin levels are low. Adiponectin is an anti-inflammatory, anti-atherosclerotic adipokine that plays a role in insulin sensitivity and metabolism. Its low serum concentration is an indicator for the future onset of cardiovascular complications (Goldstein 2004). Therefore, based on the hypothesis that TSH is inducing an inflammatory

response from human differentiated adipocytes, I hypothesized that adiponectin levels would decrease in response to TSH treatment. However, my data show that TSH had no effect on adiponectin mRNA levels in my cell model. Again, no time course or dose-dependent study was performed to thoroughly search for a TSH-dependent adiponectin response (if any). Therefore, perhaps a higher concentration and/or longer stimulation time is also required for TSH to affect adiponectin mRNA levels. Similar to RBP4 regulation, another possibility is that TSH does not affect mRNA expression of adiponectin, but may in fact affect the secretion of the protein. These possibilities need to be explored before drawing any conclusions in regards to TSH-dependent regulation of adiponectin in my cell model.

TSH-INDUCED MCP-1 REGULATION IN HUMAN DIFFERENTIATED ADIPOCYTES:

Because MCP-1 showed the greatest response to TSH, I began to elucidate the mechanism by which TSH regulates MCP-1 expression.

Effect of IKKβ inhibition on the regulation of MCP-1 production by TSH in human differentiated adipocytes

MCP-1 has been suggested to be regulated by the NF-κB pathway in various cell types. One study demonstrated an importance for NF-κB in LPS-induced MCP-1 mRNA expression in rat tubular epithelial cells (Wang 2000). Another study revealed that activation of the NF-κB pathway correlated with an increase in MCP-1 mRNA expression in mononuclear cells (Donadelli 2000). Also, activation of NF-κB in human mesangial cells by

IL-1 led to an increase in MCP-1 mRNA expression, an effect that was reversed with NF-κB inhibition (Rovin 1995). With regards to adipose tissue, angiotensin II-dependent MCP-1 gene expression is dependent on NF-κB in rat preadipocytes (Tsuchiya 2006). Furthermore, NF-κB is important for the TNFα and IL-1β-dependent MCP-1 protein release in human visceral adipose tissue (Fain 2005). Finally, MCP-1 upregulation in response to obesity in a mouse model was found to be dependent on IKKβ and NF-κB in isolated adipocytes (Jiao 2009).

In my studies, I noted an increase in both MCP-1 mRNA expression and protein release in response to TSH. This increase was significantly blunted with sc-514 pretreatment, indicating an importance for IKKβ activity and likely NF-κB in TSH-dependent MCP-1 regulation. These results were similar to those I noted for TSH-dependent regulation of IL-6 mRNA expression and the data obtained by the laboratory on TSH-dependent regulation of IL-6 protein release (Antunes 2008). Although the IL-6 response to TSH was greater than that for MCP-1, TSH-dependent regulation of both MCP-1 and IL-6 were IKKβ-dependent.

Effect of PKA inhibition on the regulation of MCP-1 production by TSH in human differentiated adipocytes

There are some discrepancies in the literature regarding MCP-1 regulation by PKA. One study found that the activation of PKA was needed for maximal MCP-1 secretion in human peripheral monocytes (Fietta 2002). More recently, prostaglandin E2 was found to mediate the TNF-α-dependent increase in MCP-1 synthesis and secretion from pancreatic acinar cells and this synergistic effect was dependent on PKA activity (Sun 2007).

However, another study using human mesangial cells demonstrated that inhibition of PKA had no effect on IL-1-induced MCP-1 mRNA expression. Furthermore, both increasing cAMP levels, an upstream event in the activation of PKA, and activating PKA itself, actually led to a decrease in basal MCP-1 mRNA expression as well as IL-1-induced MCP-1 mRNA expression (Rovin 1994). While, to my knowledge, there is no literature directly implicating CREB in the regulation of MCP-1 in adipocytes, there is a study examining the role of its upstream regulator PKA. In 2006, Kralisch *et al.* demonstrated that isoproterenol-induced MCP-1 expression is mediated via PKA in 3T3-L1 adipocytes (Kralisch 2006b).

In my studies, upon TSH stimulation of human adipocytes, I noted an increase in MCP-1 mRNA expression and protein release, responses that were significantly blunted with the PKA inhibitor H89. This indicates a role for PKA in TSH-stimulated MCP-1 regulation. However, I have shown that PKA is not important for IKKβ phosphorylation or IκBα degradation. Perhaps TSH-induced PKA-dependent activation of CREB is important for MCP-1 transcription and subsequently its protein release independent of the NF-κB pathway. For instance, it was shown that CREB binding protein (CBP) increased TNF induction of MCP-1 in NIH3T3 cells, an induction that was inhibited with CBP inhibition. Furthermore, NF-κB was also found to be important in TNF-dependent induction of MCP-1 in this cell model (Boekhoudt 2003). This could also be the case in human differentiated adipocytes in response to TSH treatment.

