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Signaling Pathways Employed by Thyroid Stimulating Hormone to Induce Interleukin-6 Release From Human and 3T3-L1 Adipocytes

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SIGNALING PATHWAYS EMPLOYED BY THYROID STIMULATING HORMONE TO INDUCE INTERLEUKIN-6 RELEASE FROM HUMAN AND 3T3-L1 ADIPOCYTES

by

Tayze Tatiana Antunes

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry

Department of Biochemistry, Microbiology and Immunology

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ABSTRACT

Subclinical hypothyroidism, a condition characterized by elevated thyroid stimulating hormone (TSH) levels, is a risk factor for cardiovascular disease (CVD). Adipose tissue contributes significantly to the circulating levels of interleukin-6 (IL-6), an atherogenic and pro-inflammatory cytokine. Adipose cells express TSH receptors and are responsive to TSH. The overall hypothesis of my Ph.D. project is that TSH acts on adipose cells to stimulate the release of IL-6.

In this thesis, I studied TSH-induced IL-6 release and the related signaling mechanisms using the mouse 3T3-L1 adipose cell line. I also evaluated human primary adipose cells, and examined the influence of stage of differentiation and fat depot localization on the effect of TSH on IL-6 release.

I first showed that in 3T3-L1 cells, TSH induces IL-6 release from differentiated adipocytes, but not from precursor cells (preadipocytes). Differentiated 3T3-L1 adipocytes increased IL-6 release in response to TSH via activation of the cAMP-dependent protein kinase (PKA) pathway. Following analysis of the TSH effect on 3T3-L1 adipose cell line, I proceeded to examine the effect of TSH on human primary adipose cells.

For the abdominal subcutaneous depot, I found that preadipocytes did not release IL-6 in response to TSH, but their differentiated adipocytes counterparts were TSH-responsive. In contrast, for the omental depot, I showed that neither preadipocytes nor the differentiated adipocytes increased IL-6 release in response to TSH.

Signaling studies on the human abdominal subcutaneous differentiated adipocytes revealed that TSH activates the nuclear factor kappa-B pathway to induce IL-6 release. Furthermore, PKA activation by TSH does not result in increased NF-κB signaling.

To demonstrate that TSH could act in an extra-thyroidal fashion *in vivo,* I analyzed serum IL-6 levels in thyroidectomized patients with a past history of thyroid cancer undergoing recombinant human (rh)TSH administration. Injection with rhTSH increased IL-6 serum levels, supporting the notion that TSH has extra-thyroidal actions *in vivo*.

In summary, this thesis demonstrates that TSH is capable of regulating inflammatory responses on adipose cells and of elevating IL-6, a biomarker of inflammation, in the circulation of thyroidectomized patients.

DEDICATION

I would like to dedicate this thesis to my husband, Rodrigo Trevizan Peretti. Thanks for being so supportive and understanding in every single step of my study. Thanks for listening to my presentations even though they did not make sense to you. Thanks for reading my research proposals and still find a way to give me comments. Thanks for going with me to the laboratory on weekends and being happy to read a book while I worked. Certainly, this thesis could not have been done without you... Above all, thank you for being a wonderful person that I am happy to share my life and dreams with, I love you!

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My family's support was definitely fundamental for the success of my study. Despite the distance, they were always there to cheer for me, to listen to my worries when experiments were not going well and when courses or presentations were stressful. Thank you for saying comforting words when they were needed. I would like to thank my dad, Eloi, for being such a strong person who has shown us a lot can be accomplished in life, if you try. I thank my sisters Fernanda and Lais for being so nice, funny, smart and fashionable. I would like to especially thank my mom, Diane, who has been extremely supportive. She would make endless phone calls just to make sure I was really fine. Thank you mom for always telling me that life should be fun, even when things were hard.

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LIST OF ABBREVIATIONS

AC: adenylyl cyclase

AP: activator protein

AT1: angiotensin II type 1

ATP: adenosine-5'-triphosphate

Bcl10: B cell chronic lymphocytic lymphoma 10

BMI: body mass index

C: catalytic

cAMP: cyclic adenosine monophosphate

C/EBP: CCAAT enhancer binding protein

CG: chorionic gonadotropin

CHO: Chinese hamster ovarian

CI: confidential interval

CREB: cAMP response element-binding

CRP: C-reactive protein

CS: calf serum

CVD: cardiovascular disease

DAG: diacylglycerol

DMEM: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

ELISA: Enzyme-Linked Immunosorbent Assay

Epac: exchange protein directly activated by cAMP

FBS: fetal bovine serum

FFA: free fatty acids

FSH: follicle stimulating hormone

GDP: guanosine diphosphate

GPCR: G protein-coupled receptor

GTP: guanosine triphosphate

HSL: hormone-sensitive lipase

^{131}I: radioiodine

IκB: inhibitor of κB

IKK: IκB kinase

IOD: integrated optical density

IL-1β: interleukin-1β

IL-6: interleukin-6

IL-6 R: IL-6 receptor

IP_3: inositol trisphosphate

KRH: Krebs Ringer Hepes

LH: luteinizing hormone

LPA: lysophosphatidic acid

LPS: lipopolysaccharide

L-T4: levothyroxine

MALT1: mucosa-associated lymphoid tissue lymphoma translocation gene 1

MAPK: mitogen-activated protein kinase

MCE: mitotic clonal expansion

MCP-1: monocyte chemoattractant protein-1

MEFs: mouse embryonic fibroblasts

NF-κB: nuclear factor-kappa B

OR: odds ratio

PAI: plasminogen activator inhibitor

PCR: Polymerase chain reaction

PI3K: phosphatidylinositol 3-kinase

PIP_2: phosphatidylinositol-4,5-bisphosphate

PKA: cAMP-dependent protein kinase

PKC: protein kinase C

PLC: phospholipase C

PPAR: peroxisome proliferator-activated receptor

Pref-1: protein preadipocyte factor 1

R: regulatory

RBP-4: retinol binding protein-4

Rh: recombinant human

RQ: relative quantification

S6K1: ribosomal S6 kinase 1

siRNA: small interfering RNA

T3: triiodothyronine

T4: thyroxine

TG: triacylglycerol

TNF-α: tumor necrosis factor

TSH: thyroid stimulating hormone

TSHR: thyroid stimulating hormone receptor

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1.0 GENERAL INTRODUCTION

SUBCLINICAL HYPOTHYROIDISM

Subclinical hypothyroidism is a disorder characterized by a thyroid stimulating hormone (TSH) serum level above the reference interval (0.45 to 4.5 mU/L) when thyroid hormone serum levels of thyroxine (T4) and triiodothyronine (T3) are normal. TSH production from thyrotrophs within the anterior pituitary increases to counteract the early stage of mild thyroid gland failure (Biondi and Cooper, 2008). This leads to a compensated state of normal thyroid hormone levels at the expense of an elevated TSH level. Patients are often asymptomatic and consequently diagnosed solely through laboratory tests, thus the term subclinical. Clinical symptoms will develop if patients progress to overt hypothyroidism, when thyroid hormone production falls below normal despite the high TSH levels.

Subclinical hypothyroidism is caused chiefly by chronic autoimmune thyroiditis, also known as Hashimoto's thyroiditis. In this condition, factors such as viral or bacterial infection, stress or genetic predisposition lead to lymphocyte infiltration of the thyroid gland. This infiltration induces the release of cytokines that cause the thyroid cells to express major histocompatibility complex molecules and therefore to initiate a self-immune attack to the thyroid gland. Thyroid proteins that become immunogenic are thyroglobulin and thyroid peroxidase (Arrigo et al., 2008; Wang and Baker, 2007).

The prevalence of subclinical hypothyroidism in the general population is in the range of 4-10%; it increases with age, with a higher prevalence (20%) in women who are over 60 years of age (Arrigo et al., 2008; Cooper, 2004). Despite being a common disorder,

mass population screening is not formally recommended. Rather, it is encouraged to perform serum TSH and free T4 testing only in high-risk patients, such as women older than 60 years, women planning a pregnancy or who are pregnant, and anyone with the following: previous thyroid dysfunction, symptoms suggestive of thyroid gland malfunction, associated autoimmune disease, a family history of thyroid disease (Arrigo et al., 2008; Cooper, 2004).

Treating or not treating subclinical hypothyroid patients with levothyroxine (L-T4) is a subject of intense debate. Some subclinical hypothyroid patients spontaneously improve their thyroid gland function over time with TSH levels re-entering the normal reference interval (Diez et al., 2005), while others will have stable subclinical hypothyroidism and never progress into hypothyroidism. On the other hand, a considerable proportion (5% per year, see below) of patients eventually progress to thyroid gland failure and become hypothyroid (Arrigo et al., 2008; Biondi and Cooper, 2008). Hence, it has been suggested by some authors, that asymptomatic patients with TSH levels between 4.5 to 10 mU/L should not be treated but instead undergo repeat TSH testing every 6 months, whereas those with symptoms compatible with hypothyroidism should be treated to ameliorate the symptoms. Many authorities agree that patients presenting with TSH levels under 10 mU/L but positive for antithyroid antibodies and that patients with TSH levels above 10 mU/L should receive L-T4 therapy, since up to 5% per year will become hypothyroid (Arrigo et al., 2008; Biondi and Cooper, 2008; Surks et al., 2004).

An important observation attracting attention is that subclinical hypothyroid patients may be at higher risk of developing cardiovascular disease (CVD). Such an association could influence the debate on the treatment of subclinical hypothyroidism.

SUBCLINICAL HYPOTHYROIDISM AND CVD: META-ANALYSIS STUDIES

Meta-analysis studies have recently pointed out the potential risk of subclinical hypothyroid patients to develop CVD. Rodondi *et al* (Rodondi et al., 2006) analyzed 14 cross-sectional, longitudinal and case-control studies from 1966 to 2004, and concluded that subclinical hypothyroid patients were at increased risk of CVD (odds ratio (OR): 1.65; confidential interval (CI): 1.28-2.12) compared to euthyroid control patients. Analyzing specific subgroups by type of research design revealed that only analysis from case-control and cross-sectional (OR: 1.72; CI: 1.25-2.38), but not from longitudinal studies (OR: 1.42; CI: 0.91-2.21), showed a clearly significant association between subclinical hypothyroidism and CVD. Singh *et al* (Singh et al., 2008) pooled 6 studies published between 2000-2006 and concluded that the rate of CVD risk was higher in the group with subclinical hypothyroidism in both cross-sectional (OR: 1.53; CI: 1.31-1.79) and follow-up (OR: 1.18; CI: 1.02-1.38) studies.

To incorporate more recent studies, Rodondi and colleagues (Ochs et al., 2008) re-examined the question by performing a meta-analysis of longitudinal studies only. Examination of 10 follow-up studies, ranging from 2 to 20 years, revealed that a small effect of subclinical hypothyroidism on CVD risk (OR: 1.2; CI: 0.97-1.49) and mortality (OR: 1.12; CI: 0.99-1.26) exists, although this did not quite reach statistical significance. Since one of these studies (Gussekloo et al., 2004) had indicated that very elderly subclinical hypothyroid patients were protected from CVD, Rodondi *et al* re-analysed their own data by age, and found that subclinical hypothyroid patients <65 years (OR: 1.51, CI: 1.09-2.09), but not older, were at increased CVD risk.

Razvi *et al* (Razvi et al., 2008) performed a new meta-analysis of 15 cross-sectional and longitudinal studies examining the influence of age on CVD and subclinical hypothyroidism. In agreement with the Rodondi group, Razvi *et al* reported that patients <65 years old are at increased risk of CVD (OR: 1.68; CI: 1.27-2.23) and mortality (OR: 1.37; CI: 1.04-1.79) compared to euthyroid controls, whereas no statistically significant association was observed in patients > 65 years old. Thus, age appears to be an important factor to consider when analyzing the influence of subclinical hypothyroidism on CVD. It has been postulated that older subjects might develop resistance to the vascular damage caused by subclinical hypothyroidism; or that they may be protected by a slower metabolism associated with subclinical hypothyroidism; (Ochs et al., 2008; Razvi et al., 2008).

SUBCLINICAL HYPOTHYROIDISM AND NOVEL CVD RISK FACTORS

Identifying the mechanisms by which subclinical hypothyroidism may cause CVD is a subject of interest as it could assist with better patient selection for therapy. As evidenced by some studies, well-established CVD risk factors, such as body mass index (BMI), blood pressure, age and smoking, do not seem to account for this association (Hak et al., 2000; Kvetny et al., 2004; Walsh et al., 2005), so alternate mechanisms should be considered.

Reports are now emerging in the literature concerning a link between subclinical hypothyroidism and non-traditional biomarkers. Several studies (Christ-Crain et al., 2003;Sengul et al., 2004; Taddei et al., 2006; Tuzcu et al., 2005), but not all (Hueston et al., 2005), report that levels of interleukin-6 (IL-6), C-reactive protein (CRP) and homocysteine, independent predictors of CVD (Danesh et al., 2008; Lobbes et al., 2006; Moat, 2008; Ridker, 2003), are elevated in subclinical hypothyroid patients compared to euthyroid

controls. Additional factors suggestive of an inflammatory or atherogenic state, such as free fatty acids (FFA), hyperinsulinemia, and retinol binding protein-4 (RBP-4), are also reported to be higher in subclinical hypothyroid patients compared to healthy controls (Al Sayed et al., 2006; Caraccio et al., 2005; Choi et al., 2008; Tuzcu et al., 2005).

Endothelial dysfunction, an early step in the progression of atherosclerosis and CVD complication, is observed in subclinical hypothyroid patients. Acetylcholine-induced vasodilatation is impaired in subclinical hypothyroid patients compared to euthyroid subjects, a fact that is attributed to the lower nitric oxide content of endothelial cells in subclinical hypothyroidism. L-T4 therapy of subclinical hypothyroid patients increased nitric oxide availability, and improved the endothelium-dependent relaxation response to acetylcholine (Taddei et al., 2003). Coronary flow reserve, an indicator of coronary microvascular function, is lower in subclinical hypothyroid patients than in euthyroid subjects (Baycan et al., 2007; Oflaz et al., 2007). Thickened carotid artery intima-media, a sign of early atherosclerosis, is observed in subclinical hypothyroid patients compared to euthyroid subjects. Restoration of euthyroidism with L-T4 substantially decreased intima-media thickness to levels close to those observed in euthyroid patients (Monzani et al., 2004).

All these data suggest that inflammation and early cardiovascular remodeling is present in subclinical hypothyroidism and that these factors possibly underlie the increased CVD risk associated with this condition.

TSH AS AN INDUCER OF ADIPOCYTE INFLAMMATION: *IN VIVO* HUMAN STUDIES

The use of recombinant human (rh)TSH in clinical practice is an invaluable tool for the treatment and monitoring of thyroid cancer. Prior to the development of rhTSH, patients needed to undergo a prolonged withdrawal from L-T4 therapy in order to increase endogenous TSH production (>30 mU/L) for proper thyroid cancer treatment and monitoring. However, induction of hypothyroidism leads to discomfort and decreased quality of life (Duntas and Cooper, 2008; Fugazzola, 2007). With the use of rhTSH, patients do not need to suspend L-T4 administration and a decline in quality of life is thus avoided.

The established procedure for treatment of many thyroid cancers consists of total thyroidectomy by surgery, followed by rhTSH administration to induce a high uptake of radioiodine (^{131}I) for the ablation of any residual thyroid tissue. L-T4 therapy is instituted to provide thyroid hormone requirements, and to suppress endogenous TSH at or just below the lower limit of its reference interval. For ongoing monitoring, to screen for any early recurrence, patients undergo rhTSH administration (0.9 mg/ dose) on 2 consecutive days, and blood is drawn for serum thyroglobulin and a whole body scan after ^{131}I uptake is performed. Since only thyroid cells can produce thyroglobulin and take up iodine in response to TSH, these thyroidectomized patients are considered cured if thyroglobulin serum levels remain low, and if there is no evidence of ^{131}I uptake (Schlumberger et al., 2007).

Several researchers are currently using the rhTSH protocol on thyroidectomized patients as a new and promising paradigm to demonstrate the physiological role of TSH on extrathyroidal targets in the setting of normal thyroid hormone levels. TSH as an

inflammatory and atherogenic mediator was reported in a study in which 24 thyroidectomized patients received rhTSH to screen for recurrence of thyroid cancer without stopping L-T4 therapy. Administration of rhTSH in these patients impaired endothelial function and increased oxidative stress, as well as serum levels of interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α (Dardano et al., 2006). A recent study, in which a short period (4h) rhTSH infusion potentiated the effect of acetylcholine to stimulate endothelium-dependent vasodilatation in both healthy and thyroidectomized patients, questioned the view of TSH as an atherogenic hormone. It was pointed out that a very short duration (4h) versus a long term rhTSH administration (2 injections over 2 days) might have different effects (Napoli et al., 2008). More research is needed to answer this question.

TSH: SYNTHESIS, FUNCTION AND STRUCTURE

TSH is produced by the pituitary gland and is under the regulation of thyrotropin releasing hormone from the hypothalamus. Upon secretion, TSH acts on its receptor, the TSH receptor (TSHR), a member of the G protein-coupled receptor (GPCR) family, and stimulates the thyroid gland to synthesize and release T4 and T3 to regulate body metabolism. Circulating levels of T3 and T4 will signal, via negative feedback, to both the hypothalamus and the pituitary, to produce the appropriate amount of thyrotropin releasing hormone and TSH, respectively, to maintain a state of euthyroidism. Besides controlling thyroid hormone synthesis and secretion, TSH is an important factor in the control of thyroid cell proliferation and survival (Grossmann et al., 1997).

TSH is a 30 kDa glycoprotein that belongs to the glycoprotein hormone family, together with the luteinizing hormone (LH), follicle stimulating hormone (FSH) and

chorionic gonadotropin (CG). Each of these hormones is a heterodimer comprised of two noncovalent linked subunits consisting of a common α subunit and a unique β subunit that provides the specificity for binding to its own receptor. The α and β subunit alone are not functional and only the assembled α/β heterodimer is able to induce physiological responses. Stabilization between α/β is conferred by a segment of the β subunit termed the seat-belt region that encircles the α subunit (Szkudlinski et al., 2002).

The human α subunit has 92 amino acid residues (14 kDa) with 30% of its molecular weight attributed to the presence of two N-linked glycosylation chains, whereas the human TSH β subunit has 112 amino acid residues (16 kDa) with one N-linked glycosylation chain that accounts for 15% of its mass (Hearn and Gomme, 2000). Glycosylation of both subunits is essential for hormone stability, secretion, and induction of signal transduction (Thotakura et al., 1992).

TSHR: STRUCTURE AND SYNTHESIS

TSH controls physiological responses via interaction with its receptor, the TSHR, which is present in the plasma membrane of the target cells. The TSHR, together with the FSH, LH and CG receptors, constitute the family of glycoprotein hormone receptors. Each of these receptors is characterized by a large N-terminal extracellular region, a seven transmembrane domain joined by three extracellular and three intracellular loops, and an intracellular C-tail (Rapoport et al., 1998). A high degree of amino acid identity (~70%) is seen among their transmembrane domains whereas a lower degree (~40%) exists among their N-terminal extracelular region and their C-tails (Gudermann et al., 1992). The TSHR has the unique feature of possessing extra amino acid residues (insertion of 8 and 50 amino

acids between residues 38-45 and 317-366, respectively) in its extracellular region in comparison with its receptor family members. The TSHR, composed of 764 amino acid residues with over half of its total amino acid content (418) forming the extracellular domain, has a predicted molecular weight of 100 kDa (Rapoport et al., 1998). The 9 leucine repeats in the extracellular domain, which do not encompass the unique amino acid insertions, make it adopt a horseshoe-shaped conformation, are highly involved in the interaction with TSH (Davies et al., 2005).

The synthesis and structure of the TSHR is still not understood well. The receptor is synthesized as a single polypeptide chain and transported to the plasma membrane surface. In the membrane, some receptors, but not all, undergo a type of post-translational maturation, whereby cleavage of the single chain occurs at two separate sites. These cleavages result in the loss of a small part of the receptor (~ 5 kDa, named C peptide) and in the formation of a TSHR containing two subunits, α (extracellular) and β (transmembrane and intracellular). After cleavage, the α and the β subunit still remain held together by disulfide bridges. The physiological significance of having a cleaved and an uncleaved receptor at the cell membrane is still an enigma, since TSH binds and induces signal in both forms (Chazenbalk et al., 1999; Chazenbalk et al., 1997; Rapoport et al., 1998). Further post-translational maturation events include N-linked glycosylation, sulfation and palmitoylation. These modifications have a significant effect on receptor folding, cell-surface expression, and ligand binding (Kursawe and Paschke, 2007).

TSH SIGNALING IN THYROID CELLS

TSHR-Gα_s coupling

TSHR in the thyroid gland allows TSH to control proliferation, differentiation, survival, iodine uptake, and T3/T4 production and release. For most of these actions, the TSHR predominantly couples with the Gα_s subunit (Figure 1.1). Activated Gα_s, after exchanging guanosine diphosphate (GDP) for guanosine triphosphate (GTP), dissociates from the G$\beta\gamma$ subunits. Free Gα_s binds to adenylyl cyclase (AC) at the plasma membrane and stimulates the production of cyclic adenosine monophosphate (cAMP). cAMP, in turn, binds to cAMP-dependent protein kinase (PKA), a tetrameric protein composed of 2 regulatory (R) and 2 catalytic (C) subunits. The cAMP-bound to the R subunits of PKA leads to the dissociation of the C subunits that, once free, phosphorylate target proteins either in the cytoplasm or in the nucleus such as cAMP response element-binding (CREB) (Rivas and Santisteban, 2003).

It was originally thought that all intracellular effects of cAMP were mediated solely by PKA, since TSH-induced translocation of free catalytic subunit of PKA to the nucleus and CREB phosphorylation could be mimicked with pharmacological activator of AC (forskolin), activator of Gα_s (cholera toxin), cAMP analogs, or blocked by the PKA inhibitor H89 (Dremier et al., 2002). However, a change in this concept was made upon the discovery that in most cell types, including thyroid cells, some of the cAMP actions do not involve PKA activation.

The discovery that cAMP binds to exchange protein directly activated by cAMP (Epac) 1 and 2 opened up new possibilities to explain some of the cAMP-dependent but PKA-independent actions (Figure 1.1). Epac proteins, once stimulated by cAMP, activate

the small GTPase Rap by promoting the exchange of GDP for GTP (Kawasaki et al., 1998). Epac1, but not Epac2, is highly expressed in thyroid cells and its expression is upregulated by TSH treatment (Dremier et al., 2007), consistent with a physiological function.

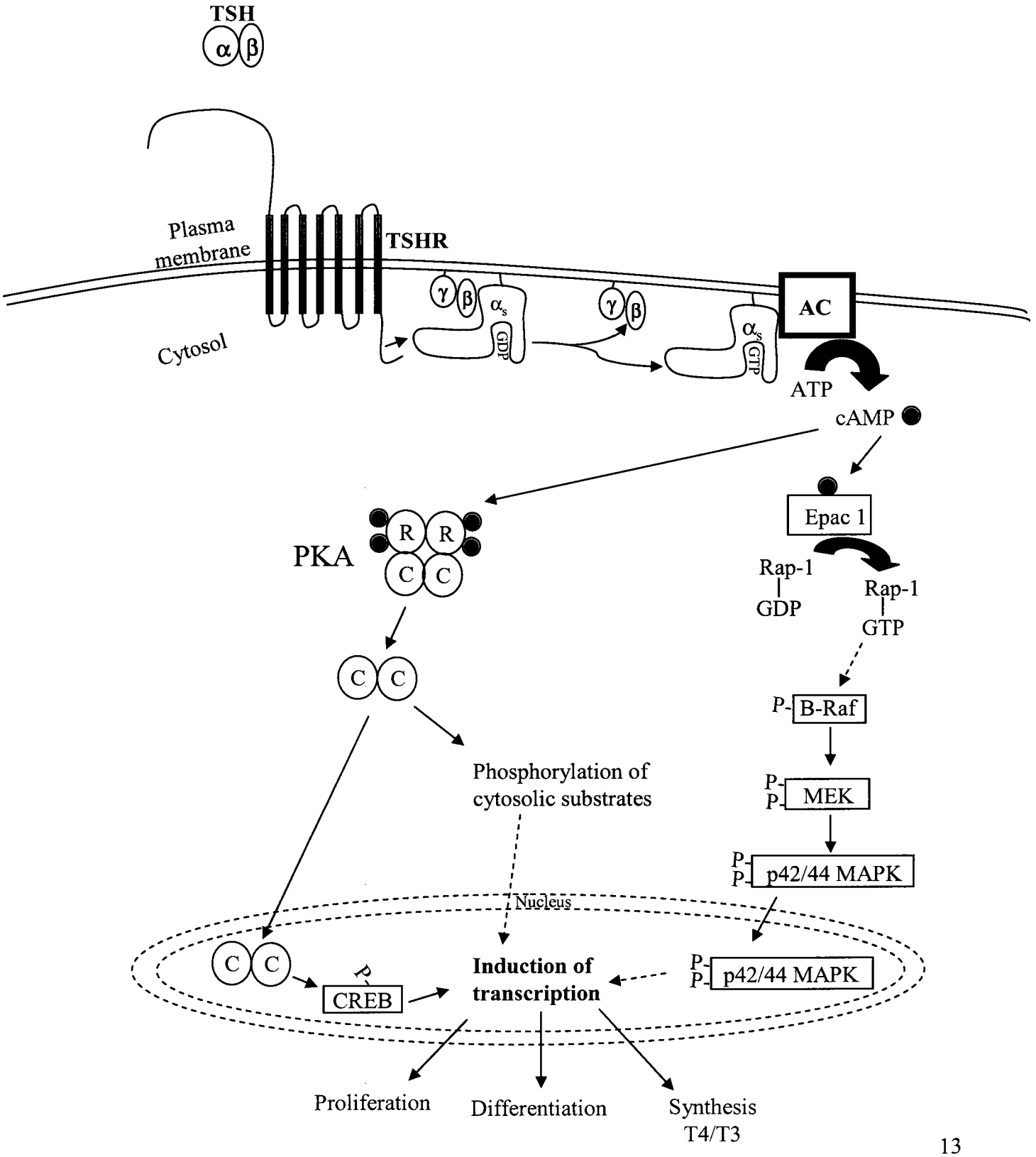
Epac's role as a mediator of some of the cAMP signaling was suggested in a rat thyroid cell line, based on the evidence that TSH-induced activation of Rap-1 and its downstream targets, B-Raf and p42/44 mitogen-activated protein kinase (MAPK), occurred in a cAMP-dependent but a PKA-independent mechanism (Iacovelli et al., 2001). Epac's function in thyroid cells was later demonstrated in a study in which transfection of a rat thyroid cell line with a dominant-negative Epac mutant reduced Rap1 activation by cAMP and TSH-induced deoxyribonucleic acid (DNA) synthesis. Moreover, it was shown that TSH requires cAMP to use both PKA and Epac signaling to induce proliferation (Hochbaum et al., 2008).

In primary dog thyroid cells, use of a cAMP analog that is incapable of activating PKA but a is potent activator of Epac showed that activation of Epac alone did not result in DNA synthesis, cytoskeletal remodeling, T3 secretion or expression of genes important for proper thyroid function and development (Dremier et al., 2007). The requirement for Epac for TSH-induced responses in dog thyroid cells still needs to be addressed with specific Epac inhibition.