Neither inhibition of IKKβ nor PKA lead to a complete reversal of TSH-induced MCP-1 mRNA expression or protein secretion to basal levels. As mentioned above, this could be due to sub-optimal inhibitor concentrations and/or incubation times, MCP-1 may be regulated by more than one pathway, or it could be a combination of the two. Again,

inhibitor studies using both sc-514 and H89 would be advantageous to perform in our cell model in the future.

Other MCP-1 regulation

Another regulator of MCP-1 expression that has specifically been found in 3T3-L1 adipocytes is the transcription factor activating protein 2β (AP-2β). AP-2β is preferentially expressed in adipose tissue and its overexpression has been linked to increased lipid accumulation and insulin resistance in 3T3-L1 adipocytes. This particular study demonstrated that overexpression of AP-2β resulted in increased activity of the MCP-1 promotor and consequently led to an increase in MCP-1 mRNA expression and protein secretion. Correspondingly, knockdown of this transcription factor resulted in a decrease in MCP-1 expression both at the mRNA and protein level (Kondo 2009). AP-2β is therefore a promising target to be explored by our lab in the future with regards to TSH-dependent MCP-1 regulation.

Another point to consider is the fact that IL-6 can stimulate MCP-1 secretion from adipose tissue (Fasshauer 2004). However, our TSH stimulation time of 4 hours for protein secretion (a time point previously determined by our lab) seems to be too short for it to be an IL-6-dependent increase in MCP-1 expression. Although IL-6 induced MCP-1 mRNA expression has been shown to be upregulated as early as 1 hour following stimulation in 3T3-L1 adipocytes (Fasshauer 2004), most studies look at MCP-1 protein secretion at least 4 hours after stimulation (Skurk 2007; Strande 2009). Furthermore, in one study, LPS induced IL-6 mRNA expression around the 3 hour mark in human adipose tissue but IL-6 secretion was examined after 24 hours after stimulation (Hoch 2008). Therefore, it seems as though it

would take much longer than our 4 hour time point for IL-6 to be transcribed, translated, secreted and then act through its receptor to increase MCP-1 transcription, followed by its translation and subsequently its release into the media. Ideally, one way to verify if it is in fact TSH inducing MCP-1 secretion rather than TSH inducing IL-6 secretion which is in turn inducing MCP-1 secretion would be to develop an adipocyte-specific IL-6 receptor knockout and then stimulate the adipocytes with TSH to see if the same increase in MCP-1 mRNA and protein release are noted. It is however difficult to do in human primary differentiated adipocytes as mentioned above. An alternative would be to use a neutralizing antibody against IL-6 (Uno 2008) and then determine the effect of TSH on MCP-1 regulation in human differentiated adipocytes. This would eliminate the possibility of IL-6 signaling being responsible for the MCP-1 response observed.

LIMITATIONS:

It was surprising to observe the differences in TSH-induced expression of adipokines whether at the mRNA or protein level between the different experiments performed. For instance, TSH increased IL-6 mRNA expression by almost 71-fold in the H89 experiment whereas it was induced by almost 130-fold in the sc-514 experiment. These differences seen in response to TSH treatment can be explained by patient variability, one of the difficulties faced when working with human adipocytes in primary culture. One reason for the differences in TSH response could be due to the BMI of the patients used. In one experiment, a patient had a very high BMI value of 37, which suggests a state of low-grade inflammation in the adipose tissue and in fact, her basal IL-6 mRNA levels were very high,

leading to only a 12-fold increase in IL-6 mRNA expression in response to TSH. This response was low compared to the average 100-fold increase observed in response to TSH. Furthermore, the differences in the expression of TSHR between patients might also explain the discrepancies. I examined TSHR mRNA expression levels of the control samples of four patients. There was indeed a large variation in TSHR mRNA expression between patient samples. For example, one patient had a 10-fold higher TSHR mRNA expression than another. However, it was difficult to detect TSHR mRNA reliably in all patients, so this was not used to normalize adipokine responses.