TSH-stimulated $G\alpha_s$ also leads to the activation of the phosphatidylinositol 3-kinase (PI3K) signaling in thyrocytes. In rat thyrocytes, TSH and cAMP elevating compounds induce PI3K activation and Akt phosphorylation. PI3K activation was implicated to be important for thyroid cell proliferation since wortmannin (inhibitor of PI3K) blocked the above mentioned agents to promote DNA synthesis (Cass et al., 1999). In another rat thyroid

cell line, TSH-stimulated TSHR tyrosine phosphorylation led to the docking of the PI3K p85α subunit with the TSHR and in increased PI3K activity. PI3K activation by TSH resulted in ribosomal S6 kinase 1 (S6K1) activation and thyroid cell proliferation. Since these events were blocked by H89, it was postulated that $G\alpha_s$/cAMP/PKA is linked to the activation of the PI3K pathway (Suh et al., 2003).

Figure 1.1: TSHR predominantly couples with $G\alpha_s$ in thyrocytes to induce cell responses: Upon TSH binding to the TSHR, the receptor undergoes a conformational change and activates $G\alpha_s$ protein on the plasma membrane. Activated $G\alpha_s$ exchanges GDP for GTP and then binds to and stimulates AC to convert ATP into cAMP. cAMP activates PKA mainly, but can also target Epac. To activate PKA, cAMP binds to the R subunits of PKA, reduces the affinity of the R subunits to the C subunits, causing the liberation of active C subunits. Free active C subunits either stay on the cytosol or enter the nucleus to phosphorylate cytosolic and nuclear protein targets. To activate Epac in thyroid cells, cAMP binds to Epac 1, which in turn allows Rap1 to exchange GDP for GTP. Activated Rap1-GTP leads to B-Raf, MEK and p42/44 MAPK phosphorylation. p42/44 MAPK subsequently translocates to the nucleus and activates transcription factors that will control thyroid cell functions.


TSH
α
β
Plasma membrane
TSHR
Cytosol
γ
β
α_s
GDP
γ
β
α_s
GTP
AC
ATP
cAMP
PKA
R
R
C
C
C
C
Epac 1
Rap-1
GDP
Rap-1
GTP
P-
B-Raf
P-
P-
MEK
P-
P-
p42/44 MAPK
Phosphorylation of cytosolic substrates
Nucleus
C
C
P
CREB
Induction of transcription
P-
P-
p42/44 MAPK
Proliferation
Differentiation
Synthesis T4/T3

TSHR-Gα_q coupling

TSH-regulated thyroid function can also be achieved through activation of Gα_q proteins (Rivas and Santisteban, 2003). TSHR coupling to Gα_q is seen in humans and rats, but not in dog thyroid cells (Allgeier et al., 1994; Raspe et al., 1992). TSH-induced activation of Gα_q, as in Gα_s, promotes a GDP-GTP exchange and further dissociation from the $\beta\gamma$ subunits. Once free, Gα_q-GTP activates the enzyme phospholipase C (PLC) β isoform in the plasma membrane. Activated PLCβ, in turn, cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into two second messengers, diacylglycerol (DAG) and inositol trisphosphate (IP_3). DAG remains bound to the plasma membrane while IP3 diffuses into the cytosol where it binds to its receptors on the endoplasmic reticulum to induce calcium release. The main result of DAG formation and increased cytosolic calcium is activation of protein kinase C (PKC).

A higher TSH concentration is needed to achieve Gα_q activation compared to Gα_s, suggesting that TSHR has a lower sensitivity for the PKC pathway than for the PKA cascade (Allgeier et al., 1994; Wang et al., 1994). This observation resulted in some uncertainty about the precise physiological function of Gα_q. However, a recent study using mice lacking Gα_q specifically in thyroid cells strongly supports the notion for the requirement of Gα_q on TSH actions. Thyroid-specific Gα_q knockout mice showed blunted response to TSH on the induction of thyroid cell growth, T4 synthesis and secretion. Moreover, as these mice aged, they developed high serum TSH concentrations concomitant with low serum T4, characteristic of hypothyroidism (Kero et al., 2007).

TSHR-Gα_i, Gα_{12} and Gα_{13} coupling

TSHR, besides coupling to Gα_s and Gα_q, also couples with the other members of the G protein subfamily Gα_i, Gα_{12} and Gα_{13} in human thyroid cells (Laugwitz et al., 1996). Since most published studies focus on Gα_s and Gα_q, little information exists regarding TSHR coupling with these other G proteins. Experiments showing that TSH blocking antibody can inhibit TSH-induced αi activation on thyroid membranes, and that TSH-induced increase in cAMP levels is potentiated by the Gα_i inhibitor pertussis toxin together indicate that TSHR couples not only to Gα_s, but also to Gα_i (Laugwitz et al., 1996). TSH-induced p42/44 MAPK activation can be achieved upon TSHR-Gα_{13} coupling. In addition, in thyroid carcinoma, but not in normal thyroid cells, Gα_{13}-induced signaling by TSH was dependent on epidermal growth factor receptor transactivation (Buch et al., 2008). Understanding the biological significance of the activation of a wide variety of G proteins as well as tyrosine kinase receptors by TSH to mediate signaling will be an interesting and complex task.

TSH ACTIONS ON EXTRATHYROIDAL CELLS

TSHR expression is not confined to the thyroid gland, but also occurs in adipose cells (Michalek et al., 2009), bone marrow cells (Wang et al., 2003), osteoblasts and osteoclasts progenitors (Abe et al., 2003), as well as in a variety of cells that belong to the cardiovascular system, such as endothelial cells (Donnini et al., 2003), vascular smooth muscle cells (Sellitti et al., 2000) and cardiomyocytes (Drvota et al., 1995).

A number of extra-thyroidal biological roles for TSH have thus been suggested. For example, mice lacking TSHR show a high rate of bone remodeling and develop osteoporosis

(Abe et al., 2003). The mechanism may be that TSH, through binding to TSHR on osteoclasts, decreases TNF-α secretion and, as a consequence, attenuates osteoclast differentiation to avoid bone loss (Hase et al., 2006). Another group, on the other hand, demonstrated that despite the fact that both osteoblasts and osteoclasts express TSHR mRNA and protein, TSH neither activates cAMP, nor alters the expression of markers of differentiation in these cells (Bassett et al., 2008). Studies on whether TSH affects bone metabolism *in vivo* have also been performed. Mazziotti G *et al* reported that rhTSH administration in thyroidectomized postmenopausal women, as seen by serum levels of specific bone markers, causes decreased bone resorption and increased bone formation (Mazziotti et al., 2005) while Martini G *et al* observed that rhTSH increases the serum level of markers of bone formation but not of markers of bone resorption in postmenopausal women (Martini et al., 2008). Thus, a conclusive answer regarding the effect of TSH on bone has yet to come. Surprisingly, in bone marrow cells, TNF-α production is enhanced by TSH (Wang et al., 2003). The opposite role of TSH on modulation of TNF-α might be explained by the capacity of TSHR to couple to different G proteins and activate varied signaling pathways.

In aortic human endothelial cells, TSH and TSH stimulating antibody are capable of increasing intracellular cAMP and modulate the expression of factors involved in coagulation, vasorelaxation and vasoconstriction (Donnini et al., 2003). In vascular smooth muscle cells as well as in cardiomyocytes, TSH increases cAMP production (Drvota et al., 1995; Sellitti et al., 2000), but the physiological significance of this effect has not yet been demonstrated.

Several studies have documented the presence of TSHR in adipose cells from different species (rat, mouse, guinea pig, and human) (Endo et al., 1995, Shimura et al., 1997, Roselli-Rehfuss et al., 1992, Janson et al., 1998). In humans, TSHR is expressed in both preadipocytes and adipocytes from the subcutaneous, visceral, and the orbital depot (Bell et al., 2000; Crisp et al., 1997; Davies et al., 1977; Janson et al., 1998). TSH-induced responses in the adipose tissue merits attention for several reasons. It has been well established that dysfunctional adipose tissue, through release of a variety of molecules into the circulation, can significantly contribute to systemic inflammation (Berg and Scherer, 2005). Since TSH modulates secretion of atherogenic/pro-inflammatory cytokines (Bell et al., 2003; Dardano et al., 2006), inflamed adipose tissue caused by TSH could therefore be a potential mechanism by which subclinical hypothyroid patients are at a higher risk for CVD.

AN OVERVIEW OF ADIPOSE TISSUE

There are two distinct types of adipose tissue: brown and white. These tissues not only originate from distinct cell lineages but they also have very distinct functions (Seale et al., 2009). In humans, brown adipose tissue, a tissue specialized in thermogenesis, is abundantly present in newborns, but it decreases early in childhood and is found in lesser amounts during adulthood (Virtanen et al., 2009; Wolf, 2009). White adipose tissue, on the other hand, a tissue whose primary function is energy storage, is widely distributed throughout the body and is present and remodeled throughout life (Rosen and MacDougald, 2006; Seale et al., 2009).

Morphologically and molecularly, adipocytes from brown adipose tissue are characterized by the presence of multiple small lipid droplets distributed throughout the

cytoplasm with numerous mitochondria containing uncoupling protein-1, the protein that leads to heat production instead of adenosine-5'-triphosphate (ATP). The adipocytes from white adipose tissue are distinguished by a large unilocular lipid droplet that occupies nearly all of the cytoplasmic volume and a nucleus located in the periphery of the cell (Gesta et al., 2007).

The focus of my thesis was on the effect of TSH on white adipocytes. White adipose tissue has a heterogeneous cell composition containing, besides adipocytes (50-70%), a stromal vascular fraction that consists of mesenchymal stem cells, preadipocytes (20-40%), fibroblasts, macrophages (1-30%) and endothelial cells (1-10%) (Hauner, 2005). Adipocytes are specialized in the storage of high amounts of circulating FFA in the form of triacylglycerol (TG) during periods of energy surplus, and in the rapid release of FFA for use in other tissues when there is an energy demand (Bays et al., 2008).

Release of FFA, termed lipolysis, by adipose tissue is a process that is highly regulated by catecholamines (stimulation) and by insulin (suppression) (Yang and Smith, 2007). During times of energy need, such as exercise, stress or extended fasting, catecholamines act on G protein-coupled β–adrenergic receptors to increase cAMP levels and activate PKA. PKA phosphorylates perilipin and hormone-sensitive lipase (HSL). Perilipin is a unique coating protein in the adipocytes situated around the lipid droplet, that, once phosphorylated, either undergoes a conformational change or leaves the lipid droplet surface and moves to the cytoplasm. Subsequently, phosphorylated HSL accesses the lipid core to hydrolyze TG to yield FFA and glycerol. In periods of energy surplus, insulin inhibits TG breakdown by signaling through the insulin receptor to stimulate

phosphodiesterase 3B and decrease cAMP levels and therefore its downstream effects on perilipin and HSL (Jocken and Blaak, 2008).

Adipose tissue as an endocrine tissue

Traditionally, white adipose tissue has been known for its fundamental role in maintaining energy homeostasis. However, in recent years, adipose tissue has emerged as an endocrine tissue. Subsequent to the discovery of leptin as an adipose-derived protein with endocrine functions (Zhang et al., 1994), a growing list of bioactive proteins, as exemplified by adiponectin, IL-6, TNF-α, RBP-4, monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor (PAI) 1 have been found to be produced and secreted by adipose tissue. Proteins exclusively produced by the adipocytes, leptin and adiponectin, are termed adipokines whereas the ones not exclusively produced by the adipocytes but also by other cell types in the adipose tissue are termed cytokines or chemokines (Fantuzzi, 2005). These bioactive proteins may act locally to influence preadipocyte growth, differentiation and angiogenesis or may be released into the circulation to control metabolic functions on different tissues. More importantly, adipose tissue-deregulated cytokine secretion initiates a state of low-grade-inflammation believed to contribute to the development of a constellation of diseases such as insulin resistance, type 2 diabetes, dyslipidemia, and CVD (Trayhurn and Wood, 2004).

Anatomical distribution of adipose tissue

White adipose tissue is not uniformly distributed throughout the body, it is situated in specific locations in which it exerts distinct physiological and molecular functions. The

adipose tissue located under the skin, which constitutes 80% of the adipose tissue mass, is named subcutaneous. Subcutaneous adipose tissue is further subdivided into smaller depots according to the anatomical location: abdominal and gluteofemoral depots (Arner, 1997). The adipose tissue located deep in the abdominal cavity, surrounding the internal organs, represents approximately 6-20% of total body fat and is named visceral (Lafontan and Berlan, 2003). The visceral adipose tissue depot is composed of omental and mesenteric depots. In addition to the subcutaneous and visceral depots, there is a distinct, although small, adipose tissue existent in the orbital area defined as orbital adipose tissue depot (Arner, 1997).

It is now clear that adipose cells from distinct anatomical locations behave differently. For example, the ability of catecholamines to stimulate lipolysis has been shown to be lower in the subcutaneous than in the visceral depot, whereas insulin-mediated inhibition of lipolysis is shown to be higher in the subcutaneous compared to the visceral depot (Lafontan and Berlan, 2003). In addition, adipose cells from distinct depots exert different endocrine functions. Secretion of leptin is 3 times higher in the subcutaneous compared to the omental depot. IL-6, adiponectin, PAI-1, MCP-1 and interleukin-8 (IL-8) secretion, on the other hand, is higher in the visceral compared to the subcutaneous depot (Bruun et al., 2005; Yang and Smith, 2007).

It has been repeatedly shown that excessive adipose tissue accumulation in the visceral depot plays a significant role in the appearance of health problems, particularly CVD and type 2 diabetes (Pi-Sunyer, 2006). The visceral depot is unique, as it drains directly into the portal vein. Thus, it had been believed that, with excess visceral adipose tissue accumulation, elevated efflux of FFA into the liver would interfere with its normal function,

leading to an impairment of insulin-mediated suppression of glucose production and disturbed lipid metabolism via altered lipoprotein synthesis (Yang and Smith, 2007). However, this view was put into question with a recent study showing that most of the FFA that reaches the liver are derived from the systemic circulation and only a minimal contribution (5-30%) exists from visceral depot lipolysis (Nielsen et al., 2004). It is now suggested that not only increased FFA, but also the high levels of atherogenic/pro-inflammatory proteins released by the visceral depot, such as IL-6, IL-8, PAI-1, angiotensinogen, might explain the hepatic insulin resistance and altered circulating lipid profile seen in patients with greater visceral fat content (Yang and Smith, 2007). Proinflammatory proteins are able to negatively regulate insulin signaling by either inducing serine phosphorylation of insulin receptor substrate-1 or by promoting expression of suppressor or cytokine signaling (Lagathu et al., 2003; Schenk et al., 2008).

Moreover, it is thus suggested that the contribution of subcutaneous depot to metabolic complications merits attention (Jensen, 2006). The first compelling indication of the importance of the subcutaneous adipose tissue on maintaining whole body homeostasis is seen in individuals with lipodystrophy, a disease in which body fat, mainly the subcutaneous adipose tissue, is severely diminished. In the absence of subcutaneous adipose tissue, FFA will be stored in organs such as liver and muscle. As a consequence of the ectopic fat deposition, lipotoxicity and malfunction of these organs, these patients develop insulin resistance (Herranz et al., 2008). It is also believed that when healthy individuals start consuming more energy than they expend, the expanding subcutaneous adipose tissue, once it reaches its storage capacity limit, will send FFA to muscle and liver. Overaccumulation of

FFA in ectopic tissue leads to insulin resistance and its related complications (Despres and Lemieux, 2006).

Adipogenesis

The conversion of preadipocyte into adipocytes, a process by which preadipocytes lose their fibroblastic morphology and acquire a spherical shape filled with lipid droplets, is named adipogenesis (Farmer and Spiegelman, 2007; Spiegelman and Flier, 2001). The amount of adipocytes, in humans, is established during the early years of life. Once adulthood is reached, it is shown that either lean or obese subjects who have been obese since childhood have their adipocyte number maintained constant, with a yearly turnover of 10%, due to a fine balance between preadipocyte proliferation, differentiation and cell death (Spalding et al., 2008). It remains to be investigated whether this is true when weight gain occurs during adulthood, or in those with insulin resistance versus insulin sensitivity. Adipogenesis is a multi-step process initiated by the multipotent mesenchymal stem cells that reside in the stromal fraction, specifically in the blood vessels that run throughout the adipose tissue (Rodeheffer et al., 2008; Tang et al., 2008).

Recently, bone morphogenetic protein 4 has been implicated as the signal responsible for mesenchymal stem cells to become committed to the preadipocytes lineage (Bowers and Lane, 2007). Committed preadipocytes, which can be identified by the presence of the unique protein preadipocyte factor 1 (pref-1) (Wang et al., 2006), will proliferate until cell-cell contact is reached and become growth-arrested at confluence. This temporary quiescent state appears to be a prerequisite for preadipocytes to undergo terminal differentiation (Avram et al., 2007). Following growth arrest, and upon mitogenic and

adipogenic inducers, preadipocytes synchronously re-enter the cell cycle and undergo one or two rounds of cell division, an event known as mitotic clonal expansion (MCE) (Otto and Lane, 2005). During MCE, cells express a cascade of transcription factors that act cooperatively and sequentially to allow differentiation to progress. Major transcription factors that play a role in adipogenesis are the CCAAT enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor (PPAR) family members. Early regulators of MCE are C/EBPδ and C/EBPβ. Their expression is induced in the first hours of induction of differentiation and soon after they acquire the capacity to bind DNA and drive transcription of other factors (Otto and Lane, 2005). Overexpression of C/EBPδ and particularly C/EBPβ accelerates the conversion of preadipocytes to adipocytes (Yeh et al., 1995). The crucial role of C/EBPβ in the adipogenic program is evidenced by the fact that its disruption abrogates both MCE and terminal differentiation (Tang et al., 2003a) and, conversely, forced expression induces differentiation in fibroblasts that would otherwise not be committed to adipogenesis (Yeh et al., 1995).

Once C/EBPδ and β acquire DNA-binding activity, they induce transcription of two key transcription factors, C/EBPα and PPARγ. As C/EBPα and PPARγ both have anti-mitotic effects, their activation results in the termination of MCE (Tang et al., 2003b). Preadipocytes thus undergo a second and final growth-arrest stage to then terminally differentiate. Terminal differentiation is maintained by C/EBPα and PPARγ that, together, coordinately activate a large group of genes responsible for the adipocyte phenotype. Genes regulated by these proteins include those involved in insulin sensitivity (insulin-dependent glucose transporter, adiponectin), fatty acid binding (fatty acid-binding

protein), uptake (lipoprotein lipase, fatty acid transport protein) and storage (acyl coenzyme A synthase) (Auwerx, 1999; Farmer, 2005).

Experimental models of adipogenesis

The understanding of adipogenesis has long been a goal of researchers in adipose tissue biology. Most of what has been learned from adipogenesis is derived from the 3T3-L1 and 3T3-F442A immortalized preadipocyte cell lines obtained from mouse embryo fibroblasts (MacDougald and Mandrup, 2002). These lines were selected from disaggregated 3T3 Swiss embryos cells for their capacity to become spherical and accumulate lipid droplets in the cytoplasm upon exposure to insulin or to high concentration of serum and, conversely, to show decreased lipid accumulation in the presence of catecholamines (Green and Kehinde, 1975; Green and Meuth, 1974). The proof that these lines faithfully represent preadipocytes was obtained with *in vivo* implantation studies. Subcutaneous injection of 3T3-F442A preadipocytes into athymic mice differentiate into adipocytes and form fat pads with characteristics comparable to those of the normal adipose tissue (Green and Kehinde, 1979; Mandrup et al., 1997). The implantation of 3T3-L1 preadipocytes into subcutaneous adipose tissue also results in the development of normal fat pads, however, only if Wnt signaling, a pathway that inhibits C/EBPα and PPARγ expression, is suppressed (Ross et al., 2000).

Both 3T3-L1 and 3T3-F442A cell lines are easily and quickly (~6-8 days) differentiated *in vitro*. In culture, shortly after the preadipocytes become confluent and growth arrested, they undergo differentiation when placed in medium supplemented with fetal bovine serum and insulin (for both 3T3-L1 and 3T3-F442A cells), dexamethasone and isobutylmethylxanthine (only for 3T3-L1 cells) (Otto and Lane, 2005). Insulin, by acting

through the insulin growth factor-1 receptor or the insulin receptor, induces activation of pro-adipogenic transcription factors, such as CREB, and represses anti-adipogenic transcription factors, such as those of the forkhead and GATA family (Rosen and MacDougald, 2006). Dexamethasone, through binding to the glucocorticoid receptor, represses the antiadipogenic protein pref-1 and induces C/EBPδ synthesis (Smas et al., 1999). Isobutylmethylxanthine increases cAMP by inhibiting phosphodiesterase activity, which results in CREB activation and subsequently induction of C/EBPβ expression (Zhang et al., 2004).

Despite the fact that preadipose cell lines mimic preadipocyte differentiation *in vivo*, several aspects must be taken into consideration when extrapolating data obtained from preadipose cell lines to cultures of human primary preadipocytes. Preadipose cell lines have an aneuploid rather than a diploid status with a limited life span (Ailhaud, 1982). Whereas cell lines and human primary preadipocytes are both committed to the adipocyte lineage, they represent distinct stages of development since preadipose cell lines are obtained during the prenatal period whereas human primary preadipocytes are obtained at postnatal to adult ages (Gregoire et al., 1998). MCE is a mandatory requirement for cell lines to undergo adipogenesis whereas human primary preadipocytes differentiate directly in the absence of MCE in culture. It is believed that human primary preadipocytes have already proceeded through the requisite MCE *in vivo* and therefore represent a later stage of the adipocytic program (Entenmann and Hauner, 1996). Importantly, studies on cell lines do not allow the analysis of regional anatomic differences with respect to lipolytic and antilipolytic hormones or profiles of cytokine secretion (Rosen et al., 2000).

Primary culture of human preadipocytes is therefore often used to strengthen the findings obtained with preadipose cell line and to obtain information on the behavior of the anatomical sites of fat depots. Human primary preadipocytes are separated from the other cells of the adipose tissue through a collagenase digestion, filtration and centrifugation process (Hauner et al., 1989). Care must be taken to ensure that contamination of other cell types is kept to a minimum. It has been reported that at the end of this process, the presence of endothelial cells in the preadipocyte culture is minimal (Hauner et al., 1989). Still, macrophages can be also present in the preadipocyte culture, the extent of which is dependent on BMI (Curat et al., 2004).

Differentiation is accomplished with the use of the adipogenic cocktail described above for 3T3-L1 cell line, with the addition of a PPARγ agonist (Adams et al., 1997). The cascade of events and the genes expressed during the differentiation process mirror those of the preadipocyte cell lines except that, as mentioned above, human preadipocytes in culture do not undergo MCE to terminally differentiate. Disadvantages also exist with the use of primary human preadipocytes: access to adipose tissue for preadipocyte isolation is dependent on surgical procedures; preadipocytes can only be passaged for a limited number of times (~ 3), the differentiation process is time consuming (~14-18 days) and the degree of differentiation is not uniform, varying according to donor characteristics, such as age (Avram et al., 2007; Cartwright et al., 2007). Nonetheless, culture of human primary preadipocytes represents an invaluable tool for the adipose tissue field.

TSH FUNCTION AND SIGNALING IN THE ADIPOSE TISSUE

TSH action in adipose tissue can be traced back to studies in 1964 that were conducted by a Nobel Prize winner in Physiology or Medicine in the year of 1994; Martin Rodbell. His studies revealed that catecholamines, as well as TSH, were able to induce lipolysis in isolated rat fat cells (Rodbell, 1964). The lipolytic effect of TSH is also observed in humans. Marcus *et al* showed that in newborns, TSH can act as a major lipolytic hormone. With aging, TSH becomes a weaker inducer of lipolysis compared to catecholamines (Marcus et al., 1988). TSH-induced lipolysis is mimicked with TSHR stimulating antibody, whereas TSHR blocking antibody depressed the lipolytic effect, suggesting that TSH mediates signaling through TSHR binding (Janson et al., 1995; Marcus et al., 1988). Although TSH increases cAMP levels in fat cells, it is uncertain whether it is the operative pathway for the induction of lipolysis (Janson et al., 1995).

Subsequent studies have further demonstrated that not only adipocytes, but also preadipocytes, are TSH responsive. On primary culture of rat preadipocytes, TSH induces proliferation, while inhibiting the differentiation into adipocytes (Haraguchi et al., 1999). Conversely, TSH appears to have a different role on the earliest step of adipose development since embryonic stem cells showed enhanced differentiation when TSH was added in the culture medium either alone or in combination with adipogenic inducers (Lu and Lin, 2008).

TSH effects on human and 3T3-L1 preadipocytes has also been investigated by our laboratory. Primary human preadipocytes isolated from either abdominal subcutaneous or the orbital depot showed increased p70 S6 kinase activity in response to TSH. This effect appears to depend on PI3K signaling, since wortmannin decreased TSH-induced p70 S6 kinase activation (Bell et al., 2000). In 3T3-L1 preadipocytes, similar to human primary

cells, TSH-induced protein kinase B/p70 S6 kinase activation requires PI3K activation. As in thyroid cells, TSH also acts as a survival factor in preadipocytes. TSH-inhibited apoptosis in preadipocytes appears to be mediated by suppression of caspase-3 activation (Bell et al., 2002).

TSH actions on adipose tissue include modulation of leptin, however with mixed actions reported. For instance, TSH decreased leptin secretion from isolated rat adipocytes while it increased secretion from tissue fragments obtained from the omental adipose depot (Menendez et al., 2003; Shintani et al., 1999). A recent in vivo study has suggested that TSH does not regulate leptin release since acute rhTSH administration into thyroidectomized females did not have any effect on leptin blood levels (Cecoli et al., 2008).

Recently, our laboratory discovered that TSH increases the secretion of the atherogenic/pro-inflammatory cytokine IL-6 from 3T3-L1, 3T3-F442A as well as from primary human abdominal subcutaneous differentiated adipocytes (Bell et al., 2003). As explained below, IL-6 released from adipose tissue reaches the systemic circulation, and high levels of circulating IL-6 are a strong predictor of CVD.

AN OVERVIEW OF IL-6

Human IL-6 is synthesized as a 212 amino acid protein, but the mature, secreted form of the protein has 184 amino acids due to cleavage of the signal peptide (Wong et al., 2003). Mouse IL-6, which is composed of 187 amino acids, has 42% amino acid identity with the human sequence (Hammacher et al., 1994; Wong et al., 2003). IL-6 from both species has a variable molecular mass ranging between 23 to 30 kDa as a result of

differential post-translational modifications such as glycosylation and phosphorylation (Keller et al., 1996; Santhanam et al., 1989).

IL-6 is released by a broad range of cells, such as osteoblasts, myocytes, thyrocytes, monocytes, including those that compose the adipose tissue, such as preadipocytes, adipocytes, endothelial cells and macrophages (Hoene and Weigert, 2008; Weetman et al., 1990). IL-6 has a plethora of actions; it regulates haematopoiesis, immune response, inflammation and metabolism (Hoene and Weigert, 2008).

The biological activities of IL-6 are initiated by binding to the IL-6 receptor (IL-6 R) that is either present on the cell surface or circulating as a soluble form (Rose-John et al., 2006). Since IL-6R does not possess any signal transduction capacity, signal into the cell is dependent on recruitment of another receptor, the gp130. After association with the IL-6/IL-6R complex, gp130 homodimerizes, recruits janus kinase proteins to tyrosine phosphorylate gp130 in the cytoplasmic tail, thereby providing docking sites for the association of molecules able to initiate signal transduction (Heinrich et al., 2003). Due to the existence of IL-6R in the circulation, IL-6 is capable of signaling in cells that lack membrane-bound IL-6R but express gp130 which, of note, is present in almost every cell in the body (Rose-John et al., 2006).

ADIPOSE TISSUE AS A SOURCE OF IL-6

As mentioned above, cells of the adipose tissue release IL-6. Using different culture systems, such as 3T3-L1, adipose tissue explants, primary adipose cells from lean and obese mouse, it was demonstrated that IL-6 release from cells of the stromal-vascular fraction

accounts for most of the adipose tissue-derived IL-6, with a small contribution (~10%) coming from the adipocytes (Fain et al., 2004; Fried et al., 1998; Harkins et al., 2004).

Adipose tissue overproduces IL-6 in conditions such as obesity, insulin resistance and diabetes (Bastard et al., 2000; Bastard et al., 2002; Mohamed-Ali et al., 1997; Rotter et al., 2003). *In vitro* studies have shown that insulin, catecholamines, as well as pro-inflammatory factors, such as TNF-α, interleukin-1β (IL-1β), lipopolysaccharide (LPS), angiotensin II, and IL-6 itself induce adipose tissue IL-6 production (Flower et al., 2003; Harkins et al., 2004; Hoene and Weigert, 2008; Skurk et al., 2004).

Interstitial IL-6 concentration in the adipose tissue is several times higher than that of the systemic circulation, suggesting that IL-6 may have an autocrine and/or paracrine mechanism of action in this tissue (Sopasakis et al., 2004). *In vitro* studies support the notion that local IL-6 modulate adipose tissue metabolic function. Studies using either differentiated adipocytes or adipose tissue explants, showed that IL-6 inhibits the expression of markers of terminal differentiation, impairs insulin signaling, decrease lipoprotein lipase activity and increase lipolysis (Greenberg et al., 1992; Lagathu et al., 2003; Rotter et al., 2003; Sopasakis et al., 2004; Trujillo et al., 2004). IL-6, by acting locally, may also alter adipose tissue endocrine function. IL-6 treatment on adipose cells not only increases its own secretion, but it also increases the release of pro-inflammatory proteins such as MCP-1 and PAI-1, and it decreases the release of adiponectin, an adipokine known to improve glucose metabolism (Fasshauer et al., 2004; Fasshauer et al., 2003; Kralisch et al., 2005, Kralisch et al., 2006; Lagathu et al., 2003).

Aside from its autocrine and paracrine actions, IL-6 from adipose tissue is able to exert endocrine functions, since 10 to 35% of the circulating IL-6 is derived from the adipose

tissue (Mohamed-Ali et al., 1997). Since clinical studies have repeatedly reported that circulating IL-6 levels increase with weight gain, insulin resistance, type 2 diabetes and CVD, it is postulated that IL-6 from the adipose tissue contributes to the low-grade inflammation present in these conditions (Kern et al., 2001; Pi-Sunyer, 2006; Pradhan et al., 2001). One possible target for adipose-tissue derived IL-6 to have pro-inflammatory action is the liver. *In vitro*, IL-6 treatment inhibits insulin signaling and insulin-induced glycogen storage in hepatocytes (Kanemaki et al., 1998; Senn et al., 2002). In support of this concept, IL-6 treatment to humans and mice lead to increased plasma glucose levels (Klover et al., 2003; Tsigos et al., 1997). Furthermore, IL-6 is the main mediator in the induction of the acute phase proteins in the liver, such as CRP, serum amyloid A and fibrinogen (Gabay and Kushner, 1999).

Another target of IL-6 is the endothelium, where it induces endothelial dysfunction and vascular inflammation (Huber et al., 1999). Interestingly, all of the events mediated by IL-6, such as insulin resistance, acute phase proteins and vascular inflammation, are events reported to contribute to the development of CVD (Pi-Sunyer, 2006).

IL-6 AS A CVD RISK FACTOR

Several studies have demonstrated that IL-6 serum levels are high in subjects with CVD (Biasucci et al., 1996; Miyao et al., 1993). The significance of IL-6 is demonstrated by the fact that circulating IL-6 levels are elevated in healthy men and women at baseline who have been documented to have a higher future risk of CVD events over time, and therefore IL-6 is considered to be an independent risk factor for CVD (Danesh et al., 2008; Ridker et al., 2000). The high IL-6 levels are likely to contribute to the increased CRP circulating levels observed in patients with CVD, which is also a powerful predictor of CVD

complications (Blake and Ridker, 2001). It is now recognized that IL-6 can directly participate in the development of CVD. IL-6 has direct effects on the endothelium, and can induce endothelial expression of chemoattractant proteins such as MCP-1 and interleukin-8, and promote the induction of adhesion molecules (Romano et al., 1997). Suggesting a direct role in the vascular inflammatory process, IL-6 treatment in mouse models of atherosclerosis led to increased plaque size (Huber et al., 1999). Moreover, vascular smooth muscle cells proliferation and platelet activation, events that lead to CVD complications, are increased by IL-6 (Ikeda et al., 1991; Oleksowicz et al., 1994).

Given the of role of IL-6 as an inflammatory mediator and its association with diabetes, insulin resistance and CVD, a better understanding of IL-6 synthesis and its mode of action would help to develop preventative or therapeutic approaches (Danesh et al., 2008).

TRANSCRIPITIONAL REGULATION OF IL-6 SYNTHESIS

IL-6 synthesis has been reported to be under the control of different transcription factors, namely nuclear factor-kappa B (NF-κB), activator protein (AP)-1, C/EBPβ and CREB (Hershko et al., 2002). The binding sites in the IL-6 promoter region for these transcription factors are conserved among mouse, rat and human (Funakoshi et al., 1999). Depending on the cell type and on the stimulus, activation of some of the transcription factors to induce IL-6 synthesis will be more prominent than others. For example, in rat cardiomyocytes, catecholamine-induced IL-6 secretion required both CREB and AP-1 activation, but not NF-κB and C/EBPβ (Rohrbach et al., 2007). In human osteoblasts, TNF-α induced NF-κB activation, but it inhibited C/EBPβ activity, to induce IL-6 synthesis

(Webb et al., 2002). Furthermore, in human enterocytes, IL-1β, in concert with a cAMP analog, needed all four transcription factors to induce IL-6 synthesis (Hershko et al., 2002).

Transcriptional regulation of IL-6 synthesis in preadipocytes and adipocytes

Although the roles of the transcription factors involved in IL-6 secretion in preadipocytes and adipocytes are not yet fully explored, recent studies revealed that NF-κB appears to be the particularly important in this process. NF-κB is the collective name for a family of transcription factors comprised of 5 proteins: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel (de Winther et al., 2005). Although all proteins can form different complexes of either homo or heterodimers to bind to DNA, only p65, RelB and c-Rel have the capacity to initiate transcription (Gilmore, 2006). Thus, to be transcriptionally active, one of these three proteins must be part of the NF-κB dimer. The most common active complex is the p65-p50 heterodimer.

In unstimulated cells, NF-κB dimers are stabilized in the cytoplasm by a specific family of inhibitory proteins, called inhibitor of κB (IκB) α, β, or ε, which hide the nuclear localization signal of the dimers (Gilmore, 2006; Schmid and Birbach, 2008). In response to stimuli, IκB is phosphorylated by its upstream kinase, the IκB kinase (IKK) complex, rapidly ubiquitinated and degraded. Degradation of IκB liberates NF-κB to enter the nucleus and activate transcription of a variety of genes, including IL-6 (Schmid and Birbach, 2008).

During 3T3-L1 adipocyte differentiation, the expression of NF-κB subunits and activity are increased, suggesting that NF-κB might play an important role in cytokine release in the late stages of differentiation (Berg et al., 2004). IL-6 release from preadipocytes or adipocytes through NF-κB activation has been shown to occur with the

classical NF-κB activators TNF-α, LPS and fatty acid (Berg et al., 2004; Chung et al., 2005; Chung et al., 2006; Gonzales and Orlando, 2008). Apart from the classical NF-κB agonists, GPCR are also able to activate NF-κB and induce inflammation (Ye, 2001). This concept has just begun to be explored in adipose cells. Recently, it was reported that in human differentiated adipocytes, angiotensin II activated the NF-κB signaling pathway to induce IL-6 gene expression (Skurk et al., 2004).

HYPOTHESIS

I hypothesize that TSH increases the release of the atherogenic/pro-inflammatory cytokine IL-6 from adipose cells.

RATIONALE

The rationale behind my hypothesis is the possibility that the high TSH levels associated with subclinical hypothyroidism may act on adipose cells, leading to increased IL-6 levels, and therefore placing subclinical hypothyroid patients at increased risk of developing CVD.

OBJECTIVES

Objective 1: Investigate the signaling pathway by which TSH stimulates IL-6 release from 3T3-L1 cells.

Objective 2: Determine the effect of TSH on IL-6 release from human adipose cells as a function of the stage of cell differentiation and the anatomic tissue depot.

Objective 3: Investigate the signaling pathway by which TSH stimulates IL-6 release from human abdominal subcutaneous differentiated adipocytes.

Objective 4: Analyze the effect of rhTSH administration in thyroidectomized patients on serum IL-6 levels.

REFERENCES

Abe, E., Marians, R.C., Yu, W., Wu, X.B., Ando, T., Li, Y., Iqbal, J., Eldeiry, L., Rajendren, G., Blair, H.C., *et al.* (2003). TSH is a negative regulator of skeletal remodeling. Cell *115*, 151-162.

Adams, M., Montague, C.T., Prins, J.B., Holder, J.C., Smith, S.A., Sanders, L., Digby, J.E., Sewter, C.P., Lazar, M.A., Chatterjee, V.K., *et al.* (1997). Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. J Clin Invest *100*, 3149-3153.

Ailhaud, G. (1982). Adipose cell differentiation in culture. Mol Cell Biochem *49*, 17-31.

Al Sayed, A., Al Ali, N., Bo Abbas, Y., and Alfadhli, E. (2006). Subclinical hypothyroidism is associated with early insulin resistance in Kuwaiti women. Endocr J *53*, 653-657.

Allgeier, A., Offermanns, S., Van Sande, J., Spicher, K., Schultz, G., and Dumont, J.E. (1994). The human thyrotropin receptor activates G-proteins Gs and Gq/11. J Biol Chem *269*, 13733-13735.

Arner, P. (1997). Regional adipocity in man. J Endocrinol *155*, 191-192.

Arrigo, T., Wasniewska, M., Crisafulli, G., Lombardo, F., Messina, M.F., Rulli, I., Salzano, G., Valenzise, M., Zirilli, G., and De Luca, F. (2008). Subclinical hypothyroidism: the state of the art. J Endocrinol Invest *31*, 79-84.

Auwerx, J. (1999). PPARgamma, the ultimate thrifty gene. Diabetologia *42*, 1033-1049.

Avram, M.M., Avram, A.S., and James, W.D. (2007). Subcutaneous fat in normal and diseased states 3. Adipogenesis: from stem cell to fat cell. J Am Acad Dermatol *56*, 472-492.

Bassett, J.H., Williams, A.J., Murphy, E., Boyde, A., Howell, P.G., Swinhoe, R., Archanco, M., Flamant, F., Samarut, J., Costagliola, S., *et al.* (2008). A lack of thyroid hormones rather than excess thyrotropin causes abnormal skeletal development in hypothyroidism. Mol Endocrinol *22*, 501-512.

Bastard, J.P., Jardel, C., Bruckert, E., Blondy, P., Capeau, J., Laville, M., Vidal, H., and Hainque, B. (2000). Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. J Clin Endocrinol Metab *85*, 3338-3342.

Bastard, J.P., Maachi, M., Van Nhieu, J.T., Jardel, C., Bruckert, E., Grimaldi, A., Robert, J.J., Capeau, J., and Hainque, B. (2002). Adipose tissue IL-6 content correlates with resistance to insulin activation of glucose uptake both in vivo and in vitro. J Clin Endocrinol Metab *87*, 2084-2089.

Baycan, S., Erdogan, D., Caliskan, M., Pamuk, B.O., Ciftci, O., Gullu, H., Yildirir, A., Guvener, N.D., and Muderrisoglu, H. (2007). Coronary flow reserve is impaired in subclinical hypothyroidism. Clin Cardiol *30*, 562-566.

Bays, H.E., Gonzalez-Campoy, J.M., Bray, G.A., Kitabchi, A.E., Bergman, D.A., Schorr, A.B., Rodbard, H.W., and Henry, R.R. (2008). Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. Expert Rev Cardiovasc Ther *6*, 343-368.

Bell, A., Gagnon, A., Dods, P., Papineau, D., Tiberi, M., and Sorisky, A. (2002). TSH signaling and cell survival in 3T3-L1 preadipocytes. Am J Physiol Cell Physiol *283*, C1056-1064.

Bell, A., Gagnon, A., Grunder, L., Parikh, S.J., Smith, T.J., and Sorisky, A. (2000). Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. Am J Physiol Cell Physiol *279*, C335-340.

Bell, A., Gagnon, A., and Sorisky, A. (2003). TSH stimulates IL-6 secretion from adipocytes in culture. Arterioscler Thromb Vasc Biol *23*, e65-66.

Berg, A.H., Lin, Y., Lisanti, M.P., and Scherer, P.E. (2004). Adipocyte differentiation induces dynamic changes in NF-kappaB expression and activity. Am J Physiol Endocrinol Metab *287*, E1178-1188.

Berg, A.H., and Scherer, P.E. (2005). Adipose tissue, inflammation, and cardiovascular disease. Circ Res *96*, 939-949.

Biasucci, L.M., Vitelli, A., Liuzzo, G., Altamura, S., Caligiuri, G., Monaco, C., Rebuzzi, A.G., Ciliberto, G., and Maseri, A. (1996). Elevated levels of interleukin-6 in unstable angina. Circulation *94*, 874-877.

Biondi, B., and Cooper, D.S. (2008). The clinical significance of subclinical thyroid dysfunction. Endocr Rev *29*, 76-131.

Blake, G.J., and Ridker, P.M. (2001). Novel clinical markers of vascular wall inflammation. Circ Res *89*, 763-771.

Bowers, R.R., and Lane, M.D. (2007). A role for bone morphogenetic protein-4 in adipocyte development. Cell Cycle *6*, 385-389.

Bruun, J.M., Lihn, A.S., Pedersen, S.B., and Richelsen, B. (2005). Monocyte chemoattractant protein-1 release is higher in visceral than subcutaneous human adipose tissue (AT): implication of macrophages resident in the AT. J Clin Endocrinol Metab *90*, 2282-2289.

Buch, T.R., Biebermann, H., Kalwa, H., Pinkenburg, O., Hager, D., Barth, H., Aktories, K., Breit, A., and Gudermann, T. (2008). G13-dependent Activation of MAPK by Thyrotropin. J Biol Chem *283*, 20330-20341.

Caraccio, N., Natali, A., Sironi, A., Baldi, S., Frascerra, S., Dardano, A., Monzani, F., and Ferrannini, E. (2005). Muscle metabolism and exercise tolerance in subclinical hypothyroidism: a controlled trial of levothyroxine. J Clin Endocrinol Metab *90*, 4057-4062.

Cartwright, M.J., Tchkonia, T., and Kirkland, J.L. (2007). Aging in adipocytes: potential impact of inherent, depot-specific mechanisms. Exp Gerontol *42*, 463-471.

Cass, L.A., Summers, S.A., Prendergast, G.V., Backer, J.M., Birnbaum, M.J., and Meinkoth, J.L. (1999). Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol *19*, 5882-5891.

Cecoli, F., Andraghetti, G., Ghiara, C., Briatore, L., Cavallero, D., Mussap, M., Minuto, F., and Giusti, M. (2008). Absence of thyrotropin-induced increase in leptin levels in patients with history of differentiated thyroid carcinoma undergoing recombinant human thyrotropin testing. J Endocrinol Invest *31*, 888-892.

Chazenbalk, G.D., Tanaka, K., McLachlan, S.M., and Rapoport, B. (1999). On the functional importance of thyrotropin receptor intramolecular cleavage. Endocrinology *140*, 4516-4520.

Chazenbalk, G.D., Tanaka, K., Nagayama, Y., Kakinuma, A., Jaume, J.C., McLachlan, S.M., and Rapoport, B. (1997). Evidence that the thyrotropin receptor ectodomain contains not one, but two, cleavage sites. Endocrinology *138*, 2893-2899.

Choi, S.H., Lee, Y.J., Park, Y.J., Kim, K.W., Lee, E.J., Lim, S., Park, D.J., Kim, S.E., Park, K.S., Jang, H.C., *et al.* (2008). Retinol Binding Protein-4 Elevation Is Associated with Serum TSH Level Independently of Obesity in Elderly Subjects with Normal Glucose Tolerance. J Clin Endocrinol Metab.

Christ-Crain, M., Meier, C., Guglielmetti, M., Huber, P.R., Riesen, W., Staub, J.J., and Muller, B. (2003). Elevated C-reactive protein and homocysteine values: cardiovascular risk factors in hypothyroidism? A cross-sectional and a double-blind, placebo-controlled trial. Atherosclerosis *166*, 379-386.

Chung, S., Brown, J.M., Provo, J.N., Hopkins, R., and McIntosh, M.K. (2005). Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production. J Biol Chem *280*, 38445-38456.

Chung, S., Lapoint, K., Martinez, K., Kennedy, A., Boysen Sandberg, M., and McIntosh, M.K. (2006). Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. Endocrinology *147*, 5340-5351.

Cooper, D.S. (2004). Subclinical thyroid disease: consensus or conundrum? Clin Endocrinol (Oxf) *60*, 410-412.

Crisp, M.S., Lane, C., Halliwell, M., Wynford-Thomas, D., and Ludgate, M. (1997). Thyrotropin receptor transcripts in human adipose tissue. J Clin Endocrinol Metab *82*, 2003-2005.

Curat, C. A., Miranville, A., Sengenes, C., Diehl, M., Tonus, C., Busse, R., and Bouloumie, A. (2004). From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes *53*, 1285-92.

Danesh, J., Kaptoge, S., Mann, A.G., Sarwar, N., Wood, A., Angleman, S.B., Wensley, F., Higgins, J.P., Lennon, L., Eiriksdottir, G., *et al.* (2008). Long-term interleukin-6 levels and subsequent risk of coronary heart disease: two new prospective studies and a systematic review. PLoS Med *5*, e78.

Dardano, A., Ghiadoni, L., Plantinga, Y., Caraccio, N., Bemi, A., Duranti, E., Taddei, S., Ferrannini, E., Salvetti, A., and Monzani, F. (2006). Recombinant human thyrotropin reduces endothelium-dependent vasodilation in patients monitored for differentiated thyroid carcinoma. J Clin Endocrinol Metab *91*, 4175-4178.

Davies, T.F., Ando, T., Lin, R.Y., Tomer, Y., and Latif, R. (2005). Thyrotropin receptor-associated diseases: from adenomata to Graves disease. J Clin Invest *115*, 1972-1983.

Davies, T.F., Smith, B.R., and Hall, R. (1977). Thyrotropin receptors in human fat. N Engl J Med *296*, 759-760.

de Winther, M.P., Kanters, E., Kraal, G., and Hofker, M.H. (2005). Nuclear factor kappaB signaling in atherogenesis. Arterioscler Thromb Vasc Biol *25*, 904-914.

Despres, J.P., and Lemieux, I. (2006). Abdominal obesity and metabolic syndrome. Nature *444*, 881-887.

Diez, J.J., Iglesias, P., and Burman, K.D. (2005). Spontaneous normalization of thyrotropin concentrations in patients with subclinical hypothyroidism. J Clin Endocrinol Metab *90*, 4124-4127.

Donnini, D., Ambesi-Impiombato, F.S., and Curcio, F. (2003). Thyrotropin stimulates production of procoagulant and vasodilative factors in human aortic endothelial cells. Thyroid *13*, 517-521.

Dremier, S., Coulonval, K., Perpete, S., Vandeput, F., Fortemaison, N., Van Keymeulen, A., Deleu, S., Ledent, C., Clement, S., Schurmans, S., *et al.* (2002). The role of cyclic AMP and its effect on protein kinase A in the mitogenic action of thyrotropin on the thyroid cell. Ann N Y Acad Sci *968*, 106-121.

Dremier, S., Milenkovic, M., Blancquaert, S., Dumont, J.E., Doskeland, S.O., Maenhaut, C., and Roger, P.P. (2007). Cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinases, but not exchange proteins directly activated by cAMP (Epac), mediate thyrotropin/cAMP-dependent regulation of thyroid cells. Endocrinology *148*, 4612-4622.

Drvota, V., Janson, A., Norman, C., Sylven, C., Haggblad, J., Bronnegard, M., and Marcus, C. (1995). Evidence for the presence of functional thyrotropin receptor in cardiac muscle. Biochem Biophys Res Commun *211*, 426-431.

Duntas, L.H., and Cooper, D.S. (2008). Review on the occasion of a decade of recombinant human TSH: prospects and novel uses. Thyroid *18*, 509-516.

Endo, T., Ohta, K., Haraguchi, K. and Onaya, T. (1995). Cloning and functional expression of a thyrotropin receptor cDNA from rat fat cells. J Biol Chem *270,* 10833-7.

Entenmann, G., and Hauner, H. (1996). Relationship between replication and differentiation in cultured human adipocyte precursor cells. Am J Physiol *270*, C1011-1016.

Fain, J.N., Madan, A.K., Hiler, M.L., Cheema, P., and Bahouth, S.W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology *145*, 2273-2282.

Fantuzzi, G. (2005). Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol *115*, 911-919; quiz 920.

Farmer, S.R. (2005). Regulation of PPARgamma activity during adipogenesis. Int J Obes (Lond) *29 Suppl 1*, S13-16.

Farmer, S.R., and Spiegelman, B.M. (2007). Cell Differentiation. Curr Opin Cell Biol *19*, 603-604.

Fasshauer, M., Klein, J., Kralisch, S., Klier, M., Lossner, U., Bluher, M., and Paschke, R. (2004). Monocyte chemoattractant protein 1 expression is stimulated by growth hormone and interleukin-6 in 3T3-L1 adipocytes. Biochem Biophys Res Commun *317*, 598-604.

Fasshauer, M., Kralisch, S., Klier, M., Lossner, U., Bluher, M., Klein, J., and Paschke, R. (2003). Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. Biochem Biophys Res Commun *301*, 1045-1050.

Flower, L., Gray, R., Pinkney, J., and Mohamed-Ali, V. (2003). Stimulation of interleukin-6 release by interleukin-1beta from isolated human adipocytes. Cytokine *21*, 32-37.

Fried, S.K., Bunkin, D.A., and Greenberg, A.S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab *83*, 847-850.

Fugazzola, L. (2007). Expanding use of recombinant hTSH. Ann Endocrinol (Paris) *68*, 220-223.

Funakoshi, Y., Ichiki, T., Ito, K., and Takeshita, A. (1999). Induction of interleukin-6 expression by angiotensin II in rat vascular smooth muscle cells. Hypertension *34*, 118-125.

Gabay, C., and Kushner, I. (1999). Acute-phase proteins and other systemic responses to inflammation. N Engl J Med *340*, 448-454.

Gesta, S., Tseng, Y.H., and Kahn, C.R. (2007). Developmental origin of fat: tracking obesity to its source. Cell *131*, 242-256.

Gilmore, T.D. (2006). Introduction to NF-kappaB: players, pathways, perspectives. Oncogene *25*, 6680-6684.

Gonzales, A.M., and Orlando, R.A. (2008). Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes. Nutr Metab (Lond) *5*, 17.

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

Green, H., and Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. J Cell Physiol *101*, 169-171.

Green, H., and Meuth, M. (1974). An established pre-adipose cell line and its differentiation in culture. Cell *3*, 127-133.

Greenberg, A.S., Nordan, R.P., McIntosh, J., Calvo, J.C., Scow, R.O., and Jablons, D. (1992). Interleukin 6 reduces lipoprotein lipase activity in adipose tissue of mice in vivo and in 3T3-L1 adipocytes: a possible role for interleukin 6 in cancer cachexia. Cancer Res *52*, 4113-4116.

Gregoire, F.M., Smas, C.M., and Sul, H.S. (1998). Understanding adipocyte differentiation. Physiol Rev *78*, 783-809.

Grossmann, M., Weintraub, B.D., and Szkudlinski, M.W. (1997). Novel insights into the molecular mechanisms of human thyrotropin action: structural, physiological, and therapeutic implications for the glycoprotein hormone family. Endocr Rev *18*, 476-501.

Gudermann, T., Birnbaumer, M., and Birnbaumer, L. (1992). Evidence for dual coupling of the murine luteinizing hormone receptor to adenylyl cyclase and phosphoinositide breakdown and Ca2+ mobilization. Studies with the cloned murine luteinizing hormone receptor expressed in L cells. J Biol Chem *267*, 4479-4488.

Gussekloo, J., van Exel, E., de Craen, A.J., Meinders, A.E., Frolich, M., and Westendorp, R.G. (2004). Thyroid status, disability and cognitive function, and survival in old age. JAMA *292*, 2591-2599.

Hak, A.E., Pols, H.A., Visser, T.J., Drexhage, H.A., Hofman, A., and Witteman, J.C. (2000). Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study. Ann Intern Med *132*, 270-278.

Hammacher, A., Ward, L.D., Weinstock, J., Treutlein, H., Yasukawa, K., and Simpson, R.J. (1994). Structure-function analysis of human IL-6: identification of two distinct regions that are important for receptor binding. Protein Sci *3*, 2280-2293.

Haraguchi, K., Shimura, H., Kawaguchi, A., Ikeda, M., Endo, T., and Onaya, T. (1999). Effects of thyrotropin on the proliferation and differentiation of cultured rat preadipocytes. Thyroid *9*, 613-619.

Harkins, J.M., Moustaid-Moussa, N., Chung, Y.J., Penner, K.M., Pestka, J.J., North, C.M., and Claycombe, K.J. (2004). Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. J Nutr *134*, 2673-2677.

Hase, H., Ando, T., Eldeiry, L., Brebene, A., Peng, Y., Liu, L., Amano, H., Davies, T.F., Sun, L., Zaidi, M., *et al.* (2006). TNFalpha mediates the skeletal effects of thyroid-stimulating hormone. Proc Natl Acad Sci U S A *103*, 12849-12854.

Hauner, H. (2005). Secretory factors from human adipose tissue and their functional role. Proc Nutr Soc *64*, 163-169.

Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R., and Pfeiffer, E.F. (1989). Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. J Clin Invest *84*, 1663-1670.

Hearn, M.T., and Gomme, P.T. (2000). Molecular architecture and biorecognition processes of the cystine knot protein superfamily: part I. The glycoprotein hormones. J Mol Recognit *13*, 223-278.

Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G., and Schaper, F. (2003). Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J *374*, 1-20.

Herranz, P., de Lucas, R., Perez-Espana, L., and Mayor, M. (2008). Lipodystrophy syndromes. Dermatol Clin *26*, 569-578, ix.

Hershko, D.D., Robb, B.W., Luo, G., and Hasselgren, P.O. (2002). Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells. Am J Physiol Regul Integr Comp Physiol *283*, R1140-1148.

Hochbaum, D., Hong, K., Barila, G., Ribeiro-Neto, F., and Altschuler, D.L. (2008). Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis. J Biol Chem *283*, 4464-4468.

Hoene, M., and Weigert, C. (2008). The role of interleukin-6 in insulin resistance, body fat distribution and energy balance. Obes Rev *9*, 20-29.

Huber, S.A., Sakkinen, P., Conze, D., Hardin, N., and Tracy, R. (1999). Interleukin-6 exacerbates early atherosclerosis in mice. Arterioscler Thromb Vasc Biol *19*, 2364-2367.

Hueston, W.J., King, D.E., and Geesey, M.E. (2005). Serum biomarkers for cardiovascular inflammation in subclinical hypothyroidism. Clin Endocrinol (Oxf) *63*, 582-587.