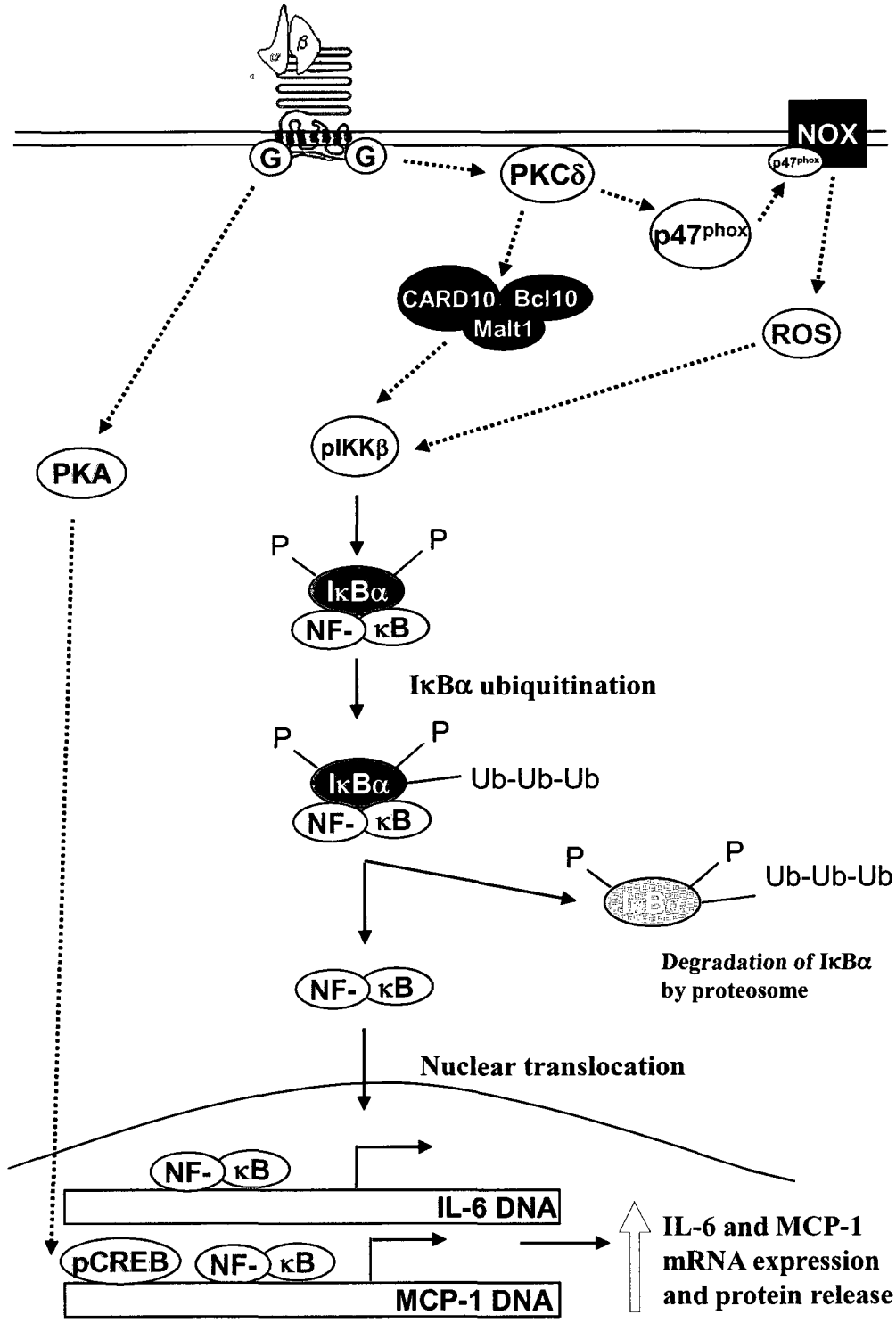
Another limitation of my project is that the dose of TSH used is significantly higher (1000 x) than physiological TSH concentrations. However, the dose used is comparable to that used in studies stimulating thyrocytes in culture with TSH (Shirokawa 2000). The reason such high doses are required even for the main cellular target of TSH is unknown but it has been suggested that it may be because TSH requires other growth factors or proteins for its optimal response.

MODEL OF TSH-INDUCED IL-6 AND MCP-1 REGULATION IN HUMAN DIFFERENTIATED ADIPOCYTES:

To summarize, I have shown that TSH induces the expression of the pro-inflammatory adipokines IL-6, MCP-1 and VEGF in human abdominal subcutaneous differentiated adipocytes. I have also begun to elucidate the signaling mechanisms by which TSH activates the IKKβ/NF-κB pathway and in turn regulates IL-6 and MCP-1 mRNA expression and protein release. Specifically, I have shown that NADPH oxidase (specific

isoform not known) and PKCδ both are upstream of TSH-induced IKKβ/NF-κB activation and that IKKβ activity is important for TSH-induced IL-6 and MCP-1 regulation. I have also shown that PKA activity is important for TSH-induced MCP-1 regulation but not for IL-6 regulation (Figure 20).

Figure 20. Model of the signaling mechanisms through which TSH stimulates adipokine production.