Iacovelli, L., Capobianco, L., Salvatore, L., Sallese, M., D'Ancona, G.M., and De Blasi, A. (2001). Thyrotropin activates mitogen-activated protein kinase pathway in FRTL-5 by a cAMP-dependent protein kinase A-independent mechanism. Mol Pharmacol *60*, 924-933.

Ikeda, U., Ikeda, M., Oohara, T., Oguchi, A., Kamitani, T., Tsuruya, Y., and Kano, S. (1991). Interleukin 6 stimulates growth of vascular smooth muscle cells in a PDGF-dependent manner. Am J Physiol *260*, H1713-1717.

Janson, A., Karlsson, F.A., Micha-Johansson, G., Bolme, P., Bronnegard, M., and Marcus, C. (1995). Effects of stimulatory and inhibitory thyrotropin receptor antibodies on lipolysis in infant adipocytes. J Clin Endocrinol Metab *80*, 1712-1716.

Janson, A., Rawet, H., Perbeck, L., and Marcus, C. (1998). Presence of thyrotropin receptor in infant adipocytes. Pediatr Res *43*, 555-558.

Jensen, M.D. (2006). Is visceral fat involved in the pathogenesis of the metabolic syndrome? Human model. Obesity (Silver Spring) *14 Suppl 1*, 20S-24S.

Jocken, J.W., and Blaak, E.E. (2008). Catecholamine-induced lipolysis in adipose tissue and skeletal muscle in obesity. Physiol Behav *94*, 219-230.

Kanemaki, T., Kitade, H., Kaibori, M., Sakitani, K., Hiramatsu, Y., Kamiyama, Y., Ito, S., and Okumura, T. (1998). Interleukin 1beta and interleukin 6, but not tumor necrosis factor alpha, inhibit insulin-stimulated glycogen synthesis in rat hepatocytes. Hepatology *27*, 1296-1303.

Kawasaki, H., Springett, G.M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D.E., and Graybiel, A.M. (1998). A family of cAMP-binding proteins that directly activate Rap1. Science *282*, 2275-2279.

Keller, E.T., Wanagat, J., and Ershler, W.B. (1996). Molecular and cellular biology of interleukin-6 and its receptor. Front Biosci *1*, d340-357.

Kern, P.A., Ranganathan, S., Li, C., Wood, L., and Ranganathan, G. (2001). Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am J Physiol Endocrinol Metab *280*, E745-751.

Kero, J., Ahmed, K., Wettschureck, N., Tunaru, S., Wintermantel, T., Greiner, E., Schutz, G., and Offermanns, S. (2007). Thyrocyte-specific Gq/G11 deficiency impairs thyroid function and prevents goiter development. J Clin Invest *117*, 2399-2407.

Klover, P.J., Zimmers, T.A., Koniaris, L.G., and Mooney, R.A. (2003). Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. Diabetes *52*, 2784-2789.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M., and Fasshauer, M. (2005). Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab *289*, E586-590.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M., and Fasshauer, M. (2006). Plasminogen activator inhibitor-1 expression and secretion are stimulated by growth hormone and interleukin-6 in 3T3-L1 adipocytes. Mol Cell Endocrinol *253*, 56-62.

Kursawe, R., and Paschke, R. (2007). Modulation of TSHR signaling by posttranslational modifications. Trends Endocrinol Metab *18*, 199-207.

Kvetny, J., Heldgaard, P.E., Bladbjerg, E.M., and Gram, J. (2004). Subclinical hypothyroidism is associated with a low-grade inflammation, increased triglyceride levels and predicts cardiovascular disease in males below 50 years. Clin Endocrinol (Oxf) *61*, 232-238.

Lafontan, M., and Berlan, M. (2003). Do regional differences in adipocyte biology provide new pathophysiological insights? Trends Pharmacol Sci *24*, 276-283.

Lagathu, C., Bastard, J.P., Auclair, M., Maachi, M., Capeau, J., and Caron, M. (2003). Chronic interleukin-6 (IL-6) treatment increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. Biochem Biophys Res Commun *311*, 372-379.

Laugwitz, K.L., Allgeier, A., Offermanns, S., Spicher, K., Van Sande, J., Dumont, J.E., and Schultz, G. (1996). The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. Proc Natl Acad Sci U S A *93*, 116-120.

Lobbes, M.B., Lutgens, E., Heeneman, S., Cleutjens, K.B., Kooi, M.E., van Engelshoven, J.M., Daemen, M.J., and Nelemans, P.J. (2006). Is there more than C-reactive protein and fibrinogen? The prognostic value of soluble CD40 ligand, interleukin-6 and oxidized low-density lipoprotein with respect to coronary and cerebral vascular disease. Atherosclerosis *187*, 18-25.

Lu, M., and Lin, R.Y. (2008). TSH stimulates adipogenesis in mouse embryonic stem cells. J Endocrinol *196*, 159-169.

MacDougald, O.A., and Mandrup, S. (2002). Adipogenesis: forces that tip the scales. Trends Endocrinol Metab *13*, 5-11.

Mandrup, S., Loftus, T.M., MacDougald, O.A., Kuhajda, F.P., and Lane, M.D. (1997). Obese gene expression at in vivo levels by fat pads derived from s.c. implanted 3T3-F442A preadipocytes. Proc Natl Acad Sci U S A *94*, 4300-4305.

Marcus, C., Ehren, H., Bolme, P., and Arner, P. (1988). Regulation of lipolysis during the neonatal period. Importance of thyrotropin. J Clin Invest *82*, 1793-1797.

Martini, G., Gennari, L., De Paola, V., Pilli, T., Salvadori, S., Merlotti, D., Valleggi, F., Campagna, S., Franci, B., Avanzati, A., *et al.* (2008). The effects of recombinant TSH on bone turnover markers and serum osteoprotegerin and RANKL levels. Thyroid *18*, 455-460.

Mazziotti, G., Sorvillo, F., Piscopo, M., Cioffi, M., Pilla, P., Biondi, B., Iorio, S., Giustina, A., Amato, G., and Carella, C. (2005). Recombinant human TSH modulates in vivo C-telopeptides of type-1 collagen and bone alkaline phosphatase, but not osteoprotegerin production in postmenopausal women monitored for differentiated thyroid carcinoma. J Bone Miner Res *20*, 480-486.

Menendez, C., Baldelli, R., Camina, J.P., Escudero, B., Peino, R., Dieguez, C., and Casanueva, F.F. (2003). TSH stimulates leptin secretion by a direct effect on adipocytes. J Endocrinol *176*, 7-12.

Michalek, K., Morshed, S.A., Latif, R., and Davies, T.F. (2009). TSH receptor autoantibodies. Autoimmun Rev.

Miyao, Y., Yasue, H., Ogawa, H., Misumi, I., Masuda, T., Sakamoto, T., and Morita, E. (1993). Elevated plasma interleukin-6 levels in patients with acute myocardial infarction. Am Heart J *126*, 1299-1304.

Moat, S.J. (2008). Plasma total homocysteine: instigator or indicator of cardiovascular disease? Ann Clin Biochem *45*, 345-348.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., and Coppack, S.W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab *82*, 4196-4200.

Monzani, F., Caraccio, N., Kozakowa, M., Dardano, A., Vittone, F., Virdis, A., Taddei, S., Palombo, C., and Ferrannini, E. (2004). Effect of levothyroxine replacement on lipid profile and intima-media thickness in subclinical hypothyroidism: a double-blind, placebo-controlled study. J Clin Endocrinol Metab *89*, 2099-2106.

Mozaffarian, D. (2007). Free fatty acids, cardiovascular mortality, and cardiometabolic stress. Eur Heart J *28*, 2699-2700.

Napoli, R., Biondi, B., Guardasole, V., D'Anna, C., De Sena, A., Pirozzi, C., Terracciano, D., Mazzarella, C., Matarazzo, M., and Sacca, L. (2008). Enhancement of vascular endothelial function by recombinant human thyrotropin. J Clin Endocrinol Metab *93*, 1959-1963.

Nielsen, S., Guo, Z., Johnson, C.M., Hensrud, D.D., and Jensen, M.D. (2004). Splanchnic lipolysis in human obesity. J Clin Invest *113*, 1582-1588.

Ochs, N., Auer, R., Bauer, D.C., Nanchen, D., Gussekloo, J., Cornuz, J., and Rodondi, N. (2008). Meta-analysis: Subclinical Thyroid Dysfunction and the Risk for Coronary Heart Disease and Mortality. Ann Intern Med.

Oflaz, H., Kurt, R., Cimen, A., Elitok, A., Onur, I., Golcuk, E., Demirturk, M., Batmaz, S., and Kasikcioglu, E. (2007). Coronary flow reserve is also impaired in patients with subclinical hypothyroidism. Int J Cardiol *120*, 414-416.

Oleksowicz, L., Mrowiec, Z., Zuckerman, D., Isaacs, R., Dutcher, J., and Puszkin, E. (1994). Platelet activation induced by interleukin-6: evidence for a mechanism involving arachidonic acid metabolism. Thromb Haemost *72*, 302-308.

Otto, T.C., and Lane, M.D. (2005). Adipose development: from stem cell to adipocyte. Crit Rev Biochem Mol Biol *40*, 229-242.

Pi-Sunyer, F.X. (2006). The relation of adipose tissue to cardiometabolic risk. Clin Cornerstone *8 Suppl 4*, S14-23.

Pradhan, A.D., Manson, J.E., Rifai, N., Buring, J.E., and Ridker, P.M. (2001). C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA *286*, 327-334.

Rapoport, B., Chazenbalk, G.D., Jaume, J.C., and McLachlan, S.M. (1998). The thyrotropin (TSH) receptor: interaction with TSH and autoantibodies. Endocr Rev *19*, 673-716.

Raspe, E., Reuse, S., Roger, P.P., and Dumont, J.E. (1992). Lack of correlation between the activation of the Ca(2+)-phosphatidylinositol cascade and the regulation of DNA synthesis in the dog thymocyte. Exp Cell Res *198*, 17-26.

Razvi, S., Shakoor, A., Vanderpump, M., Weaver, J.U., and Pearce, S.H. (2008). The Influence of Age on the Relationship between Subclinical Hypothyroidism and Ischemic Heart Disease: A Meta-Analysis. J Clin Endocrinol Metab.

Ridker, P.M. (2003). Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation *107*, 363-369.

Ridker, P.M., Rifai, N., Stampfer, M.J., and Hennekens, C.H. (2000). Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation *101*, 1767-1772.

Rivas, M., and Santisteban, P. (2003). TSH-activated signaling pathways in thyroid tumorigenesis. Mol Cell Endocrinol *213*, 31-45.

Rodbell, M. (1964). Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. J Biol Chem *239*, 375-380.

Rodeheffer, M.S., Birsoy, K., and Friedman, J.M. (2008). Identification of white adipocyte progenitor cells in vivo. Cell *135*, 240-249.

Rodondi, N., Aujesky, D., Vittinghoff, E., Cornuz, J., and Bauer, D.C. (2006). Subclinical hypothyroidism and the risk of coronary heart disease: a meta-analysis. Am J Med *119*, 541-551.

Rohrbach, S., Engelhardt, S., Lohse, M.J., Werdan, K., Holtz, J., and Muller-Werdan, U. (2007). Activation of AP-1 contributes to the beta-adrenoceptor-mediated myocardial induction of interleukin-6. Mol Med *13*, 605-614.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., van Hinsbergh, V., Sozzani, S., *et al.* (1997). Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity *6*, 315-325.

Rose-John, S., Scheller, J., Elson, G., and Jones, S.A. (2006). Interleukin-6 biology is coordinated by membrane-bound and soluble receptors: role in inflammation and cancer. J Leukoc Biol *80*, 227-236.

Roselli-Rehfuss, L., Robbins, L. S., and Cone, R. D. (1992). Thyrotropin receptor messenger ribonucleic acid is expressed in most brown and white adipose tissues in the guinea pig. Endocrinology *130,* 1857-61.

Rosen, E.D., and MacDougald, O.A. (2006). Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol *7*, 885-896.

Rosen, E.D., Walkey, C.J., Puigserver, P., and Spiegelman, B.M. (2000). Transcriptional regulation of adipogenesis. Genes Dev *14*, 1293-1307.

Ross, S.E., Hemati, N., Longo, K.A., Bennett, C.N., Lucas, P.C., Erickson, R.L., and MacDougald, O.A. (2000). Inhibition of adipogenesis by Wnt signaling. Science *289*, 950-953.

Rotter, V., Nagaev, I., and Smith, U. (2003). Interleukin-6 (IL-6) induces insulin resistance in 3T3-L1 adipocytes and is, like IL-8 and tumor necrosis factor-alpha, overexpressed in human fat cells from insulin-resistant subjects. J Biol Chem *278*, 45777-45784.

Santhanam, U., Ghrayeb, J., Sehgal, P.B., and May, L.T. (1989). Post-translational modifications of human interleukin-6. Arch Biochem Biophys *274*, 161-170.

Schenk, S., Saberi, M., and Olefsky, J. M. (2008). Insulin sensitivity: modulation by nutrients and inflammation. J Clin Invest 118, 2992-3002.

Schlumberger, M., Ricard, M., De Pouvourville, G., and Pacini, F. (2007). How the availability of recombinant human TSH has changed the management of patients who have thyroid cancer. Nat Clin Pract Endocrinol Metab *3*, 641-650.

Schmid, J.A., and Birbach, A. (2008). IkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB. Cytokine Growth Factor Rev *19*, 157-165.

Seale, P., Kajimura, S., and Spiegelman, B.M. (2009). Transcriptional control of brown adipocyte development and physiological function--of mice and men. Genes Dev *23*, 788-797.

Sellitti, D.F., Dennison, D., Akamizu, T., Doi, S.Q., Kohn, L.D., and Koshiyama, H. (2000). Thyrotropin regulation of cyclic adenosine monophosphate production in human coronary artery smooth muscle cells. Thyroid *10*, 219-225.

Sengul, E., Cetinarslan, B., Tarkun, I., Canturk, Z., and Turemen, E. (2004). Homocysteine concentrations in subclinical hypothyroidism. Endocr Res *30*, 351-359.

Senn, J.J., Klover, P.J., Nowak, I.A., and Mooney, R.A. (2002). Interleukin-6 induces cellular insulin resistance in hepatocytes. Diabetes *51*, 3391-3399.

Shimura, H., Haraguchi, K., Endo, T., and Onaya, T. (1997). Regulation of thyrotropin receptor gene expression in 3T3-L1 adipose cells is distinct from its regulation in FRTL-5 thyroid cells. Endocrinology *138,* 1483-90.

Shintani, M., Nishimura, H., Akamizu, T., Yonemitsu, S., Masuzaki, H., Ogawa, Y., Hosoda, K., Inoue, G., Yoshimasa, Y., and Nakao, K. (1999). Thyrotropin decreases leptin production in rat adipocytes. Metabolism *48*, 1570-1574.

Singh, S., Duggal, J., Molnar, J., Maldonado, F., Barsano, C.P., and Arora, R. (2008). Impact of subclinical thyroid disorders on coronary heart disease, cardiovascular and all-cause mortality: a meta-analysis. Int J Cardiol *125*, 41-48.

Skurk, T., van Harmelen, V., and Hauner, H. (2004). Angiotensin II stimulates the release of interleukin-6 and interleukin-8 from cultured human adipocytes by activation of NF-kappaB. Arterioscler Thromb Vasc Biol *24*, 1199-1203.

Smas, C.M., Chen, L., Zhao, L., Latasa, M.J., and Sul, H.S. (1999). Transcriptional repression of pref-1 by glucocorticoids promotes 3T3-L1 adipocyte differentiation. J Biol Chem *274*, 12632-12641.

Sopasakis, V.R., Sandqvist, M., Gustafson, B., Hammarstedt, A., Schmelz, M., Yang, X., Jansson, P.A., and Smith, U. (2004). High local concentrations and effects on differentiation implicate interleukin-6 as a paracrine regulator. Obes Res *12*, 454-460.

Spalding, K.L., Arner, E., Westermark, P.O., Bernard, S., Buchholz, B.A., Bergmann, O., Blomqvist, L., Hoffstedt, J., Naslund, E., Britton, T., *et al.* (2008). Dynamics of fat cell turnover in humans. Nature *453*, 783-787.

Spiegelman, B.M., and Flier, J.S. (2001). Obesity and the regulation of energy balance. Cell *104*, 531-543.

Suh, J.M., Song, J.H., Kim, D.W., Kim, H., Chung, H.K., Hwang, J.H., Kim, J.M., Hwang, E.S., Chung, J., Han, J.H., *et al.* (2003). Regulation of the phosphatidylinositol 3-kinase, Akt/protein kinase B, FRAP/mammalian target of rapamycin, and ribosomal S6 kinase 1 signaling pathways by thyroid-stimulating hormone (TSH) and stimulating type TSH receptor antibodies in the thyroid gland. J Biol Chem *278*, 21960-21971.

Surks, M.I., Ortiz, E., Daniels, G.H., Sawin, C.T., Col, N.F., Cobin, R.H., Franklyn, J.A., Hershman, J.M., Burman, K.D., Denke, M.A., *et al.* (2004). Subclinical thyroid disease: scientific review and guidelines for diagnosis and management. JAMA *291*, 228-238.

Szkudlinski, M.W., Fremont, V., Ronin, C., and Weintraub, B.D. (2002). Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. Physiol Rev *82*, 473-502.

Taddei, S., Caraccio, N., Virdis, A., Dardano, A., Versari, D., Ghiadoni, L., Ferrannini, E., Salvetti, A., and Monzani, F. (2006). Low-grade systemic inflammation causes endothelial dysfunction in patients with Hashimoto's thyroiditis. J Clin Endocrinol Metab *91*, 5076-5082.

Taddei, S., Caraccio, N., Virdis, A., Dardano, A., Versari, D., Ghiadoni, L., Salvetti, A., Ferrannini, E., and Monzani, F. (2003). Impaired endothelium-dependent vasodilatation in subclinical hypothyroidism: beneficial effect of levothyroxine therapy. J Clin Endocrinol Metab *88*, 3731-3737.

Tang, Q.Q., Otto, T.C., and Lane, M.D. (2003a). CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. Proc Natl Acad Sci U S A *100*, 850-855.

Tang, Q.Q., Otto, T.C., and Lane, M.D. (2003b). Mitotic clonal expansion: a synchronous process required for adipogenesis. Proc Natl Acad Sci U S A *100*, 44-49.

Tang, W., Zeve, D., Suh, J.M., Bosnakovski, D., Kyba, M., Hammer, R.E., Tallquist, M.D., and Graff, J.M. (2008). White fat progenitor cells reside in the adipose vasculature. Science *322*, 583-586.

Thotakura, N.R., Desai, R.K., Szkudlinski, M.W., and Weintraub, B.D. (1992). The role of the oligosaccharide chains of thyrotropin alpha- and beta-subunits in hormone action. Endocrinology *131*, 82-88.

Trayhurn, P., and Wood, I.S. (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr *92*, 347-355.

Trujillo, M.E., Sullivan, S., Harten, I., Schneider, S.H., Greenberg, A.S., and Fried, S.K. (2004). Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro. J Clin Endocrinol Metab *89*, 5577-5582.

Tsigos, C., Papanicolaou, D.A., Kyrou, I., Defensor, R., Mitsiadis, C.S., and Chrousos, G.P. (1997). Dose-dependent effects of recombinant human interleukin-6 on glucose regulation. J Clin Endocrinol Metab *82*, 4167-4170.

Tuzcu, A., Bahceci, M., Gokalp, D., Tuzun, Y., and Gunes, K. (2005). Subclinical hypothyroidism may be associated with elevated high-sensitive c-reactive protein (low grade inflammation) and fasting hyperinsulinemia. Endocr J *52*, 89-94.

Virtanen, K.A., Lidell, M.E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N.J., Enerback, S., *et al.* (2009). Functional brown adipose tissue in healthy adults. N Engl J Med *360*, 1518-1525.

Walsh, J.P., Bremner, A.P., Bulsara, M.K., O'Leary, P., Leedman, P.J., Feddema, P., and Michelangeli, V. (2005). Subclinical thyroid dysfunction as a risk factor for cardiovascular disease. Arch Intern Med *165*, 2467-2472.

Wang, H.C., Dragoo, J., Zhou, Q., and Klein, J.R. (2003). An intrinsic thyrotropin-mediated pathway of TNF-alpha production by bone marrow cells. Blood *101*, 119-123.

Wang, S.H., and Baker, J.R. (2007). The role of apoptosis in thyroid autoimmunity. Thyroid *17*, 975-979.

Wang, X.D., Kiang, J.G., and Smallridge, R.C. (1994). A phospholipase C inhibitor, U-73122, blocks TSH-induced inositol trisphosphate production, Ca2+ increase and arachidonic acid release in FRTL-5 thyroid cells. Biochim Biophys Acta *1223*, 101-106.

Wang, Y., Kim, K.A., Kim, J.H., and Sul, H.S. (2006). Pref-1, a preadipocyte secreted factor that inhibits adipogenesis. J Nutr *136*, 2953-2956.

Webb, S.J., McPherson, J.R., Pahan, K., and Koka, S. (2002). Regulation of TNF-alpha-induced IL-6 production in MG-63 human osteoblast-like cells. J Dent Res *81*, 17-22.

Weetman, A.P., Bright-Thomas, R., and Freeman, M. (1990). Regulation of interleukin-6 release by human thyrocytes. J Endocrinol *127*, 357-361.

Wolf, G. (2009). Brown adipose tissue: the molecular mechanism of its formation. Nutr Rev *67*, 167-171.

Wong, P.K., Campbell, I.K., Egan, P.J., Ernst, M., and Wicks, I.P. (2003). The role of the interleukin-6 family of cytokines in inflammatory arthritis and bone turnover. Arthritis Rheum *48*, 1177-1189.

Yang, X., and Smith, U. (2007). Adipose tissue distribution and risk of metabolic disease: does thiazolidinedione-induced adipose tissue redistribution provide a clue to the answer? Diabetologia *50*, 1127-1139.

Ye, R.D. (2001). Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. J Leukoc Biol *70*, 839-848.

Yeh, W.C., Cao, Z., Classon, M., and McKnight, S.L. (1995). Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev *9*, 168-181.

Zhang, J.W., Klemm, D.J., Vinson, C., and Lane, M.D. (2004). Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. J Biol Chem *279*, 4471-4478.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature *372*, 425-432.

2.0 MANUSCRIPT #1

Thyroid-Stimulating Hormone Stimulates Interleukin-6 Release from 3T3-L1 Adipocytes through a cAMP-Protein Kinase A Pathway

STATEMENT OF AUTHOR CONTRIBUTIONS

Tayze T. Antunes was the primary author and performed all the experimental work on the effect and underlying signaling mechanisms of TSH-induced IL-6 secretion from 3T3-L1 adipocytes. She also participated in the experimental design, data analysis, and manuscript preparation. **Dr. AnneMarie Gagnon** is a Research Associate in Dr. Alexander Sorisky's laboratory. She supervised the experimental work, participated in the experimental design, data analysis, and participated in the manuscript preparation. **Dr. Andrea Bell** is a former graduate student supervised by Dr. Alexander Sorisky. During her Ph.D. studies she performed the experimental work on the effect of TSH on IL-6 release from 3T3-L1 preadipocytes (Figure 2.2B). **Dr. Alexander Sorisky** is the Chronic Disease Program Director and a Senior Scientist at the Ottawa Hospital Research Institute. He is the supervisor of Tayze T. Antunes and contributed to the experimental design, data analysis and preparation of manuscript.

SUMMARY

The following manuscript entitled "Thyroid-Stimulating Hormone Stimulates Interleukin-6 Release from 3T3-L1 Adipocytes through a cAMP-Protein Kinase A Pathway" was published in Obesity Research [(2005) *13*, 2066-2071]. Dr. Sorisky's laboratory had previously shown that TSH increases IL-6 release from 3T3-L1, 3T3-F442A as well as from

human abdominal differentiated adipocytes in vitro [Bell A *et al.* Arterioscler Thromb Vasc Biol(2003) 23: e65-6].

The objective of manuscript #1 was therefore to further explore whether TSH effect on IL-6 release was dependent on the stage of preadipocyte differentiation, and to identify the signaling mechanisms utilized by TSH to induce IL-6 release. The 3T3-L1 cell line was chosen for this study since, as mentioned in the General Introduction, it is a reliable model for studying adipocyte biology, and can be easily grown and differentiated in culture.

TSH-induced cell responses in thyroid cells occurs mostly through activation of the cAMP/PKA pathway. Furthermore, previous studies from our laboratory had shown that TSH increases cAMP levels in the 3T3-L1 adipocytes [Bell A *et al.* Am J Physiol Cell Physiol (2002) 283: C1056-64]. It was thus postulated in manuscript #1 that cAMP/PKA activation was required for TSH to induce IL-6 secretion. To test the role of this pathway, specific pharmacological activators and inhibitors were used. Forskolin directly activates AC and H89 is an inhibitor of PKA. To investigate whether the alternative cAMP-dependent target, Epac, is involved in IL-6 release, 8-pCPT-2'-O-Me-cAMP, a specific cAMP analog for Epac, was used. To investigate whether TSH-induced IL-6 release was associated with changes in gene expression, IL-6 mRNA was assessed by semiquantitative reverse transcription polymerase chain reaction, and inhibition of gene transcription by actinomycin D was performed.

Based on this study, a cAMP/PKA-dependent pathway for TSH to induce IL-6 gene expression and release in 3T3-L1 differentiated adipocytes was proposed.

Thyroid-Stimulating Hormone Stimulates Interleukin-6 Release from 3T3-L1 Adipocytes through a cAMP-Protein Kinase A Pathway

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ABSTRACT

ANTUNES, TAYZE T., ANNEMARIE GAGNON, ANDREA BELL, AND ALEXANDER SORISKY.

Thyroid-stimulating hormone stimulates interleukin-6 release from 3T3-L1 adipocytes through a cAMP-protein kinase pathway. *Obes Res.* 2005;13:2066-2071.

Objective: Thyroid-stimulating hormone (TSH) is a novel modulator of adipokine release from human and mouse adipocytes. The aim of our study was to identify the signal transduction pathways activated by TSH that stimulate interleukin (IL)-6 production.

Research Methods and Procedures: Mouse 3T3-L1 preadipocytes and differentiated adipocyte cell cultures were studied. The effect of 0 to 1 μM TSH on IL-6 protein release into the medium over 0 to 24 hours was assessed. TSH signaling pathways responsible for regulating IL-6 were studied through the use of 1 μM forskolin, 100 μM 8-pCPT-2'-O-Me-cAMP, 10 μM H89, 50 μM PD98059, and 2 μg/ml actinomycin D.

Results: TSH stimulated IL-6 release by 2.6-fold from 3T3-L1 adipocytes at concentrations as low as 0.01 μM but did not alter IL-6 production of corresponding preadipocytes. Forskolin (elevates intracellular cAMP) stimulated IL-6 release from 3T3-L1 adipocytes ($n = 3, p < 0.005$), and H89, an inhibitor of cAMP-dependent protein kinase A (PKA), reduced TSH-stimulated IL-6 release by 66% ($n = 3, p < 0.01$), indicating a requirement for cAMP-dependent PKA. Inhibition of the mitogen-activated protein kinase pathway with PD98059 did not affect TSH-stimulated IL-6 release. Activation of an alternate cAMP target, the exchange protein of cAMP, with 8-pCPT-2'-O-Me-cAMP, had no effect on IL-6 release. TSH raised the level of IL-6 mRNA, and blocked of transcription with actinomycin D abrogated IL-6 protein release by TSH ($n = 3, p < 0.05$).

Discussion: TSH stimulates IL-6 release from differentiated 3T3-L1 adipocytes, but not preadipocytes, by signaling through cAMP-PKA to activate IL-6 gene transcription.

Key words: adipocyte, thyroid-stimulating hormone, interleukin-6, cAMP, protein kinase A

INTRODUCTION

The thyroid-stimulating hormone (TSH) receptor (TSHR), expressed in thyrocytes, can also be detected in preadipocytes and adipocytes (Davies et al., 2002; Kershaw and Flier, 2004; Prabhakar et al., 2003). TSH may act directly on extrathyroidal TSHR-bearing adipose cells (Sorisky et al., 2000). Effects of TSH on lipolysis in neonatal adipocytes have been reported (Janson et al., 1998), and more recently, changes in leptin secretion were described (Menendez et al., 2003; Shintani et al., 1999). We have observed that TSH stimulates the release of interleukin (IL)-6 from 3T3-L1, 3T3-F442A, and human differentiated adipocytes (Bell et al., 2003). Adipose tissue is an important source of IL-6 production, given that over 30% of circulating IL-6 is released by it (Mohamed-Ali et al., 1997). IL-6 either directly stimulates atheroma progression or indirectly promotes atherogenesis by elevating hepatic production of C-reactive protein (Libby, 2002).

TSH levels are elevated in subclinical hypothyroidism. Although this elevation serves to normalize thyroid hormone production in the failing thyroid gland, it has been associated with increased cardiovascular risk in epidemiological studies (Franklyn et al., 2005; Hak et al., 2000; Imaizumi et al., 2004; Kvetny et al., 2004). Traditional cardiovascular disease risk factors do not clearly explain this association, but recent work suggests that C-reactive protein levels may be high in subjects with subclinical hypothyroidism (Christ-Crain et al., 2003; Tuzcu et al., 2005). It is possible that higher levels of TSH may alter adipocyte adipokine production, such as that of IL-6, and this might underlie the changes observed in C-reactive protein levels. To gain more insight into the action of TSH, we have studied the signal transduction pathways that regulate IL-6 production, using mouse 3T3-L1 adipocytes. These immortalized cells undergo complete

differentiation in a homogeneous fashion and are used widely as an experimental adipocyte model system.

RESEARCH METHODS AND PROCEDURES

3T3-L1 Preadipocyte Studies and Adipocyte Differentiation

Murine 3T3-L1 preadipocytes were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (all from Invitrogen, Carlsbad, CA) in 35-mm dishes until confluent for 2 days. Preadipocytes were then either maintained in control medium (DMEM, 10% CS, and antibiotics) or induced to differentiate for 8 days. Differentiation medium consisted of DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1 μM of insulin for the first 4 days and with 0.25 μM dexamethasone and 0.5 mM isobutylmethylxanthine for the first 2 days. Control and differentiation media were replaced every 2 days over an 8-day period. For preadipocyte experiments, a 5 μM TSH dose was used in serum-free medium. Before stimulation with lower doses of TSH (ranging from 0.001 to 1 μM), adipocytes were placed overnight in DMEM supplemented with 1% CS. Treatment with TSH, 100 μM 8-pCPT-2'-O-Me-cAMP (Axxora, LLC, San Diego, CA; vehicle, water), 1 μM forskolin (Calbiochem, San Diego, CA; vehicle, 0.1% dimethyl sulfoxide) was performed in DMEM supplemented with 1% CS. Where indicated, adipocytes were pretreated for the specified times with 10 μM H89, 50 μM PD98059, 2 μg/ml actinomycin D (all from Calbiochem), or vehicle (final concentration, 0.1% to 0.4% dimethyl sulfoxide).

Measurement of IL-6 in the Medium and Protein Quantification

IL-6 was measured in cell culture medium by enzyme immunometric assay (Assay Designs, Ann Arbor, MI), according to the manufacture's instruction. After treatment, cells were lysed in Laemmli buffer (Laemmli, 1970), and protein concentration of the cell lysates was quantified by Lowry reaction, using bovine serum albumin as standard.

Measurement of IL-6 mRNA

After treatment of 3T3-L1 adipocytes with 0.1 μM TSH (or vehicle) for 2 hours, RNA was extracted by the acid guanidine thiocyanate-phenol/chloroform method, followed by DNase I treatment (Ambion, Austin, TX). RNA integrity was evaluated on a 1% agarose-formaldehyde gel. Total RNA (1 μg) was reversed transcribed (RT) with the Retroscript kit (Ambion) using random decamers. Polymerase chain reaction (PCR) was performed in 50-μl reaction volume consisting of 5 μl of RT reaction, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (all from Ambion), 0.4 μM mouse IL-6 primers, or 0.4 μM 18S primers (Ambion; proprietary primers, sequences not disclosed), and 1 U of recombinant Taq DNA polymerase (Invitrogen). The amplification conditions were: 2 minutes at 94 °C for template denaturation; and 40 cycles (for IL-6) or 30 cycles (for 18S) consisting of 30 seconds of 94 °C for denaturation, 30 seconds at 61 °C for annealing, 30 seconds at 72 °C for elongation; and 7-minute final elongation at 72 °C, in a GeneAmp Gene System 2400 (Applied Biosystems, Foster City, CA). Another 1 U of Taq polymerase was added to the IL-6 PCR samples at the 20^{th} cycle. The PCR products were loaded on a 2% agarose gel stained with ethidium bromide (1 μg/ml), and bands were visualized under UV light. Amplicon sizes were 414 base pairs for IL-6 and 495 base pairs for 18S.

Statistical Analysis

Data were analyzed by Student's *t* test for paired values or ANOVA with Student-Newman-Keuls post-test, as appropriate, using GraphPad Instat version 3.05 (GraphPad Software Inc., San Diego, CA), with $p < 0.05$ considered significant.

RESULTS

To advance our previous findings on IL-6 release by adipocytes (Bell et al., 2003), we performed a time course study of cells treated with 0.01 μM TSH (Figure 2.1). Basal release of IL-6 increased significantly over the 24-hour period, reaching 84 ± 6 pg/mL per milligram of protein (mean ± SE) at 4 hours and 167 ± 23 pg/mL per milligram of protein at 24 hours. IL-6 release was, therefore, expressed as fold increase over the respective unstimulated time-matched samples. The peak response was 2.6-fold ($n = 7, p < 0.05$) and occurred at the 4-hour time point (Figure 2.1). This time course is very reminiscent of the response of IL-6 release from human differentiated adipocytes stimulated by isoproterenol, in which the peak response occurred at 3 hours, persisted up to 12 hours, and then returned to control values by 24 hours (Path et al., 2001). The effect of TSH over 4 hours at different concentrations was evaluated for its effect on the release of IL-6 (Figure 2.2A). The lowest concentration of TSH that stimulated IL-6 release (2 fold) was 0.01 μM, with larger responses observed at 0.1 and 1 μM TSH (7.6- and 13.6-fold, respectively). It should be noted that these TSH concentrations, although higher than physiological levels, are in the approximate range of those used for thyrocytes in culture, a traditional TSH target cell (Kimura et al., 2001). In contrast, IL-6 release from 3T3-L1 preadipocytes did not increase

in response to TSH, even at a higher concentration of 5 μM. In fact, a slight inhibition (9 ± 2%) was noted (Figure 2.2B).

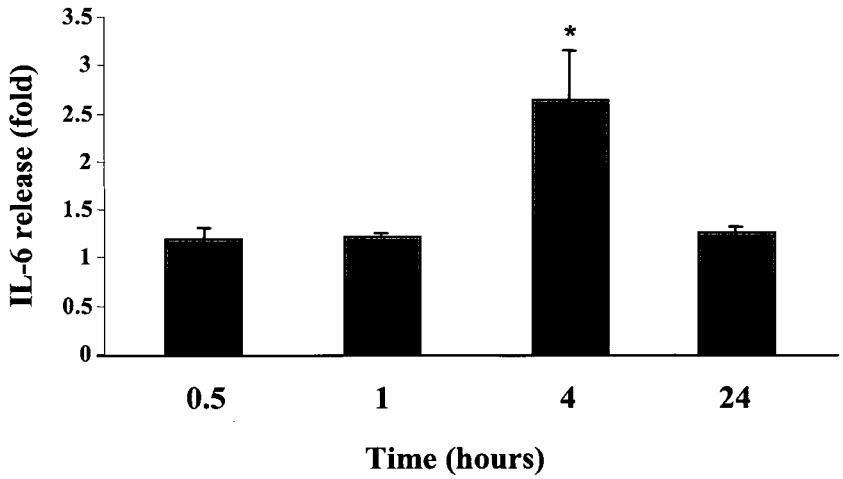
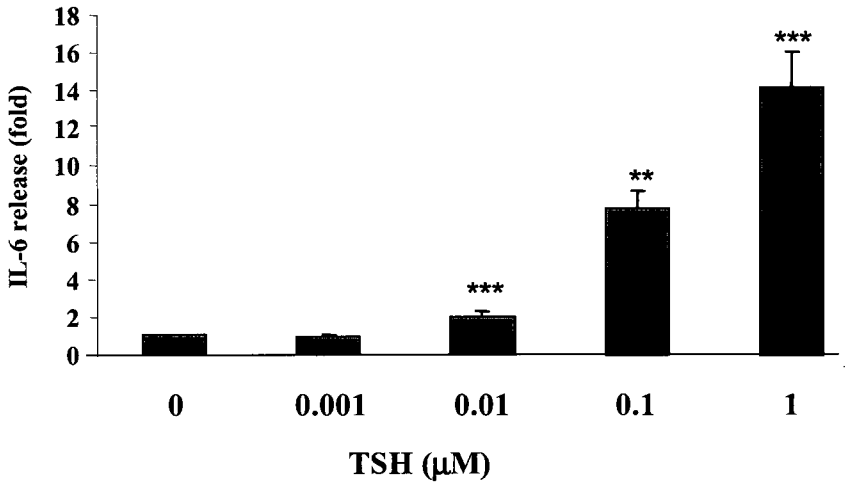


Figure 2.1: Time-course of TSH-induced IL-6 release from 3T3-L1 adipocytes. 3T3-L1 adipocytes were stimulated for up to 24 hours with or without 0.01 μM TSH. Medium was collected and IL-6 protein was measured as described. Results are expressed as the fold increase of basal secretion for each time point, and represent the means ± SE of 3 to 7 independent experiments, each performed in duplicate. * denotes $p<0.05$ compared to timed control.

A.



B.

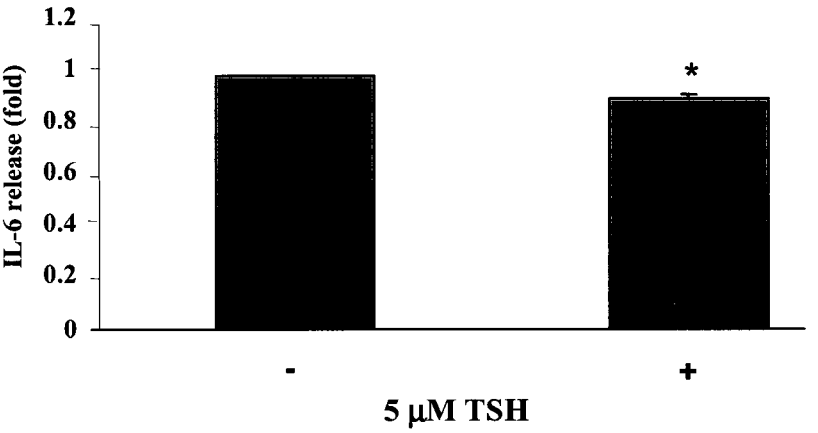


Figure 2.2: TSH-induced IL-6 release from 3T3-L1 adipocytes, but not preadipocytes. A. 3T3-L1 adipocytes were stimulated for 4 hours with 0 to 1 μM TSH. Medium was collected and IL-6 protein was measured as described. Results are expressed as fold-change of control and represent the means ± SE of 6 independent experiments, each performed in duplicate. B. 3T3-L1 preadipocytes were stimulated for 4 hours with or without 5 μM TSH. Medium was collected and IL-6 protein was measured as described. Results are expressed as fold change compared to control and represent the means ± SE of 3 independent experiments, each performed in duplicate. * $p<0.05$, ** $p<0.005$ and *** $p<0.001$, compared to control.

We have reported previously that as 3T3-L1 preadipocytes differentiate into adipocytes, they acquire the capacity to elevate intracellular cAMP in response to TSH (Bell et al., 2002). We, therefore, examined this signaling route with respect to TSH regulation of IL-6 release. cAMP can exert its effects either through activation of cAMP-dependent protein kinase A (PKA) or through an alternate target, the exchange protein activated by cAMP (EPAC) (Kopperud et al., 2003). Forskolin, which increases cAMP by directly activating adenylyl cyclase, increased IL-6 release by ~20-fold (n = 3, $p < 0.005$; Figure 2.3A). Direct and specific activation of EPAC with 100 μM 8-pCPT-2'-O-Me-cAMP (Rangarajan et al., 2003) had no effect on IL-6 release (n = 5; Figure 2.3B), suggesting that PKA is the operative cAMP target. Indeed, inhibition of PKA by 10 μM H89 reduced the stimulatory effect of TSH on IL-6 release by 66% (n = 3, $p < 0.01$; Figure 2.3C). The activation of p42/44 mitogen-activated protein kinase (MAPK) by TSH in thyrocyte cell models is controversial (Kimura et al., 2001; Vandeput et al., 2003). Pre-treatment of 3T3-L1 adipocytes with 50 μM PD98059, an inhibitor of the upstream regulatory kinase MEK1, did not alter TSH-stimulated IL-6 release (n = 3; Figure 2.3C). In the adipocyte cell model, p42/44 MAPK signaling is apparently not involved in the induction of IL-6 by TSH.

TSH might regulate IL-6 protein release by altering transcription. Consistent with this, actinomycin D (2 μg/ml) blocked IL-6 secretion completely (n = 3, $p < 0.05$; Figure 2.4A). Direct measurement of IL-6 mRNA by RT-PCR did not detect a reliable signal under basal conditions but did reveal an increase in IL-6 mRNA levels on TSH treatment (n = 4; Figure 2.4B).

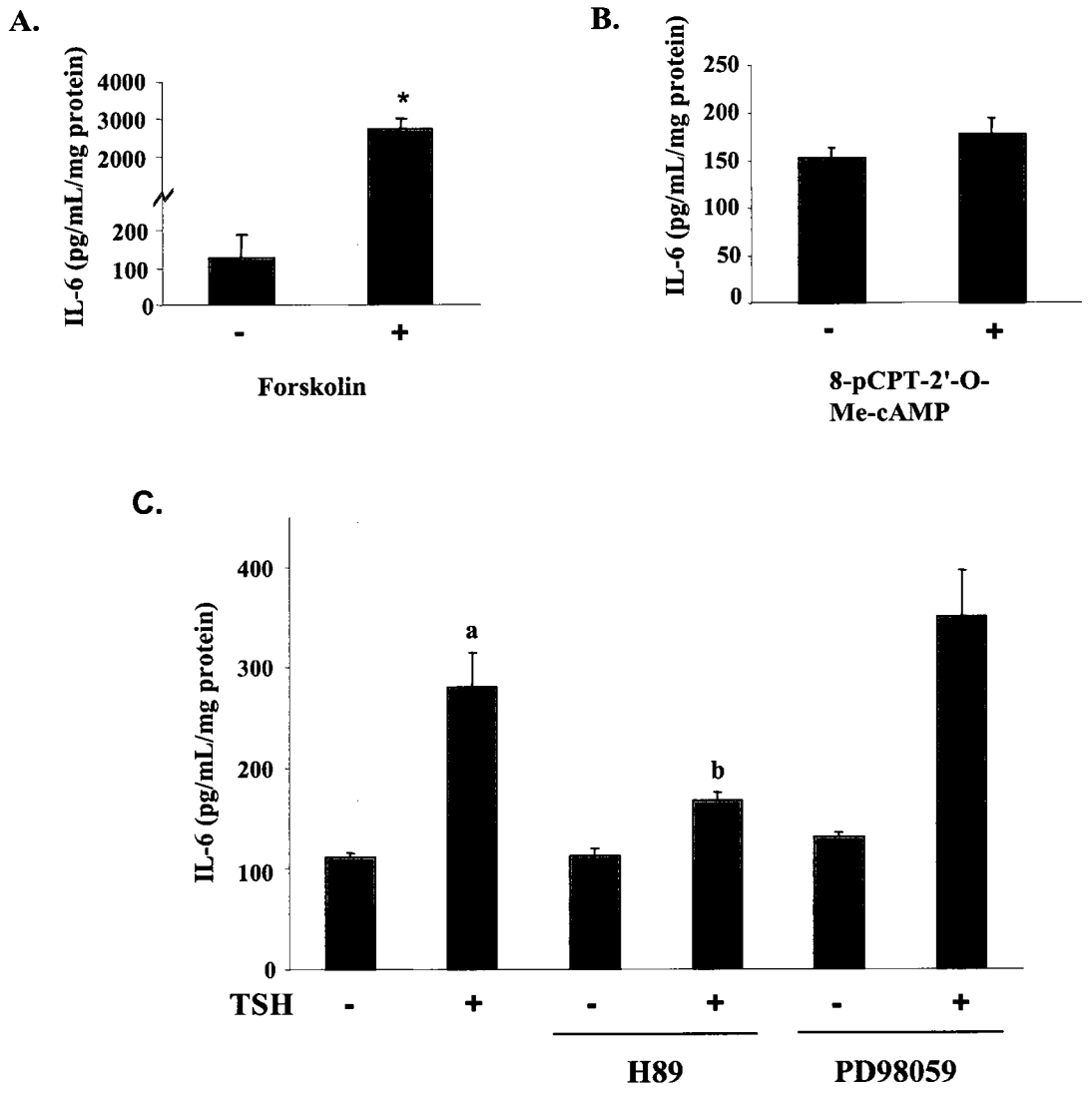


Figure 2.3: TSH induces IL-6 release from 3T3-L1 adipocytes through a cAMP-PKA signaling pathway. A and B. 3T3-L1 adipocytes were stimulated for 4 hours with or without 1 μM forskolin or 100 μM 8-pCPT-2'-O-Me-cAMP. C. 3T3-L1 adipocytes were pre-treated with 10 μM H89 or 50 μM PD98059 for 15 minutes, then stimulated with 0.01 μM TSH for 4 hours. IL-6 protein in the medium was measured as described. Results are expressed as pg/mL/mg of protein and represent the means ± SE of 3-5 independent experiments, each performed in duplicate. * denotes $p<0.005$ compared to control; a, $p<0.01$ compared to control; and b, $p<0.01$ compared to TSH.

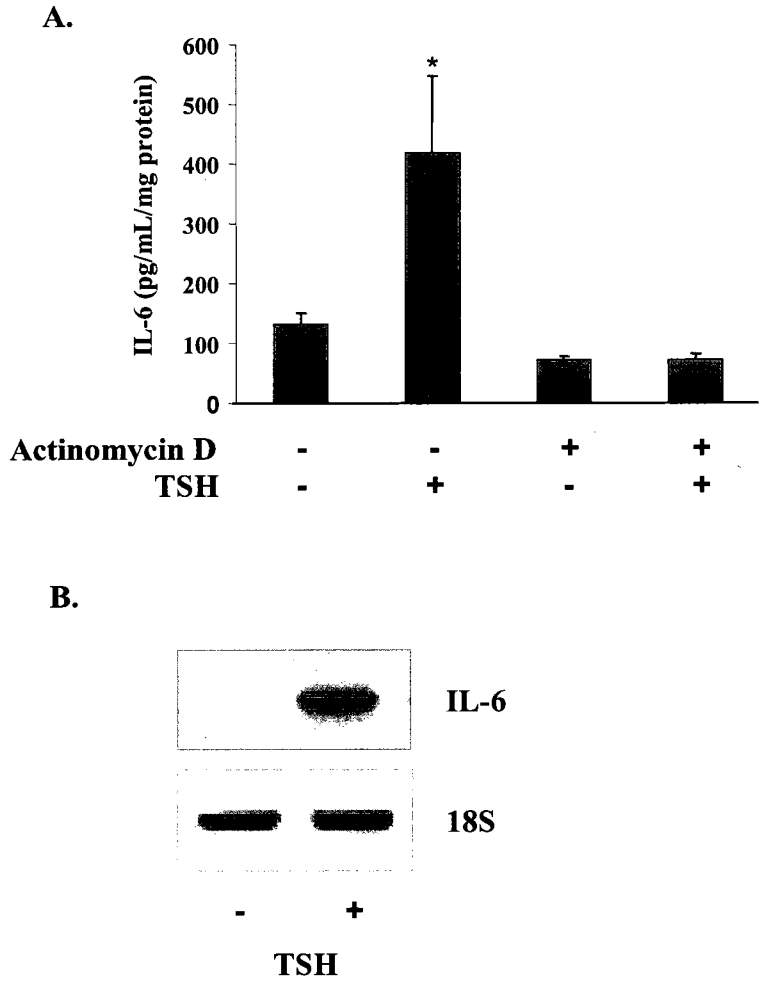


Figure 2.4: TSH increases IL-6 mRNA. A. 3T3-L1 adipocytes were pre-treated with 2 μg/mL of actinomycin D for 1 hour prior to treatment with or without 0.1 μM TSH for 4 hours. IL-6 protein in the medium was measured as described. Results are expressed as pg/mL/mg protein and represent the means ± SE of 3 independent experiments. * denotes $p<0.05$ compared to control and to actinomycin D + TSH. B. 3T3-L1 adipocytes were treated for 2 hours with or without 0.1 μM TSH. RNA was extracted, and RT-PCR was performed as described. Results are representative of 4 independent experiments.

DISCUSSION

The role of TSH action on adipocytes has been under investigation for several decades but is still elusive. Early experiments performed by Rodbell that established the connection between G-protein-coupled receptors and cAMP signaling included studies on TSH-stimulated adipocytes (Rodbell, 1964). TSH-provoked metabolic responses in adipocytes that were described at that time included carbohydrate regulation and lipolysis. Subsequent studies implicated TSH in lipolysis, but primarily in the neonatal period (Janson et al., 1998). Others have reported that TSH either affects proliferation or survival of preadipose cells (Bell et al., 2002; Haraguchi et al., 1999). Most recently, the ability of TSH to modulate the production of adipokines has been described. Leptin production was decreased by TSH in rat adipocytes but was stimulated by TSH in human adipose tissue (Menendez et al., 2003; Shintani et al., 1999). We have reported that TSH increases the release of IL-6 from differentiated human abdominal subcutaneous, mouse 3T3-L1, and 3T3-F442A adipocytes (Bell et al., 2003). Therefore, in contrast to leptin, the IL-6 response to TSH is consistent across rodent and human adipocyte models.

We now begun to identify the signal transduction pathways activated by TSH that are required for IL-6 production in 3T3-L1 adipocytes. This experimental model is an established approach for adipocyte studies because the cells differentiate in a uniform and reproducible fashion and are hormone-responsive (Ross et al., 2000). In particular, they express IL-6 and TSHR (Bell et al., 2002; Fasshauer et al., 2003; Stephens et al., 1992). It should be noted that our studies on 3T3-L1 adipocytes utilized TSH concentrations that approximate those used on thyrocytes (Kimura et al., 2001), traditional TSH target cells. The approach of our signaling studies is based on pharmacological activators and inhibitors

that are well-characterized for these purposes (Vandeput et al., 2003), but inherent limitations related to absolute specificity of these agents must always be noted with this strategy.

Previously, we reported that TSH elevates cytosolic cAMP levels in differentiated 3T3-L1 adipocytes, but not in preadipocytes (Bell et al., 2002). The release of IL-6 by 3T3-L1 preadipocytes treated with TSH did not increase, unlike what was observed for differentiated adipocytes. Because TSHR protein is expressed at the preadipocyte stage, the difference in the IL-6 response may be related to the absence of coupling of TSHR to adenylyl cyclase in preadipocytes (Bell et al., 2002). Changes in adenylyl cyclase isoform expression between preadipocytes and adipocytes that might influence TSHR signaling have also been described (Serazin-Leroy et al., 2001). TSHR signaling in 3T3-L1 and human preadipocytes is linked to activation of tyrosine kinases, PI3K, PKB/Akt, and p70 S6K (Bell et al., 2002; Bell et al., 2000). Although 3T3-L1 preadipocytes may be a significant source of IL-6, having been recently reported to release more IL-6 protein than adipocytes in response to lipopolysaccharide (Harkins et al., 2004), our data suggest that any potential TSH-dependent changes would be expected to act on adipocytes.

Consistent with the importance of cAMP-dependent signaling, direct activation of adenylyl cyclase by forskolin potently increased IL-6 release. Potential downstream effectors of cAMP include PKA and EPAC. An EPAC-specific cAMP analog failed to influence basal IL-6 secretion, and TSH-stimulated IL-6 secretion was attenuated by H89. Thus, it appears that cAMP-PKA activation represents an important signaling pathway for IL-6 release from 3T3-L1 adipocytes. This finding is consistent with studies examining TSH action in thyrocytes, which did not find a significant role of EPAC in mitogenesis (Dremier

et al., 2000; Vanvooren et al., 2001). Activation of MAPK by TSH has been reported in some, but not all, thyrocyte signaling studies related to proliferation (Kimura et al., 2001; Vandeput et al., 2003). We did not observe any effect of p42/44 MAPK inhibition on IL-6 release by 3T3-L1 adipocytes.

TSH increased IL-6 mRNA, and actinomycin D blocked TSH-stimulated IL-6 release. These data are consistent with TSH acting at the transcriptional level to regulate IL-6 secretion. Control at the mRNA level of IL-6 expression seems to be an important phenomenon in adipocytes because it has also been observed in response to isoproterenol, tumor necrosis factor-α, insulin and growth hormone (Fasshauer et al., 2003; Path et al., 2001).

In summary, our data show that adipocytes release IL-6 in a TSH-responsive manner through a cAMP-PKA pathway that acts at the transcriptional level. Understanding how TSH acts to regulate adipokine production may provide new insights on the physiological impact of thyroid function.

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REFERENCES

Bell, A., Gagnon, A., Dods, P., Papineau, D., Tiberi, M., and Sorisky, A. (2002). TSH signaling and cell survival in 3T3-L1 preadipocytes. Am J Physiol Cell Physiol *283*, C1056-1064.

Bell, A., Gagnon, A., Grunder, L., Parikh, S.J., Smith, T.J., and Sorisky, A. (2000). Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. Am J Physiol Cell Physiol *279*, C335-340.

Bell, A., Gagnon, A., and Sorisky, A. (2003). TSH stimulates IL-6 secretion from adipocytes in culture. Arterioscler Thromb Vasc Biol *23*, e65-66.

Christ-Crain, M., Meier, C., Guglielmetti, M., Huber, P.R., Riesen, W., Staub, J.J., and Muller, B. (2003). Elevated C-reactive protein and homocysteine values: cardiovascular risk factors in hypothyroidism? A cross-sectional and a double-blind, placebo-controlled trial. Atherosclerosis *166*, 379-386.

Davies, T., Marians, R., and Latif, R. (2002). The TSH receptor reveals itself. J Clin Invest *110*, 161-164.

Dremier, S., Vandeput, F., Zwartkruis, F.J., Bos, J.L., Dumont, J.E., and Maenhaut, C. (2000). Activation of the small G protein Rap1 in dog thyroid cells by both cAMP-dependent and -independent pathways. Biochem Biophys Res Commun *267*, 7-11.