NOX
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Malt1
ROS
PKA
pIKKβ
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NF- κB
IκBα ubiquitination
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Ub-Ub-Ub
Degradation of IκBα
by proteosome
NF- κB
Nuclear translocation
NF- κB
IL-6 DNA
pCREB
NF- κB
MCP-1 DNA
IL-6 and MCP-1
mRNA expression
and protein release

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EDUCATION

2007-ongoing	University of Ottawa -Master of Science in Biochemistry
2003-2007	University of Ottawa -Honours Bachelor of Science in Biochemistry
1999-2003	Lo-Ellen Park Secondary School -Enriched Program/University stream -Honours Student

SCHOLASTIC AWARDS

2007-ongoing	OGSST Recipient
2007-ongoing	University of Ottawa Graduate Admissions Scholarship
2003-2007	University of Ottawa Dean's List
2003-2007	University of Ottawa Admissions Scholarship
2003-ongoing	Member of Golden Key International Honour Society
2003	Received Bilingual Certificate
2003	Lo-Ellen Park Secondary school proficiency awards: Physical Education, French, Chemistry, Anatomy, Mathematics, Biology

PUBLICATIONS AND PRESENTATIONS

2009	Langille, ML, Gagnon A, Antunes TT, Sorisky A. TSH Stimulates MCP-1 Production in Abdominal Differentiated Human Adipocytes. Manuscript Submitted.
2009	Melanie L Langille, Tayze T Antunes, AnneMarie Gagnon and Alexander Sorisky. Accepted June 2009. Pro-inflammatory actions of TSH on human abdominal differentiated adipocytes. 91st Annual Meeting – The Endocrine Society, Washington, DC. –Abstract and Poster
2008	Langille Melanie, Gagnon AnneMarie and Sorisky Alexander. TSH-dependent activation of the NF-κB pathway in human differentiated adipocytes is

PKCδ dependent. Ottawa Health Research Institute Research Day. Ottawa, Ontario. –Abstract and Poster

2008 Tayze T Antunes, Melanie L Langille, AnneMarie Gagnon and Alexander Sorisky. Accepted June 2008. Thyroid Stimulating Hormone Activates the NF-κB Pathway to Induce Interleukin-6 Release From Human Adipocytes. 90th Annual Meeting – The Endocrine Society, San Francisco, California. –Abstract and Poster

2008 Antunes TT, Gagnon A, Langille ML, Sorisky A. Thyroid-stimulating hormone induces interleukin-6 release from human adipocytes through activation of the nuclear factor-kappaB pathway. *Endocrinology.* June 2008; 149(6): 3062-6.

2008 Langille Melanie, Gagnon AnneMarie and Sorisky Alexander. Thyroid Stimulating Hormone Stimulates Inflammatory Pathways in Human Adipocytes University of Ottawa BMI Poster Day. Ottawa, Ontario. –Abstract and Poster

2007 Langille Melanie, Gagnon AnneMarie and Sorisky Alexander. TSH-induced IKKβ phosphorylation in human differentiated adipocytes is ROS-dependent. Ottawa Health Research Institute Research Day. Ottawa, Ontario. – Abstract and Poster

EMPLOYMENT

2008-2009 University of Ottawa
-Laboratory Teaching Assistant
-Organic Chemistry 2 Laboratory

2007 University of Ottawa
-Research Analyst
-Signaling mechanisms of TSH on adipocytes

2006-2007 University of Ottawa (Human Kinetics Dept.–Dr. Imbeault)
-Research Analyst
-Metabolic effects of adiponectin

2006 Laurentian University (Chemistry Dept.–Dr. Siemann)
-Research Analyst
-Kinetics of *Bacillus anthracis*

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2005	Northern Centre for Biotechnology and Clinical Research -Biotechnology Research Assistant -Quality Control and Production Management
2002-2004	Canadian Tire Corporation -Customer Service representative/cashier -Greenhouse horticultural assistant

VOLUNTEER WORK

2008	Science Travels Student Ambassador -Promote science to youth in communities that do not have access to the same level of science programming
2007-ongoing	The Ottawa Hospital Continuing Care Unit Volunteer -Provide the basic needs for chronic care patients
2007-ongoing	Let's Talk Science Volunteer and Volunteer Advisor -Promote the field of science among the youth of our community -Perform science demonstrations in the classroom
2006-2007	University of Ottawa BioX Club Member -Liaison between the club and students regarding science career opportunities
2005-ongoing	Sudbury Samaritan House -Prepare and serve meals for the homeless

INTERESTS AND ACHIEVEMENTS

2008-2009	Ottawa Competitive Volleyball League - Tier 1 Team Member
2005	ISO13485:2003 and ISO9001:2000 certified
2003	OFSAA 'AA' volleyball champion/team captain
2003	Lo-Ellen Park S.S. Principal's Award for leadership
2003	Lo-Ellen Park S.S. Athletic Association president
2001/2003	Lo-Ellen Park S.S. Junior and Senior Athlete of the Year
2001/2002	OFSAA gymnastics award winner

2001	Lo-Ellen Park S.S. representative at the Ontario Educational Leadership Centre – for leadership training