Fasshauer, M., Klein, J., Lossner, U., and Paschke, R. (2003). Interleukin (IL)-6 mRNA expression is stimulated by insulin, isoproterenol, tumour necrosis factor alpha, growth hormone, and IL-6 in 3T3-L1 adipocytes. Horm Metab Res *35*, 147-152.

Franklyn, J.A., Sheppard, M.C., and Maisonneuve, P. (2005). Thyroid function and mortality in patients treated for hyperthyroidism. JAMA *294*, 71-80.

Hak, A.E., Pols, H.A., Visser, T.J., Drexhage, H.A., Hofman, A., and Witteman, J.C. (2000). Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study. Ann Intern Med *132*, 270-278.

Haraguchi, K., Shimura, H., Kawaguchi, A., Ikeda, M., Endo, T., and Onaya, T. (1999). Effects of thyrotropin on the proliferation and differentiation of cultured rat preadipocytes. Thyroid *9*, 613-619.

Harkins, J.M., Moustaid-Moussa, N., Chung, Y.J., Penner, K.M., Pestka, J.J., North, C.M., and Claycombe, K.J. (2004). Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. J Nutr *134*, 2673-2677.

Imaizumi, M., Akahoshi, M., Ichimaru, S., Nakashima, E., Hida, A., Soda, M., Usa, T., Ashizawa, K., Yokoyama, N., Maeda, R., *et al.* (2004). Risk for ischemic heart disease and all-cause mortality in subclinical hypothyroidism. J Clin Endocrinol Metab *89*, 3365-3370.

Janson, A., Rawet, H., Perbeck, L., and Marcus, C. (1998). Presence of thyrotropin receptor in infant adipocytes. Pediatr Res *43*, 555-558.

Kershaw, E.E., and Flier, J.S. (2004). Adipose tissue as an endocrine organ. J Clin Endocrinol Metab *89*, 2548-2556.

Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J.E., and Roger, P.P. (2001). Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models. Endocr Rev *22*, 631-656.

Kopperud, R., Krakstad, C., Selheim, F., and Doskeland, S.O. (2003). cAMP effector mechanisms. Novel twists for an 'old' signaling system. FEBS Lett *546*, 121-126.

Kvetny, J., Heldgaard, P.E., Bladbjerg, E.M., and Gram, J. (2004). Subclinical hypothyroidism is associated with a low-grade inflammation, increased triglyceride levels and predicts cardiovascular disease in males below 50 years. Clin Endocrinol (Oxf) *61*, 232-238.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Libby, P. (2002). Inflammation in atherosclerosis. Nature *420*, 868-874.

Menendez, C., Baldelli, R., Camina, J.P., Escudero, B., Peino, R., Dieguez, C., and Casanueva, F.F. (2003). TSH stimulates leptin secretion by a direct effect on adipocytes. J Endocrinol *176*, 7-12.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., and Coppack, S.W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab *82*, 4196-4200.

Path, G., Bornstein, S.R., Gurniak, M., Chrousos, G.P., Scherbaum, W.A., and Hauner, H. (2001). Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. J Clin Endocrinol Metab *86*, 2281-2288.

Prabhakar, B.S., Bahn, R.S., and Smith, T.J. (2003). Current perspective on the pathogenesis of Graves' disease and ophthalmopathy. Endocr Rev *24*, 802-835.

Rangarajan, S., Enserink, J.M., Kuiperij, H.B., de Rooij, J., Price, L.S., Schwede, F., and Bos, J.L. (2003). Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. J Cell Biol *160*, 487-493.

Rodbell, M. (1964). Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis. J Biol Chem *239*, 375-380.

Ross, S.E., Hemati, N., Longo, K.A., Bennett, C.N., Lucas, P.C., Erickson, R.L., and MacDougald, O.A. (2000). Inhibition of adipogenesis by Wnt signaling. Science *289*, 950-953.

Serazin-Leroy, V., Morot, M., de Mazancourt, P., and Giudicelli, Y. (2001). Differences in type II, IV, V and VI adenylyl cyclase isoform expression between rat preadipocytes and adipocytes. Biochim Biophys Acta *1550*, 37-51.

Shintani, M., Nishimura, H., Akamizu, T., Yonemitsu, S., Masuzaki, H., Ogawa, Y., Hosoda, K., Inoue, G., Yoshimasa, Y., and Nakao, K. (1999). Thyrotropin decreases leptin production in rat adipocytes. Metabolism *48*, 1570-1574.

Sorisky, A., Bell, A., and Gagnon, A. (2000). TSH receptor in adipose cells. Horm Metab Res *32*, 468-474.

Stephens, J.M., Butts, M.D., and Pekala, P.H. (1992). Regulation of transcription factor mRNA accumulation during 3T3-L1 preadipocyte differentiation by tumour necrosis factor-alpha. J Mol Endocrinol *9*, 61-72.

Tuzcu, A., Bahceci, M., Gokalp, D., Tuzun, Y., and Gunes, K. (2005). Subclinical hypothyroidism may be associated with elevated high-sensitive c-reactive protein (low grade inflammation) and fasting hyperinsulinemia. Endocr J *52*, 89-94.

Vandeput, F., Perpete, S., Coulonval, K., Lamy, F., and Dumont, J.E. (2003). Role of the different mitogen-activated protein kinase subfamilies in the stimulation of dog and human thyroid epithelial cell proliferation by cyclic adenosine 5'-monophosphate and growth factors. Endocrinology *144*, 1341-1349.

Vanvooren, V., Allgeier, A., Nguyen, M., Massart, C., Parma, J., Dumont, J.E., and Van Sande, J. (2001). Mutation analysis of the Epac--Rap1 signaling pathway in cold thyroid follicular adenomas. Eur J Endocrinol *144*, 605-610.

3.0 MANUSCRIPT #2

Interleukin-6 release from human abdominal adipose cells is regulated by thyroid-stimulating hormone: effect of adipocyte differentiation and anatomic depot

STATEMENT OF AUTHOR CONTRIBUTIONS

Tayze T. Antunes was the primary author and performed all the experimental work on human primary adipose cells. She also helped with the experimental approach, data analysis, and manuscript preparation. **Dr. AnneMarie Gagnon** is a Research Associate in Dr. Alexander Sorisky's laboratory. She supervised the experimental work, contributed to the experimental design, data interpretation, statistical analysis, and helped in the manuscript preparation. Ms. **Beiling Chen** is a Research Assistant in Dr. Terry Smith's laboratory at the Department of Medicine, Harbor-University of California Los Angeles Medical Center and the David Geffen School of Medicine at the University of California Los Angeles, Torrance, California. She performed serum TSH and IL-6 testing from thyroidectomized patients (Figure 3.3). **Dr. Furio Pacini** is a research collaborator of Dr. Alexander Sorisky who was in the Department of Endocrinology and Metabolism at the University of Pisa, Pisa, Italy, at the time of the study. He coordinated the recruitment of the thyroidectomized patients for the study of the rhTSH administration and blood sample collection. **Dr. Terry J Smith** is a research collaborator of Dr. Alexander Sorisky in the Department of Medicine, Harbor-University of California Los Angeles Medical Center and the David Geffen School of Medicine at the University of California Los Angeles, Torrance, California. He is the supervisor of Beiling Chen, and obtained the samples from Dr. Furio Pacini to perform the

experimental analysis on TSH and IL-6. He also participated in the data analysis of the rhTSH study. **Dr. Alexander Sorisky** is the Chronic Disease Program Director and a Senior Scientist at the Ottawa Hospital Research Institute. He is the supervisor of Tayze T. Antunes. He contributed to the experimental design, data interpretation, statistical analysis and preparation of the manuscript.

SUMMARY

The following manuscript entitled "Interleukin-6 release from human abdominal adipose cells is regulated by thyroid-stimulating hormone: effect of adipocyte differentiation and anatomic depot" was published in the American Journal of Physiology - Endocrinology and Metabolism [(2006) *290*, E1140-E1144]. In the previous chapter, manuscript #1, the effect of TSH on IL-6 release was examined in the 3T3-L1 cell line. As mentioned in the General Introduction, although the 3T3-L1 cell line represents a faithful cell model to study adipocyte biology, there are limitations with this model. Studies using human primary adipose cells are helpful to further strengthen the relevance of the findings, and also allow for the comparison of differences that may exist between anatomic regional adipose tissue sites.

Manuscript #2 thus attempts to analyze basal and TSH-induced IL-6 release according to stages of differentiation (preadipocytes versus their differentiated adipocyte counterparts), and according to the anatomical location (abdominal subcutaneous versus omental) of human primary cells. Adipose tissue samples of abdominal subcutaneous and omental adipose tissue were obtained from patients undergoing elective abdominal surgery at The Ottawa Hospital (approved by the Research Ethics Board). This study was designed

to obtain paired adipose tissue samples from each patient in order to reduce inter-patient variability.

To determine if TSH increases IL-6 circulating levels in extrathyroidal targets *in vivo*, IL-6 serum levels were measured in thyroidectomized patients prior to and following rhTSH injections.

From this study, it was proposed that differences exist between basal and TSH-stimulated IL-6 release according to the stage of differentiation and the adipose tissue depot location. Furthermore, the *in vivo* study indicates that TSH can target extrathyroidal tissues to modulate IL-6 serum levels.

Interleukin-6 release from human abdominal adipose cells is regulated by thyroid-stimulating hormone: effect of adipocyte differentiation and anatomic depot

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Running head: TSH stimulates IL-6 release from adipose cells

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ABSTRACT

Adipose cells are extrathyroidal targets of thyroid-stimulating hormone (TSH). TSH stimulates interleukin-6 (IL-6) release from adipocytes. We examined TSH responsiveness as a function of stage of differentiation or adipose tissue depot in cultured adipose cells and determined the effect of TSH on extrathyroidal IL-6 production in vivo. Stromal preadipocytes, isolated from human abdominal subcutaneous or omental adipose tissue, and their differentiated counterparts were studied. IL-6 protein concentration in the medium was measured after TSH stimulation. Basal IL-6 release was greater for preadipocytes than differentiated adipocytes, whether derived from subcutaneous or omental fat depots. A depot-dependent effect (omental > subcutaneous) on basal IL-6 release was observed for preadipocytes (1.6-fold, $P < 0.05$); a similar trend for differentiated adipocytes was not significant (6.2-fold, $P > 0.05$). IL-6 responsiveness to TSH was observed upon differentiation, but only for subcutaneous adipocytes (1.9-fold over basal, $P < 0.001$). To determine if TSH could stimulate IL-6 release from extrathyroidal tissues in vivo, we measured serum IL-6 levels from five thyroidectomized patients who received recombinant human (rh) TSH and found that levels increased by threefold on *days 3* and *4* ($P < 0.05$) after its administration. Our data demonstrate that stage of differentiation and fat depot origin affect basal and TSH-stimulated IL-6 release from adipose cells in culture. Furthermore, rhTSH elevates serum IL-6 response in thyroidectomized patients, indicating an extrathyroidal site of TSH action.

Preadipocyte; adipose tissue; thyroid; adipokine; inflammation

INTRODUCTION

It is now recognized that thyroid-stimulating hormone (TSH) does not exclusively target the thyroid gland but also acts on a variety of other cells, including preadipocytes and adipocytes (Davies et al., 2002; Davies et al., 2005). Adipose tissue, in addition to its crucial role in lipogenesis and lipolysis, also influences metabolic and vascular health through its secretion of cytokines (adipokines; see Ref. (Kershaw and Flier, 2004)). TSH-mediated preadipocyte and/or adipocyte responses include proliferation, differentiation, survival, lipolysis, and leptin secretion (Bell et al., 2002; Haraguchi et al., 1999; Janson et al., 1998; Menendez et al., 2003; Shintani et al., 1999).

Interleukin-6 (IL-6) is a proinflammatory adipokine that is associated with an elevated risk of cardiovascular disease (CVD) in longitudinal studies (Bennet et al., 2003; Pradhan et al., 2002; Ridker et al., 2000; Volpato et al., 2001). It may act directly at the vessel well or indirectly by stimulating hepatic production of C-reactive protein (CRP), a novel CVD risk marker (Libby, 2002; Ridker, 2003). Because adipose tissue makes a significant contribution to the level of IL-6 in the circulation (Mohamed-Ali et al., 1997), it is important to identify the factors that influence IL-6 secretion from this tissue. We have demonstrated that TSH stimulates IL-6 release from 3T3-L1, 3T3-F442A, and human differentiated adipocytes in culture (Bell et al., 2003).

Elevated TSH secretion in subclinical hypothyroidism (mild thyroid gland failure) serves to maintain normal thyroid hormone levels, and, interestingly, this thyroid disorder has been associated with CVD (Crapo, 2005; Franklyn et al., 2005; Hak et al., 2000; Imaizumi et al., 2004; Kvetny et al., 2004; Rodondi et al., 2005; Walsh et al., 2005). Traditional CVD risk factors do not clearly account for the association, but recently CRP

was found to be higher in patients with subclinical hypothyroidism (Christ-Crain et al., 2003; Tuzcu et al., 2005). This clinical finding is consistent with our cellular studies linking TSH to IL-6 production in adipocytes, given that IL-6 is a major regulator of CRP production (Libby, 2002).

To broaden our knowledge of TSH action on IL-6 release from human adipose cells, we have investigated the effects of stage of adipocyte differentiation and anatomical site of adipose tissue depot on this process. Stromal preadipocytes and differentiated adipocytes derived from paired samples of abdominal subcutaneous and omental fat depots were studied. In addition, serum levels of IL-6 were measured in thyroidectomized patients treated with rhTSH as a measure of extrathyroidal action of TSH in vivo.

MATERIALS AND METHODS

Isolation, culture, and differentiation of human stromal preadipocytes

Paired samples of abdominal subcutaneous and omental adipose tissue (~1-2g wet weight) were obtained from five patients (4 female, 1 male) undergoing elective abdominal surgery (3 total abdominal hysterectomies, 1 bowel resection, and 1 gastrojejunostomy) with the approval of the Ottawa Hospital Research Ethics Committee (no. 1995023-01H). Their age was 51.4 (SD 7.3) yr, and body mass index was 26.6 (SD 3.7) Kg/m^2. The hospital record noted they were not acutely ill, were weight stable, and did not have diabetes, renal, or infectious disease. Two had well-controlled hypertension, one of whom was also known to have stable coronary artery disease, and were treated with candesartan, hydrochlorothiazide, losartan, verapamil, and simvastatin. Of the four women, two were postmenopausal, one of whom was treated with estrogen (transdermal) and

medroxyprogesterone. Stromal preadipocytes were isolated from each depot, and processed in parallel, as previously described (Artemenko et al., 2005). Blood vessels and fibrous tissue were carefully removed from the adipose tissue, followed by collagenase (CLS type I; 200U/g of tissue) digestion. After centrifugation, size filtration and treatment with erythrocyte lysis buffer, equal numbers of stromal preadipocytes from each depot for each patient were subsequently seeded (the number varied between patients, averaging 7 X 10^3 cells/cm^2) and grown in DMEM supplemented with 20% Fetal Bovine Serum (FBS) and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin) for a maximum of three passages (Adams et al., 1997; Hutley et al., 2003). Stromal preadipocytes were also sometimes cryopreserved before passaging and, once thawed, were grown in DMEM supplemented with 10% FBS and antibiotics. This did not alter their ability to differentiate into adipocytes (Ort et al., 2005).

For differentiation, the passaged preadipocytes from both depots were plated at a density of 3 X 10^4 cells/cm^2 in a 24-well plate in DMEM supplemented with 10% FBS and antibiotics. The following day, cells were either induced to differentiate in DMEM supplemented with 10% FBS, 0.85 µmol/l insulin, 100 µmol/l indomethacin, 0.5 µmol/l dexamethasone, 0.25 mmol/l isobutyl methylxanthine, and antibiotics or maintained in control medium (DMEM with 10% FBS and antibiotics). After the 14-day differentiation period, both stromal preadipocytes and differentiated adipocytes from each of the two depots were placed in DMEM supplemented with 1% calf serum and treated with 1 µmol/l TSH (Sigma) or vehicle (water) for 4 h. At the end of each experiment, medium was collected and centrifuged (500 *g* for 5 min at 4°C) to eliminate cell debris. The supernatant was either

kept at 4°C and analyzed in the following day or frozen at -80°C until assayed for IL-6 protein.

Measurement of IL-6 protein in conditioned medium

IL-6 protein was measured by enzyme immunometric assay (Assay Designs, Ann Arbor, MI), according to the manufacturer's instructions. After the collection of the medium, the adherent cells were lysed in Laemmli buffer (without bromphenol blue dye; see Ref. (Laemmli, 1970), and protein concentration was determined by modified Lowry method (Bio-Rad; Mississauga, ON, Canada), using Bovine Serum Albumin (BSA) as a standard.

Measurement of IL-6 levels in human serum

The study subjects (approved by the Institutional Review Board of University of Pisa) included five men aged 43 (SD 2) yr who had undergone thyroidectomy and radioablative iodine therapy for thyroid cancer. They have been described previously (Lado-Abeal et al., 2003). None had evidence of metastatic disease and were otherwise healthy. As part of ongoing routine follow-up care, and without discontinuation of thyroid hormone therapy, they received two separate doses of rhTSH (0.9 mg im) administered 24 h apart on *days 0* and *1*. Serum samples were obtained on *days 0* (before the 1st dose), *1, 2, 3* and *4* and were then frozen. IL-6 and TSH were measured in thawed samples by an ELISA kit (Alpco Diagnostics, Windham, NH) and by chemiluminescence (Elecsys 2010; Roche Diagnostics, Indianopolis, IN), respectively.

Statistical analysis

Data were analyzed by ANOVA and the Newman-Keul's test for multiple comparisons ($P < 0.05$ considered significant).

RESULTS

Basal IL-6 release was measured in medium obtained after 4 h incubation with the cell cultures. To examine the effect of stage of differentiation and adipose tissue depot, we compared basal IL-6 secretion from stromal preadipocytes and differentiated adipocytes derived from either the abdominal subcutaneous or omental adipose tissue depots. As shown in Figure 3.1, mean basal IL-6 release was 30-fold ($P < 0.01$) and 8-fold ($P < 0.001$) higher from stromal preadipocytes vs. differentiated adipocytes from the subcutaneous and omental depots, respectively. In the case of stromal preadipocytes, those from the omental depot released 1.6-fold more IL-6 ($P < 0.05$). Omental differentiated adipocytes also released more IL-6 than subcutaneous differentiated adipocytes, but this difference did not reach significance.

The response to treatment with 1 μmol/l TSH was assessed by comparing each condition with its respective control, given the very different basal IL-6 values (Figure 3.1). TSH increased IL-6 release by differentiated subcutaneous adipocytes by 90% over basal (Figure 3.2). This approximately twofold stimulation is consistent with our previous study on subcutaneous differentiated adipocytes (Bell et al., 2003). The responses of the preadipocytes from either depot, as well as differentiated omental adipocytes, were ~ 35-40% and did not reach significance. The omental preadipocytes did not become more TSH responsive upon differentiation, in contrast to the differentiation of subcutaneous preadipocytes.

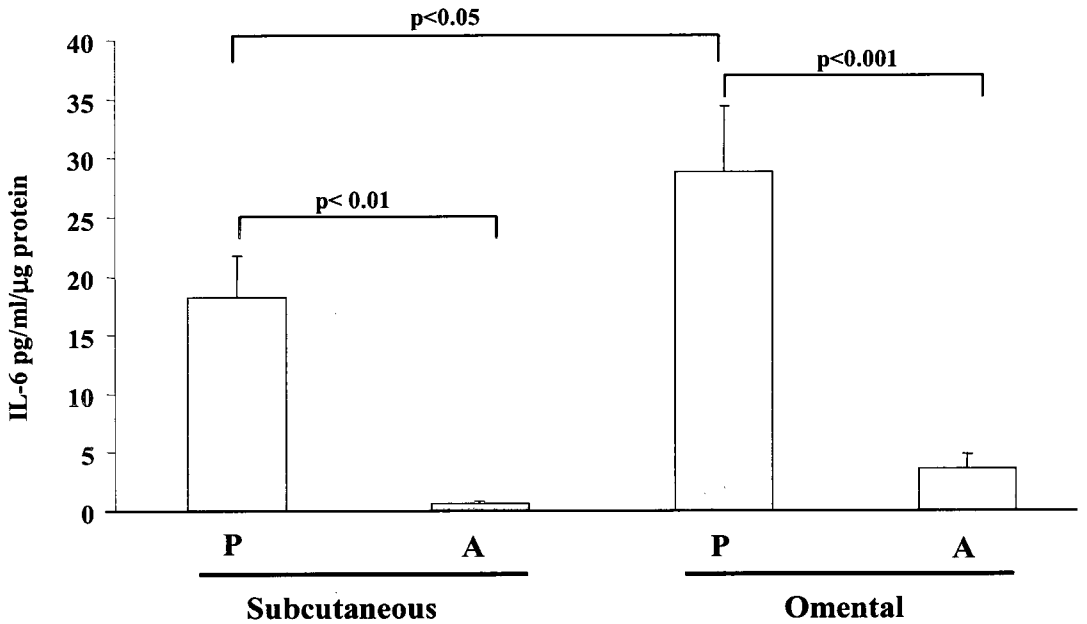

Figure 3.1: **Comparison of basal IL-6 release from human abdominal subcutaneous and omental preadipocytes and differentiated adipocytes.** Isolated preadipocytes from paired abdominal subcutaneous or omental adipose tissue from 5 patients were either kept in control medium (preadipocytes, P) or induced to differentiate (adipocytes, A) for 14 days. Basal IL-6 release over 4 h was measured as described. The effect of stage of differentiation and anatomic depot was compared by 2-way ANOVA. Results are expressed as means ± SE (n=5).

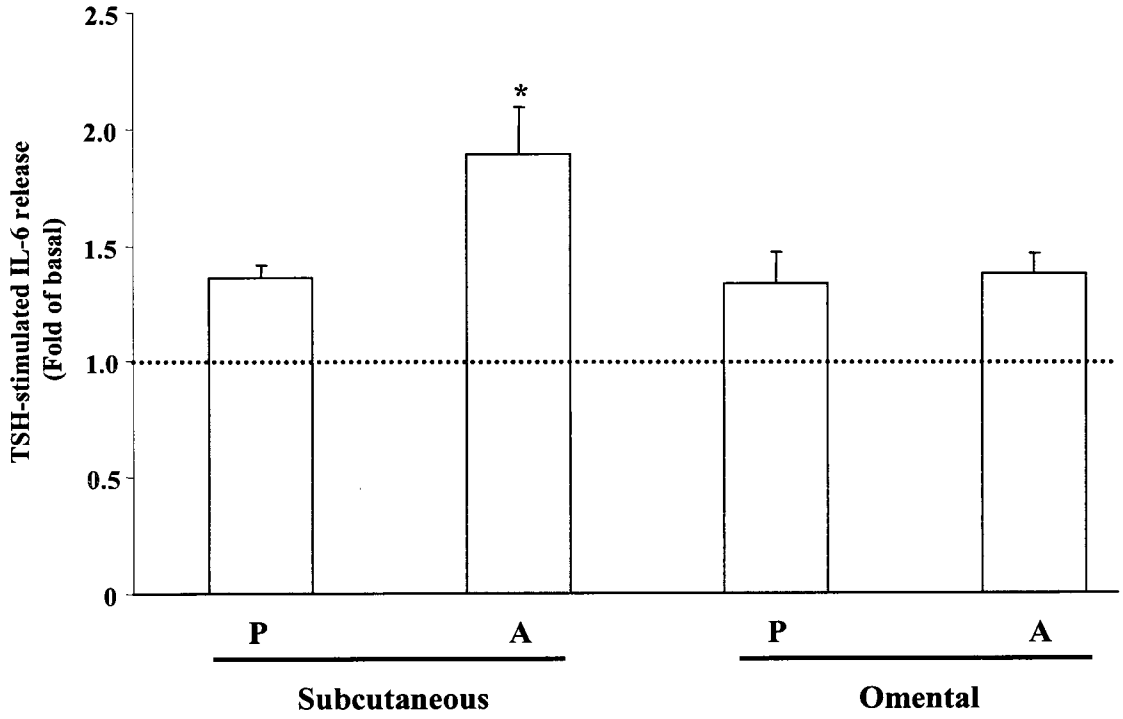

Figure 3.2: Effect of TSH on IL-6 release from abdominal subcutaneous and omental preadipocytes or differentiated adipocytes. Isolated preadipocytes from paired abdominal subcutaneous or omental adipose tissue from 5 patients were either kept in control medium (preadipocytes; P) or induced to differentiate (adipocytes; A) for 14 days. Cultures were stimulated for 4 h with 1 μmol/l TSH or vehicle. IL-6 released in the medium was measured as described. Results are expressed as fold of basal release (mean ± SE, n = 5). Two-way ANOVA was performed. *P <0.05 compared with subcutaneous and omental preadipocytes, and P <0.01 compared to omental adipocytes.

To address the question of whether TSH could act on extrathyroidal tissue targets in vivo and elicit an increase in serum levels of IL-6, we obtained serial blood samples for measurement of TSH and IL-6 from five male patients who had previously undergone surgical thyroidectomy followed by radioactive iodine ablation of any residual thyroid tissue. They received rhTSH injections in preparation for routine radioactive iodine body scans for thyroid cancer surveillance. rhTSH (0.9 mg) was administered on *days 0* and *1*; values for serum TSH and IL-6 levels on *days 0-4* are shown (Figure 3.3). The mean baseline TSH was 0.63 $\pm$ 0.05 (SE) mU/l (n = 5), and rose as expected in response to rhTSH administration. The mean baseline IL-6 value was 1.7 $\pm$ 0.9 (SE) pg/ml, with a range from undetectable to 4.6 pg/ml. IL-6 levels began to increase by *day 1*, and by *day 4* the mean value reached 4.7 $\pm$ 0.8 pg/ml. This approximately threefold response to IL-6 over the 4 days just failed to reach significance (P = 0.05).

Samples from one of the five patients, although also showing a clear response to TSH, started from a much higher baseline IL-6 concentration for unknown reasons. Reanalysis of the data without this patient results in a baseline IL-6 of 0.9 $\pm$ 0.7 pg/ml, reaching a value of 4.4 $\pm$ 0.9 pg/ml on *day 4*, an approximately fivefold rise ($P < 0.01$).

To reduce the variation between all five subjects, the data were normalized by setting the IL-6 value of the final time point, *day 4*, at 100% and comparing the earlier time points as a percentage of the respective *day 4* value for each patient. The rise in the serum IL-6 level was approximately threefold on *days 3* and *4* ($P < 0.05$, n = 5). Taken together, the data indicate that rhTSH has raised serum IL-6 in these thyroidectomized patients by acting on extrathyroidal tissue(s).

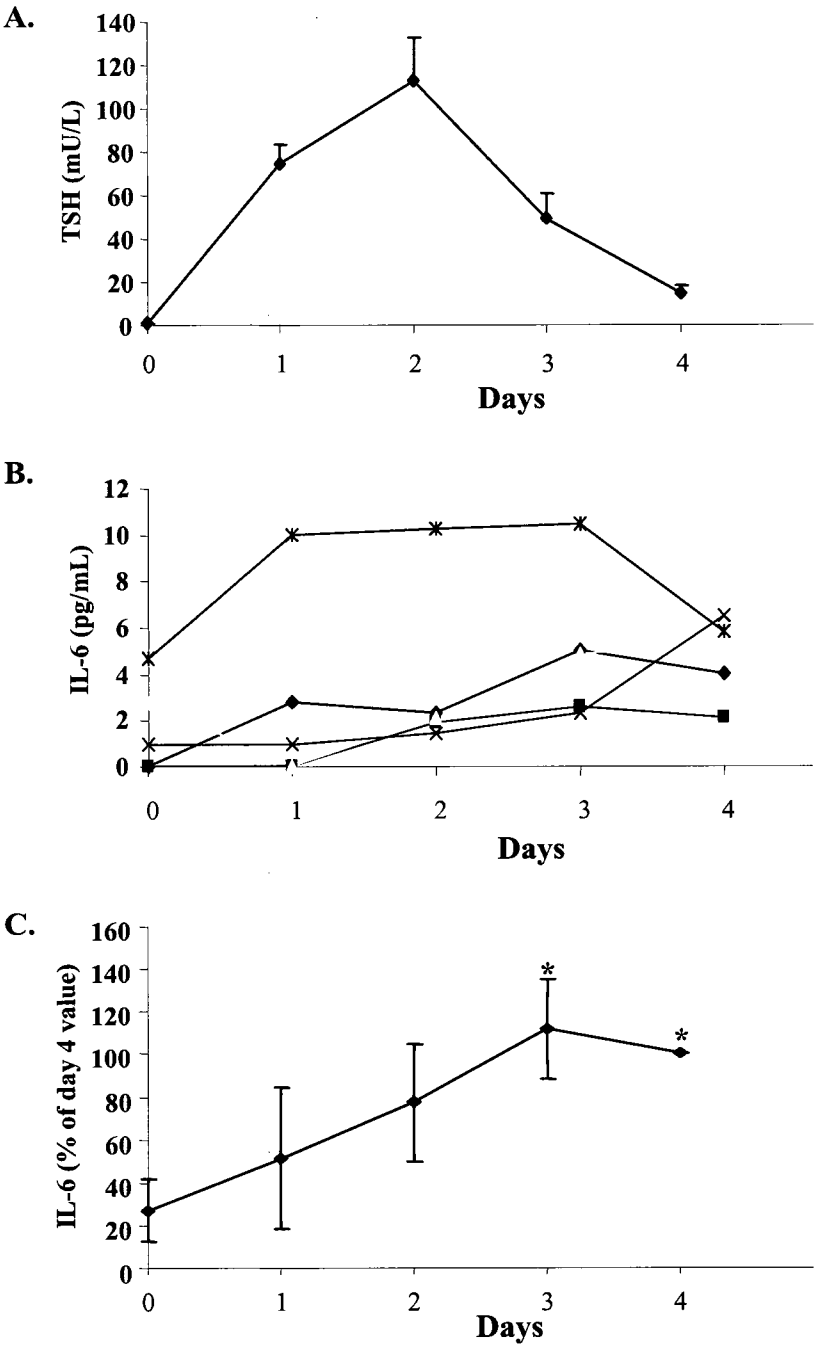

Figure 3.3: TSH increases serum IL-6 levels in thyroidectomized patients. Patients received 2 injections of rhTSH 24 h apart on *days 0* and *1*. Serum samples were collected daily (baseline = *day 0*) until *day 4*. A: serum TSH levels (mean ± SE). B: serum IL-6 levels for each patient. C: serum IL-6 levels expressed as a percentage of *day 4* responses. Repeated-measures ANOVA was performed. *P < 0.05 compared with baseline (n = 5).

DISCUSSION

We previously demonstrated that mouse (3T3-L1, 3T3-F442A) and human abdominal subcutaneous differentiated adipocytes secrete IL-6 in response to TSH (Bell et al., 2003). We now report on basal and TSH-stimulated IL-6 release in human adipose cells at different stages of differentiation (preadipocytes and differentiated adipocytes) and from different anatomical locations (subcutaneous abdominal and omental adipose tissue depots).

Our data indicating that basal IL-6 release is higher from stromal preadipocytes vs. differentiated adipocytes is consistent with several previous studies. The contribution of isolated adipocytes to IL-6 secretion was found to be only 10% compared with that of adipose tissue fragments (Fried et al., 1998). These authors therefore concluded that the stromal-vascular cellular fraction in intact adipose tissue must be an important IL-6 source. A subsequent study concurred, describing higher IL-6 secretion from stromal preadipocytes vs. isolated adipocytes from adipose tissue obtained from lean control or leptin-deficient obese *ob/ob* mice (Harkins et al., 2004). Differentiation of human subcutaneous preadipocytes into adipocytes was also recently reported to markedly reduce IL-6 mRNA expression, although there was a return to preadipocyte levels with prolonged culture time (Wang et al., 2005).

However, the literature on IL-6 production from adipose tissue cellular fractions is somewhat controversial. In a comparison of human isolated adipocytes with stromal cells, an equivalent amount of IL-6 secretion was observed; in addition, a vaguely defined undigested matrix component of adipose tissue was reported to be responsible for a large part of the IL-6 produced by adipose tissue (Fain et al., 2004). On the other hand, immunohistochemistry analysis of human breast adipose tissue did not detect any IL-6

reactivity in preadipocytes (Path et al., 2001). The reason for this is unclear, but technical limitations are a possibility, since earlier work demonstrated IL-6 mRNA expression in stromal cells from breast adipose tissue, making it unlikely that breast preadipocytes are unique compared with other preadipocytes (Crichton et al., 1996). Another study reported that IL-6 secretion increased during differentiation of human subcutaneous preadipocytes into adipocytes. However, the experimental design precluded a true assessment of IL-6 secretion from control preadipocytes, since their first measurement actually occurred after 6 days of differentiation treatment (Vicennati et al., 2002).

We found that stromal preadipocytes behaved in a depot-specific fashion, with omental cells releasing more IL-6 under basal conditions than subcutaneous cells. A parallel trend favoring the omental depot for the differentiated adipocytes was not significant. These responses correspond to those of isolated adipocytes and adipose tissue fragments, which also show this depot-related characteristic of IL-6 secretion (Fried et al., 1998). Another study also detected the same depot-specific effect of IL-6 secretion from adipose tissue samples; for isolated adipocytes, the trend was in the same direction, but not significant, perhaps because of differences in collection times (Fain et al., 2004). Furthermore, we provide novel data that stromal preadipocytes also exhibit this same depot-dependent response. The molecular basis for this depot-dependent difference in IL-6 production is not known. The previous two studies cited above used freshly isolated adipocytes, leaving open the possibility that in vivo influences before tissue removal might still have been operating during the relatively brief culture period for those cells. Because our data were derived from cells that were maintained for 2 wk in control or differentiation medium beyond the time for

the initial passages, it would seem that the depot-related difference of IL-6 release is based on inherent attributes of the adipose cells themselves.

Adipogenesis resulted in the acquisition of IL-6 responsiveness to TSH for subcutaneous, but not omental, differentiated adipocytes. In previous work, we did not detect any consistent changes in TSH receptor (TSHR) protein expression upon human adipocyte differentiation or between depots that would account for this result; larger studies to more rigorously examine TSHR expression are warranted (Bell et al., 2000). However, we have observed that, in the 3T3-L1 preadipocyte cell line model, adipogenesis is associated with the ability of TSH to elevate intracellular cAMP levels (Bell et al., 2002). Future work will need to address whether downstream differences in TSH signaling molecules may be operative between depots and/or during human adipogenesis as well. It should be noted that, although the release of IL-6 by abdominal subcutaneous differentiated adipocytes is TSH responsive, the actual amount released under these culture conditions is smaller than that released basally by stromal preadipocytes. Whether this will be the case for other adipokines implicated in metabolic dysfunction, such as tumor necrosis factor-α, is an open question. Finally, it should be noted that the number of patients in our study was small and somewhat heterogeneous (e.g., age, sex, medical history). With a larger and more homogeneous sample, more subtle regulatory aspects of IL-6 release may be revealed in the future.

To investigate the possibility of nonthyroidal action of TSH in vivo, we used the paradigm of rhTSH administration to patients who had been previously treated for thyroid cancer with surgical thyroidectomy and ablative doses of radioactive iodine. The elevation of serum IL-6 concentration in response to rhTSH injection complements and strengthens

our experiments on culture adipose cells reported here and previously (Bell et al., 2003). The design of the study, conducted in vivo, does not allow precise identification of adipose tissue as the site of nonthyroidal IL-6 production. To answer that question, adipose tissue biopsy and measurement of IL-6 expression before and after exposure to elevated TSH levels will be required.

Others have recently used rhTSH administration in thyroidectomized patients to explore extrathyroidal action of TSH. rhTSH treatment reduces serum vascular endothelial growth factor levels in such patients (Sorvillo et al., 2003). In another study, short-term elevation of serum TSH levels did not elevate serum testosterone (Lado-Abeal et al., 2003). These studies, together with our findings, suggest that extrathyroidal action of TSH and its regulation of pro-atherogenic adipokines will require careful investigation, since substantial tissue selectivity of TSH responsiveness appears to exist.

In summary, we show here for the first time that differentiated adipocytes derived from the abdominal subcutaneous, but not the omental, depot are TSH responsive in terms of IL-6 release. We further report that IL-6 release in the circulation occurs in thyroidectomized patients receiving rhTSH, indicating an extrathyroidal tissue(s) response to TSH.

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REFERENCES

Adams, M., Montague, C.T., Prins, J.B., Holder, J.C., Smith, S.A., Sanders, L., Digby, J.E., Sewter, C.P., Lazar, M.A., Chatterjee, V.K., *et al.* (1997). Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. J Clin Invest *100*, 3149-3153.

Artemenko, Y., Gagnon, A., Aubin, D., and Sorisky, A. (2005). Anti-adipogenic effect of PDGF is reversed by PKC inhibition. J Cell Physiol *204*, 646-653.

Bell, A., Gagnon, A., Dods, P., Papineau, D., Tiberi, M., and Sorisky, A. (2002). TSH signaling and cell survival in 3T3-L1 preadipocytes. Am J Physiol Cell Physiol *283*, C1056-1064.

Bell, A., Gagnon, A., Grunder, L., Parikh, S.J., Smith, T.J., and Sorisky, A. (2000). Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. Am J Physiol Cell Physiol *279*, C335-340.

Bell, A., Gagnon, A., and Sorisky, A. (2003). TSH stimulates IL-6 secretion from adipocytes in culture. Arterioscler Thromb Vasc Biol *23*, e65-66.

Bennet, A.M., Prince, J.A., Fei, G.Z., Lyrenas, L., Huang, Y., Wiman, B., Frostegard, J., and Faire, U. (2003). Interleukin-6 serum levels and genotypes influence the risk for myocardial infarction. Atherosclerosis *171*, 359-367.

Christ-Crain, M., Meier, C., Guglielmetti, M., Huber, P.R., Riesen, W., Staub, J.J., and Muller, B. (2003). Elevated C-reactive protein and homocysteine values: cardiovascular risk factors in hypothyroidism? A cross-sectional and a double-blind, placebo-controlled trial. Atherosclerosis *166*, 379-386.

Crapo, L.M. (2005). Subclinical hypothyroidism and cardiovascular disease. Arch Intern Med *165*, 2451-2452.

Crichton, M.B., Nichols, J.E., Zhao, Y., Bulun, S.E., and Simpson, E.R. (1996). Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells. Mol Cell Endocrinol *118*, 215-220.

Davies, T., Marians, R., and Latif, R. (2002). The TSH receptor reveals itself. J Clin Invest *110*, 161-164.

Davies, T.F., Ando, T., Lin, R.Y., Tomer, Y., and Latif, R. (2005). Thyrotropin receptor-associated diseases: from adenomata to Graves disease. J Clin Invest *115*, 1972-1983.

Fain, J.N., Madan, A.K., Hiler, M.L., Cheema, P., and Bahouth, S.W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology *145*, 2273-2282.

Franklyn, J.A., Sheppard, M.C., and Maisonneuve, P. (2005). Thyroid function and mortality in patients treated for hyperthyroidism. JAMA *294*, 71-80.

Fried, S.K., Bunkin, D.A., and Greenberg, A.S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab *83*, 847-850.

Hak, A.E., Pols, H.A., Visser, T.J., Drexhage, H.A., Hofman, A., and Witteman, J.C. (2000). Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study. Ann Intern Med *132*, 270-278.

Haraguchi, K., Shimura, H., Kawaguchi, A., Ikeda, M., Endo, T., and Onaya, T. (1999). Effects of thyrotropin on the proliferation and differentiation of cultured rat preadipocytes. Thyroid *9*, 613-619.

Harkins, J.M., Moustaid-Moussa, N., Chung, Y.J., Penner, K.M., Pestka, J.J., North, C.M., and Claycombe, K.J. (2004). Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. J Nutr *134*, 2673-2677.

Hutley, L.J., Newell, F.M., Joyner, J.M., Suchting, S.J., Herington, A.C., Cameron, D.P., and Prins, J.B. (2003). Effects of rosiglitazone and linoleic acid on human preadipocyte differentiation. Eur J Clin Invest *33*, 574-581.

Imaizumi, M., Akahoshi, M., Ichimaru, S., Nakashima, E., Hida, A., Soda, M., Usa, T., Ashizawa, K., Yokoyama, N., Maeda, R., *et al.* (2004). Risk for ischemic heart disease and all-cause mortality in subclinical hypothyroidism. J Clin Endocrinol Metab *89*, 3365-3370.

Janson, A., Rawet, H., Perbeck, L., and Marcus, C. (1998). Presence of thyrotropin receptor in infant adipocytes. Pediatr Res *43*, 555-558.

Kershaw, E.E., and Flier, J.S. (2004). Adipose tissue as an endocrine organ. J Clin Endocrinol Metab *89*, 2548-2556.

Kvetny, J., Heldgaard, P.E., Bladbjerg, E.M., and Gram, J. (2004). Subclinical hypothyroidism is associated with a low-grade inflammation, increased triglyceride levels and predicts cardiovascular disease in males below 50 years. Clin Endocrinol (Oxf) *61*, 232-238.

Lado-Abeal, J., Molinaro, E., DeValk, E., Pacini, F., and Refetoff, S. (2003). The effect of short-term treatment with recombinant human thyroid-stimulating hormones on Leydig cell function in men. Thyroid *13*, 649-652.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Libby, P. (2002). Inflammation in atherosclerosis. Nature *420*, 868-874.

Menendez, C., Baldelli, R., Camina, J.P., Escudero, B., Peino, R., Dieguez, C., and Casanueva, F.F. (2003). TSH stimulates leptin secretion by a direct effect on adipocytes. J Endocrinol *176*, 7-12.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., and Coppack, S.W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab *82*, 4196-4200.

Ort, T., Arjona, A.A., MacDougall, J.R., Nelson, P.J., Rothenberg, M.E., Wu, F., Eisen, A., and Halvorsen, Y.D. (2005). Recombinant human FIZZ3/resistin stimulates lipolysis in cultured human adipocytes, mouse adipose explants, and normal mice. Endocrinology *146*, 2200-2209.

Path, G., Bornstein, S.R., Gurniak, M., Chrousos, G.P., Scherbaum, W.A., and Hauner, H. (2001). Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. J Clin Endocrinol Metab *86*, 2281-2288.

Pradhan, A.D., Manson, J.E., Rossouw, J.E., Siscovick, D.S., Mouton, C.P., Rifai, N., Wallace, R.B., Jackson, R.D., Pettinger, M.B., and Ridker, P.M. (2002). Inflammatory biomarkers, hormone replacement therapy, and incident coronary heart disease: prospective analysis from the Women's Health Initiative observational study. JAMA *288*, 980-987.

Ridker, P.M. (2003). Clinical application of C-reactive protein for cardiovascular disease detection and prevention. Circulation *107*, 363-369.

Ridker, P.M., Rifai, N., Stampfer, M.J., and Hennekens, C.H. (2000). Plasma concentration of interleukin-6 and the risk of future myocardial infarction among apparently healthy men. Circulation *101*, 1767-1772.

Rodondi, N., Newman, A.B., Vittinghoff, E., de Rekeneire, N., Satterfield, S., Harris, T.B., and Bauer, D.C. (2005). Subclinical hypothyroidism and the risk of heart failure, other cardiovascular events, and death. Arch Intern Med *165*, 2460-2466.

Shintani, M., Nishimura, H., Akamizu, T., Yonemitsu, S., Masuzaki, H., Ogawa, Y., Hosoda, K., Inoue, G., Yoshimasa, Y., and Nakao, K. (1999). Thyrotropin decreases leptin production in rat adipocytes. Metabolism *48*, 1570-1574.

Sorvillo, F., Mazziotti, G., Carbone, A., Piscopo, M., Rotondi, M., Cioffi, M., Musto, P., Biondi, B., Iorio, S., Amato, G., *et al.* (2003). Recombinant human thyrotropin reduces serum vascular endothelial growth factor levels in patients monitored for thyroid carcinoma even in the absence of thyroid tissue. J Clin Endocrinol Metab *88*, 4818-4822.

Tuzcu, A., Bahceci, M., Gokalp, D., Tuzun, Y., and Gunes, K. (2005). Subclinical hypothyroidism may be associated with elevated high-sensitive c-reactive protein (low grade inflammation) and fasting hyperinsulinemia. Endocr J *52*, 89-94.

Vicennati, V., Vottero, A., Friedman, C., and Papanicolaou, D.A. (2002). Hormonal regulation of interleukin-6 production in human adipocytes. Int J Obes Relat Metab Disord *26*, 905-911.

Volpato, S., Guralnik, J.M., Ferrucci, L., Balfour, J., Chaves, P., Fried, L.P., and Harris, T.B. (2001). Cardiovascular disease, interleukin-6, and risk of mortality in older women: the women's health and aging study. Circulation *103*, 947-953.

Walsh, J.P., Bremner, A.P., Bulsara, M.K., O'Leary, P., Leedman, P.J., Feddema, P., and Michelangeli, V. (2005). Subclinical thyroid dysfunction as a risk factor for cardiovascular disease. Arch Intern Med *165*, 2467-2472.

Wang, B., Jenkins, J.R., and Trayhurn, P. (2005). Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. Am J Physiol Endocrinol Metab *288*, E731-740.

4.0 MANUSCRIPT #3

Thyroid Stimulating Hormone Induces Interleukin-6 Release from Human Adipocytes Through Activation of the NF-κB Pathway

STATEMENT OF AUTHOR CONTRIBUTIONS

Tayze T. Antunes was the primary author and performed most of the experimental work (~90%) for this manuscript. She also contributed to the experimental design, data analysis, and manuscript preparation. **Dr. AnneMarie Gagnon** is a Research Associate in Dr. Alexander Sorisky's laboratory. She supervised the experimental work and contributed to the experimental design, data analysis and to the manuscript preparation. **Melanie L. Langille** is currently a Master's Student under the supervision of Dr. Alexander Sorisky. She performed the experimental analysis of the role of PKA as a potential upstream kinase for the IKKβ/ NF-κB pathway (Figure 4.3D). **Dr. Alexander Sorisky** is the Chronic Disease Program Director and a Senior Scientist at the Ottawa Hospital Research Institute. He is the supervisor of Tayze T. Antunes and contributed to the experimental design, data analysis and preparation of manuscript.

SUMMARY

The following manuscript entitled "Thyroid Stimulating Hormone Induces Interleukin-6 Release from Human Adipocytes Through Activation of the NF-κB Pathway" was published in Endocrinology [(2008) *149*, 3062-3066]. In manuscript #2, human primary abdominal subcutaneous, but not omental, differentiated adipocytes released IL-6 in

response to TSH. Numerous studies, as mentioned in the General Introduction, have shown that the IKKβ/NF-κB is an important pathway that regulates IL-6 synthesis in many cell types, including adipocytes.

In manuscript #3, TSH-induced activation of the IKKβ/NF-κB pathway and its involvement in IL-6 release was investigated in human abdominal subcutaneous differentiated adipocytes. TSH-induced IKKβ activation, as assessed by its phosphorylation on Ser181 and IκBα degradation was measured by immunoblotting. NF-κB translocation to the nucleus upon TSH treatment was demonstrated using an ELISA-based assay that detects p65 NF-κB subunit binding to coated NF-κB oligonucleotide sequences (TransAM NFκB p65 Activation Assay; Active Motif). The requirement for IKKβ/NF-κB on TSH-stimulated IL-6 release was shown with the use of specific pharmacological inhibitors. The potential involvement of PKA on TSH-induced IKKβ-NF-κB activation was evaluated using H89. To show the specific requirement for the presence of TSHR for the ability of TSH to stimulate NF-κB signaling and IL-6 expression, Chinese hamster ovarian (CHO) control cells and CHO cells expressing human TSHR were used.

Based on this study, it was concluded that activation of the IKKβ/NF-κB signaling pathway by TSH was required to induce IL-6 release from human abdominal subcutaneous differentiated adipocytes.

Thyroid Stimulating Hormone Induces Interleukin-6 Release from Human Adipocytes Through Activation of the NF-κB Pathway

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Abbreviated title: TSH-stimulates NF-κB and IL-6 release

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Disclosure Statement: All of the authors have nothing to disclose.

ABSTRACT

Our objective was to identify the signaling pathway activated by thyroid stimulating hormone (TSH) that induces interleukin (IL)-6 secretion from human abdominal subcutaneous differentiated adipocytes. Human abdominal subcutaneous preadipocytes in culture were differentiated into adipocytes. IL-6 release stimulated by TSH was inhibited by 35% ($P<0.05$) with SN50, an inhibitor of nuclear factor κB (NF-κB) nuclear translocation, and by 60% ($P<0.01$) with sc-514, an inhibitor of inhibitory κB kinase (IKK) β. Phosphorylation of IKKβ increased upon TSH treatment (10.3-fold, $P<0.01$), and IκBα levels were reduced by 78% ($P<0.01$). TSH activated NF-κB (23-fold, $P<0.001$), a process that was inhibited (60%, $P<0.01$) by SN50. Inhibition of protein kinase A by H89 did not affect TSH-stimulated IKKβ phosphorylation or IκBα degradation. TSH-mediated NF-κB activation and IL-6 induction also specifically occurred in Chinese hamster ovarian cells expressing the human TSH receptor, resulting in a 5.9-fold ($P<0.001$) increase in IKKβ phosphorylation and a 9.5-fold increase in IL-6 mRNA expression. Our data demonstrate that the IKKβ/NF-κB pathway is a novel TSH target that is required for TSH-induced IL-6 release from human adipocytes.

Key words: TSH, adipocyte, IKKβ, NF-κB, IL-6.

INTRODUCTION

In addition to energy storage and release, adipose tissue releases a variety of cytokines (adipokines) with wide-ranging physiological effects, including appetite, energy expenditure, insulin sensitivity, and coagulation. Deregulation of adipokine production is associated with pro-inflammatory, pro-atherogenic state. Adipose tissue constitutes a significant source of circulating interleukin (IL)-6 (Mohamed-Ali et al., 1997). IL-6 has been identified as an independent risk factor for cardiovascular disease. A recent systematic review confirmed that serum IL-6 levels predict the development of cardiovascular disease (CVD) with an adjusted relative risk between 1.1-3.1 (Lobbes et al., 2006). Studies published subsequently continue to demonstrate IL-6 is a significant prognostic indicator of CVD (Fisman et al., 2006; Lee et al., 2006; Stork et al., 2006). However, our understanding of hormonal regulators of adipocyte production of IL-6 is incomplete.

In subclinical hypothyroidism, thyroid stimulating hormone (TSH) levels rise to compensate for mild thyroid gland failure, and thereby maintain thyroid hormone levels in the normal range. However, a higher risk for CVD appears to be independently associated with subclinical hypothyroidism. A recent meta-analysis concluded that subclinical hypothyroidism is associated with an elevated risk of CVD in observational studies (odds ratio 1.65, 95% confidence interval 1.28-2.12), with a similar trend noted in prospective studies (odds ratio 1.42, 95% confidence interval 0.91-2.21) (Rodondi et al., 2006). Furthermore, acute administration of TSH to biochemically euthyroid patients (healthy thyroid cancer patients on L-thyroxine treatment post thyroid gland excision and radioablation) caused endothelial dysfunction and increased serum levels of C-reactive protein, TNFα, several indices of oxidative stress, and notably, IL-6 (Antunes et al., 2006;

Dardano et al., 2006). This extra-thyroidal effect of TSH is consistent with our studies demonstrating the ability of TSH to stimulate IL-6 mRNA expression and release of IL-6 protein from 3T3-L1 and human abdominal subcutaneous differentiated adipocytes in culture (Antunes et al., 2005; Antunes et al., 2006).

IL-6 transcription is positively regulated by nuclear factor-kappa B (NF-κB) in a variety of cells, including adipocytes (Chung et al., 2006). NF-κB is retained in the cytoplasm in an inactive form bound to inhibitory κB (IκB) protein. Upon appropriate stimulation, IκB is phosphorylated by IκB kinase (IKK) β, resulting in IκB degradation and release of NF-κB. Free active NF-κB translocates to the nucleus, binds to sequence-specific DNA regions, and triggers a variety of transcriptional events and cell responses, including initiation of inflammation (Shoelson et al., 2006).

In this report, we have investigated the novel possibility that TSH activates the IKKβ/NF-κB pathway in human abdominal subcutaneous differentiated adipocytes, and whether this pathway is required for TSH-stimulated IL-6 release.

MATERIALS AND METHODS

Isolation, culture, and differentiation of human stromal preadipocytes

Human abdominal subcutaneous adipose tissue were obtained from 13 patients (10 female, 3 male), age 49 ± 7 years (mean ± SD), 28 ± 6 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 Committee (1995023-01H). The stromal preadipocytes were isolated as described (Antunes et al., 2006). Briefly, blood vessels and fibrous tissue were carefully removed, followed by collagenase digestion

(CLS type I; 600 U/g of tissue). Stromal preadipocytes were obtained following sequential centrifugation, size filtration, and treatment with erythrocytes lysis buffer. Preadipocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin). Upon reaching 80-90% confluence, cells were either 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 3×10^4 cells/cm^2 in DMEM supplemented with 10% FBS and antibiotics, and placed in differentiation medium the next day. Differentiation was induced with DMEM supplemented with 10% FBS, 0.85 µmol/l insulin, 100 µmol/l indomethacin, 0.5 µmol/l dexamethasone, 0.25 mmol/l isobutylmethylxanthine and antibiotics for 14 days. On day 14, differentiation medium was removed, cells were thawed 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 50%.

Chinese hamster ovarian (CHO) cell culture

CHO-JP02 (vector control) and CHO-JP2626 cells overexpressing the human TSH receptor (TSHR) were kindly provided by Dr. J. E. Dumont, Erasme University Hospital, Free University of Brussels, Brussels, Belgium (Van Sande et al., 2003). Cells were plated and grown in Ham's F12 medium supplemented with 10% FBS, antibiotics, and 400 µg/ml G418. Medium was changed every 2 days until cells were confluent.

Quantification of IL-6 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 μmol/l sc-514 (inhibitor of IKKβ) or vehicle (0.1% dimethyl sulfoxide), or with 50 μg/ml SN50 (inhibitor of NF-κB) or vehicle (H_2O) 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 protein was measured by enzyme immunometric assay (Assay Designs, Ann Arbor, MI), following the manufacturer's instruction. 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 released into the medium was normalized to total cell lysate protein.

Cell stimulation and western blot analysis

Human abdominal subcutaneous differentiated adipocytes or confluent CHO cells were placed in Krebs Ringer Hepes (KRH) buffer supplemented with 1% calf serum, and treated with 50 mU/ml TSH or vehicle for 30 minutes. Where indicated, cells were treated with 20 μmol/l H89 or vehicle (0.1% DMSO) for 1 hour in DMEM with 1% calf serum and antibiotics prior to addition of TSH. Following treatment, cells were lysed in Laemmli buffer (Laemmli, 1970) supplemented with 5 mmol/l EGTA, 5 mmol/l NaPPi and 50 mol/l NaF. Lysate protein concentration was determined as described above. Equal amounts of solubilised protein (10-80 μg, depending on the experiment) were resolved by SDS-PAGE,

followed by electrophoretic transfer to a nitrocellulose membrane. Non specific binding sites were blocked, and the membrane was probed with either anti-phospho IKKα ser180/IKKβ ser181 (1:500), anti-IκBα (1:500), anti-phospho CREB Ser 133 (1:1000; all from Cell Signaling Technology), or anti-IKKβ (clone 10AG2; 2 μg/ml; Upstate) followed by the appropriate horseradish peroxidase-conjugated antibody. Immunoreactivity was detected by enhanced chemiluminescence. Relative intensity of the bands was determined with AlphaEaseFC Software and data expressed as integrated optical density (IOD) units.

Preparation of nuclear extracts and measurement of NF-κB DNA binding

Human abdominal subcutaneous differentiated adipocytes were placed in DMEM supplemented with 1% calf serum and antibiotics. Cells were pretreated with 50 μg/ml SN50 or vehicle for 15 minutes, and treated with 50 mU/ml TSH or vehicle for 1 hour. Cells were washed twice with ice-cold PBS containing phosphatase inhibitors (125 mmol/l NaF, 250 mmol/l β-glycerophosphate, 250 mmol/l phenylphosphate and 25 mmol/l sodium orthovanadate), and nuclear extracts were prepared according to the TransAM NFκB p65 Activation Assay (Active motif). Briefly, cells were lysed in 1 ml hypotonic buffer pH 7.5 (20 mmol/l Hepes pH 7.5, 5 mmol/l NaF, 10 μmol/l Na_2MoO_4 and 0.1 mmol/l EDTA). Cells were scraped and allowed to swell for 15 minutes on ice. After the addition of 50 μl of 10% Nonidet P-40, the homogenates were centrifuged at 14,000 x g for 1 minute at 4°C. Supernatants were discarded, and nuclear pellets were resuspended in 50 μl of complete lysis buffer and incubated for 30 minutes at 4°C with continuous mixing. Samples were centrifuged at 14,000 x g for 10 minutes at 4°C, and the supernatants were collected. NF-κB

DNA binding was determined using the TransAM NFκB p65 Activation Assay (Active Motif) using 1 μg of the nuclear extracts.

Real Time PCR

Confluent CHO cells were placed in Ham's F12 medium supplemented with 1% calf serum, and treated with 50 mU/ml TSH or vehicle for 2 hours. Following stimulation, RNA was extracted with TRI Reagent, and treated with DNAse I, according to manufacturer's instruction (Ambion). Total RNA (1μg) was heat-denatured prior to addition of reverse transcriptase (RT). Reverse transcription was performed with Retroscript Kit (Ambion). Real time PCR was performed in 25 μl reaction volume consisting of 5 μl RT, 5 μl primers, 900 nmol/l IL-6 primers, and 10 μl SYBR Green PCR Master Mix (ABS). IL-6 specific primer pairs were forward: 5'TTGGGAAATTTGCCTACTGAA3', and reverse: 5'AGGCATGACTATTTTATCTGGA3'. 18S was used as an internal control. All samples were run in triplicate and RT-minus control assays were performed for both IL-6 and 18S. Amplification consisted of 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds (denaturation) followed by 1 minute at 60°C (annealing/extension) performed in an Applied Biosystem 7500-Real Time PCR System. mRNA expression data are expressed as relative quantification (RQ).

Statistical analysis

Statistical analysis was performed with Student's t test for paired values or ANOVA with Student-Newman-Keuls post-hoc test, as appropriate, for multiple means, using

GraphPad Instat version 3.05 (GraphPad Software Inc., San Diego, CA), with $p<0.05$ considered significant.

RESULTS

We have previously shown that human abdominal subcutaneous differentiated adipocytes in culture release IL-6 in response to TSH (Antunes et al., 2006). We have now performed dose-response studies to characterize the TSH-dependent effect. Human abdominal subcutaneous differentiated adipocytes were exposed to increasing concentrations of TSH (from 0 to 50 mU/ml) and IL-6 release into the medium was quantified. We observed a strong increase in IL-6 secretion (9.4 $\pm$ 1.6-fold, n=2) with 5 mU/ml TSH (Figure 4.1). The dose-response observed here correlates with our previous reported data on 3T3-L1 adipocytes (Antunes et al., 2005) and it also approximates the doses employed for thyroid cell studies (Kimura et al., 2001).

We investigated whether activation of the IKKβ/NF-κB pathway was required for TSH-stimulated IL-6 release. Human abdominal subcutaneous differentiated adipocytes were pretreated with 100 μmol/l sc-514, a selective inhibitor of IKKβ, followed by TSH treatment (Figure 4.2A). Sc-514 resulted in a 60% inhibition of TSH-induced IL-6 release (132.2 $\pm$ 9 pg/ml/μg protein) versus 55 $\pm$ 14.7 pg/ml/μg protein, $P<0.01$; n=3). To further implicate the NF-κB pathway in TSH-induced IL-6 release, cells were pretreated with 50 μg/ml SN50, a peptide that blocks NF-κB nuclear translocation, followed by TSH treatment. SN50 decreased TSH-induced IL-6 release by 35% (222 $\pm$ 54 pg/ml/μg protein versus 144 $\pm$ 31 pg/ml/μg protein when SN50 was present, $P<0.05$; n=4) (Figure 4.2B). Thus, both inhibitor studies indicate that NF-κB is a signaling target that is necessary for TSH to fully induce IL-6 release.

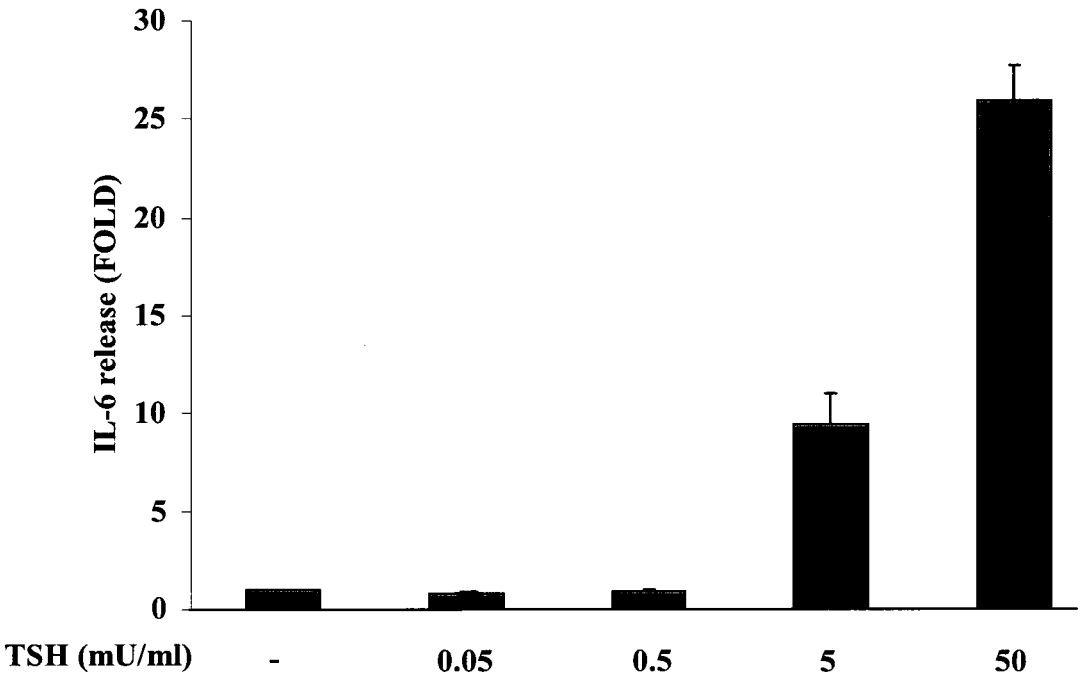


Figure 4.1: Dose-response of TSH on IL-6 release: Human abdominal subcutaneous differentiated adipocytes were stimulated for 4 hours with 0 to 50 mU/ml TSH. IL-6 protein in the medium was measured as described. Data are expressed as fold-change of control and represent means ± range of 2 separate patient samples, each performed in duplicate.

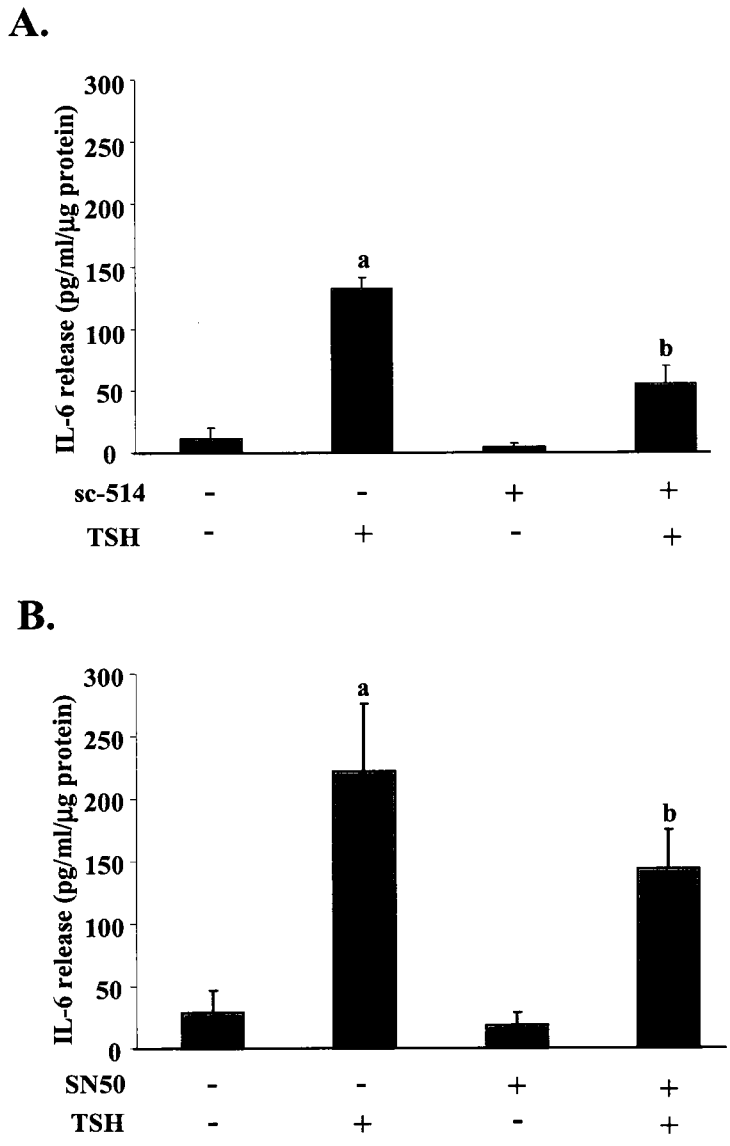


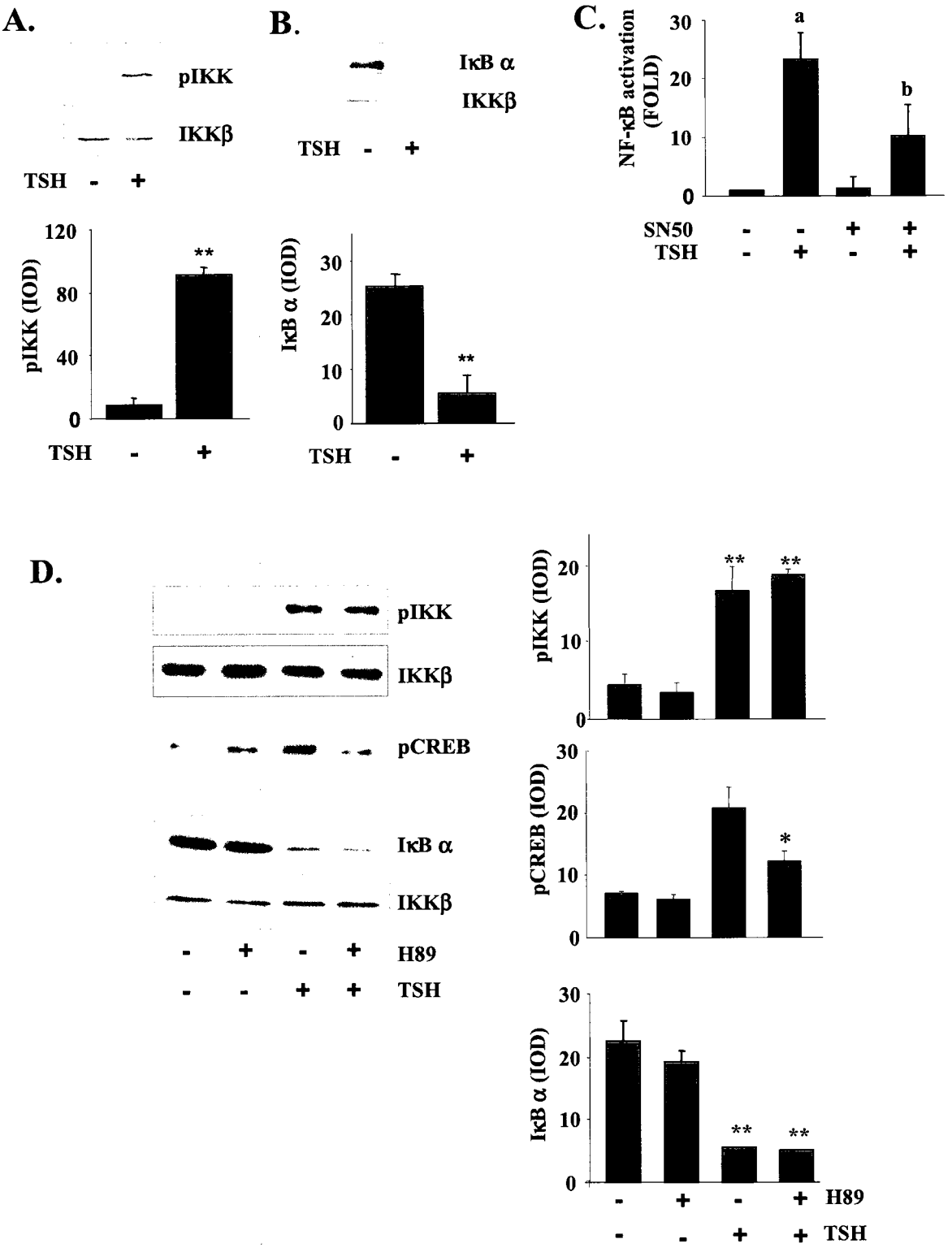
Figure 4.2: Inhibitors of the NF-κB pathway blunt TSH-induced IL-6 secretion. Cells were pretreated with 100 µmol/l sc-514 or vehicle for 1 hour (A) or with 50 µg/ml SN50 or vehicle for 15 minutes (B), then stimulated with 50 mU/ml TSH or vehicle for 4 hours. IL-6 protein in the medium was measured as described. Data are expressed as mean ± SE of 3 (A) or 4 (B) separate patient samples, each performed in duplicate. a denotes $P<0.001$ compared to control and to sc-514 or SN50, and $P<0.01$ compared to sc-514-TSH, and $P<0.05$ compared to SN50-TSH. b denotes $P<0.05$ compared to control and to sc-514 (both for A), and $P<0.01$ compared to control and to SN50 (both for B).

We investigated whether TSH could stimulate the phosphorylation of IKKβ, the reduction of IκBα, and lead to NF-κB activation. TSH treatment substantially increased IKKβ phosphorylation (10.3-fold, $P<0.01$; n=4) and reduce the levels of IκBα (by 78%, $P<0.01$; n=3) (Figure 4.3A and B). We next determined if NF-κB translocates to the nucleus and is activated in response to TSH. Human abdominal subcutaneous differentiated adipocytes were pretreated with 50 μg/ml SN50, and then stimulated with TSH. Nuclear lysates were evaluated for specific binding of the activated p65 subunit of NF-κB to DNA. TSH increased NF-κB (p65 subunit) binding to DNA by 23-fold ($P<0.001$; n=3). SN50 reduced TSH-induced NF-κB DNA binding by 60% ($P<0.01$, n=3) (Figure 4.3C). TSH-stimulated IKKβ phosphorylation was not dependent on protein kinase A (PKA). Pretreatment with 20 μmol/l H89 did inhibit TSH-stimulated phosphorylation of CREB, a known PKA-dependent event, but did not alter the IKKβ response (Figure 4.3D). Similarly, the TSH-induced IκBα degradation was not affected by H89.

To further demonstrate our novel observation that TSH activates NF-κB, we utilized a well-known cell model for studying TSH actions, the CHO cell line stably transfected with either human TSHR (JP2626) or with a control vector (JP02) (Van Sande et al., 2003). We assessed whether the NF-κB pathway is activated by TSH by monitoring IKKβ phosphorylation. JP02 and JP2626 cells were both exposed to TSH. IKKβ phosphorylation increased by 5.9-fold in JP2626 cells specifically ($P<0.001$, n=3), with no effect in JP02 cells (Figure 4.4A). To determine whether TSH could activate IL-6 expression in this model system, the cells were treated with TSH and IL-6 induction was measured by real-time PCR.

TSH increased IL-6 mRNA expression by 9.5-fold (n=2) in JP2626 cells, with no response in the control JP02 cells (Figure 4.4B).

Figure 4.3: TSH stimulates the IKKβ/NF-κB pathway, independently of PKA. A, B and D. Human abdominal subcutaneous differentiated adipocytes were stimulated for 30 minutes with 50 mU/ml TSH or vehicle. Where indicated, cells were pretreated with 20 μmol/l H89 or vehicle for 1 hour prior to TSH treatment. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibody against phospho-IKK (pIKK), IKKβ, IκBα, or phospho-CREB (pCREB). Representative immunoblots from a single experiment are shown. Densitometric data from 4 (A) or 3 (B and D) separate patient samples are expressed as means ± SE: * $P<0.05$ compared to TSH alone, and ** $P<0.01$ compared to control (A and B) or to control and H89 (D). IOD: integrated optical density. C. Cells were pretreated with 50 μg/ml SN50 or vehicle for 15 minutes, then stimulated with 50 mU/ml TSH or vehicle for 1 hour. p65 nuclear translocation was measured as described. Data are expressed as fold-change of control and represent means ± SD of 3 separate patients samples. a denotes $P<0.001$ compared to control and to SN50 and $P<0.01$ compared to SN50-TSH. b denotes $P<0.01$ compared to SN50 and $P<0.05$ compared to control.


A.
pIKK
IKKβ
TSH - +
pIKK (IOD)
120
80
40
0
**
TSH - +
B.
IκB α
IKKβ
TSH - +
IκB α (IOD)
30
20
10
0
**
TSH - +
C.
NF-κB activation (FOLD)
30
20
10
0
a
b
SN50 - - + +
TSH - + - +
D.
pIKK
IKKβ
pCREB
IκB α
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pIKK (IOD)
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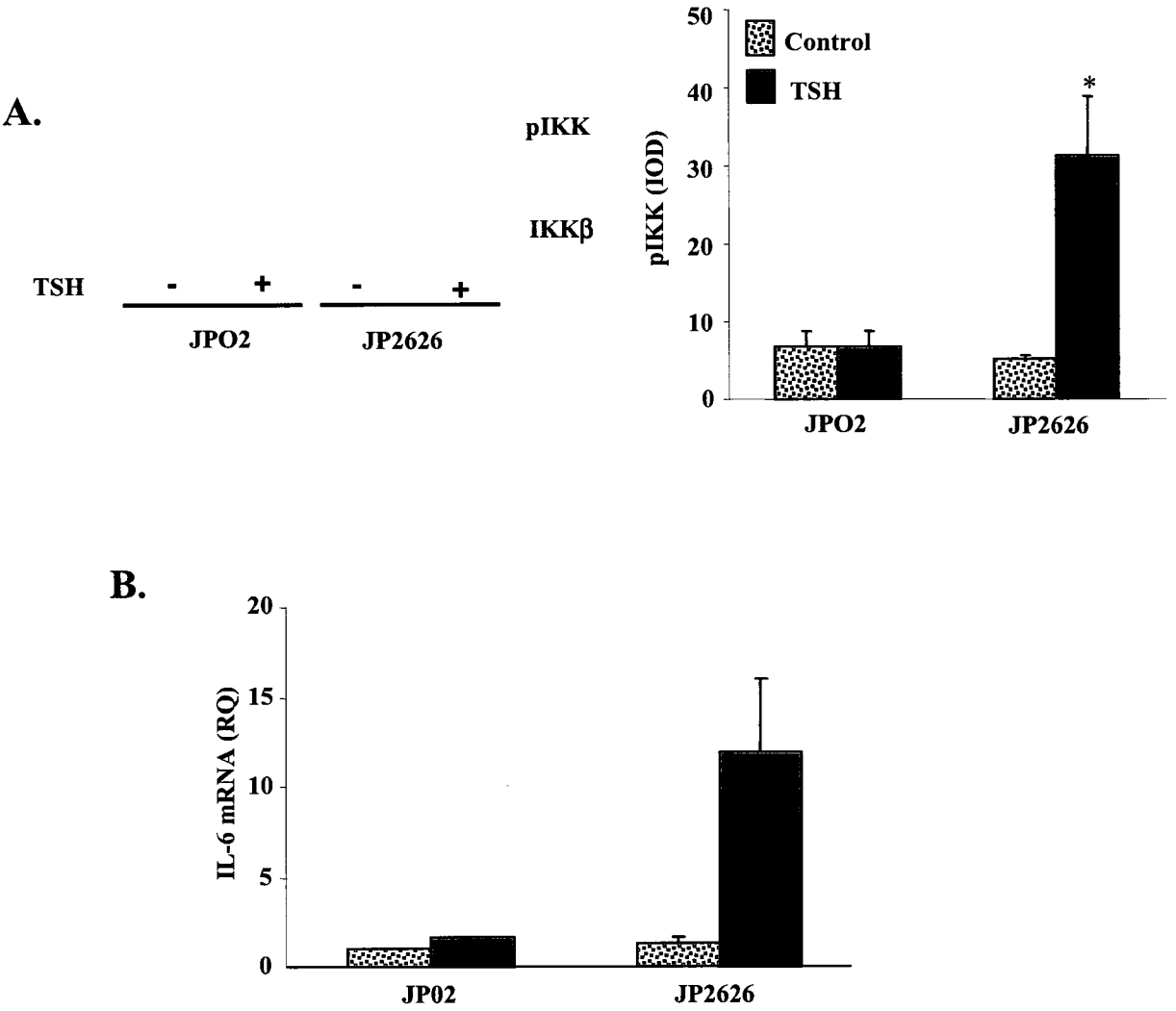

Figure 4.4: TSH stimulates IKKβ phosphorylation and increases IL-6 mRNA in TSHR overexpressing cells. JP02 and JP2626 cells were stimulated with or without 50 mU/ml TSH for 30 minutes (A) or 2 hours (B). A. Equal amounts of solubilized protein were separated by SDS-PAGE and immunoblotted with antibody against phospho-IKK (pIKK). Blots were stripped and reprobed with antibody against IKKβ. Representative immunoblots from a single experiment are shown. Densitometric data analyzing pIKK, derived from 3 separate experiments, are expressed as means ± SE.* denotes $p<0.001$ compared to other conditions. IOD: integrated optical density. B. RNA was extracted, and quantified by real time PCR. Data are expressed relative to 18S rRNA levels (RQ) and represent the means ± range of 2 separated experiments.

DISCUSSION

We have recently reported that TSH induces IL-6 release from human abdominal subcutaneous differentiated adipocytes (Antunes et al., 2006). In the present study, we have clarified the signaling mechanism of this TSH action by identifying an important role for NF-κB in this process. Upon TSH stimulation, IKKβ was phosphorylated, and there was an increase in NF-κB nuclear activation. Either inhibiting the activity of IKKβ or blocking the translocation of NF-κB to the nucleus reduced TSH-mediated IL-6 release.

The TSHR is a member of the G protein-coupled receptor (GPCR) family. In thyrocyte –based models, TSHR has been found to stimulate two principal signaling pathways (Davies et al., 2002). When coupled to Gs, TSHR leads to activation of adenylyl cyclase and elevation of cAMP, and this route of signaling in thyrocytes has been linked to thyroid hormone secretion. TSHR coupling to Gq triggers activation of PLC, and the generation of inositol phosphates and diacylglycerol, and these events are required for thyroid hormone synthesis (Van Sande et al., 2006).

However, the ability of TSHR to activate NF-κB has not been reported previously in either adipocytes or thyrocytes. TSH has been shown to enhance the ability of TNFα to activate NF-κB and upregulate IL-6 expression in rat thyroid follicular cells; this effect was proposed to be due to the formation of a complex consisting of the PKA catalytic subunit with IκB in the presence of TNFα (Cao et al., 2005). However, in that experimental system, an independent effect of TSH on NF-κB on IL-6 expression was not described in the absence of TNFα. Our data demonstrate that in human differentiated adipocytes, TSH alone is sufficient to activate the NF-κB pathway and does not depend on PKA, based on the

observations that H89 did inhibit CREB phosphorylation, but had no effect either on IKKβ phosphorylation or IκBα degradation.

The residual release of IL-6 in response to TSH in the presence of SN50 or sc-514 might be because of incomplete pharmacological inhibition of IKKβ/NF-κB pathway, or might indicate the operation of other TSH signaling routes that regulate this event. Further analysis of candidate TSH-stimulated signaling targets will be helpful to provide a more comprehensive view of TSH action in this regard.

The classical cell-surface receptors that activate NF-κB and induce inflammation are cytokine receptors, such as those for TNFα. Recently, the GPCRs have been also shown to activate the NF-κB pathway in a variety of cell types (Ye, 2001). However, there is only a single report of NF-κB activation in adipocytes by a GPCR in the literature, specifically, the angiotensin II type 1 (AT1) receptor. Angiotensin II was shown to act on the AT1 receptor to increase IL-6 and IL-8 release from human adipocytes, and this depended on NF-κB activation (Skurk et al., 2004). Our data show that NF-κB signaling may be activated in adipocytes by another GPCR, the TSHR. TSH-induced NF-κB activation was also confirmed in CHO cells expressing human TSHR, an established cell model to explore TSHR signaling (Van Sande et al., 2003; Van Sande et al., 2006). TSH treatment clearly augmented IKKβ phosphorylation and IL-6 mRNA expression in a TSHR-specific fashion.

As with the studies on angiotensin II noted above, we monitored NF-κB activity in the differentiated adipocytes using an assay that traces p65 subunit nuclear translocation and its activated state through its ability to bind DNA in a sequence-specific manner. Our studies with SN50, which blocks p50 nuclear import, suggest that the p65-p50 dimer is the NF-κB form regulated by TSH. The p50-p65 form is mainly regulated by IKKβ (Karin, 2006), and

this is consistent with our studies using sc-514. It is of course possible that other NF-κB components are operative in human adipocytes (Berg et al., 2004).

In summary, we show that TSH modulates NF-κB signaling to induce IL-6 release in human abdominal subcutaneous differentiated adipocytes. Low-grade inflammation induced by adipocyte hypertrophy has been proposed to explain how either obesity or lipodystrophy predisposes to metabolic and vascular dysfunction (Hotamisligil, 2006). Our results suggest that TSH may also trigger a pro-inflammatory and pro-atherogenic adipocyte response. Elevated IL-6 and TNFα serum levels, as well as impaired endothelial physiology, have been observed in patients with exposed to high TSH levels (Antunes et al., 2006; Dardano et al., 2006). Learning more about the mechanism by which TSH acts on adipocytes may enlarge our understanding of inflammation and cardiovascular disease in subclinical hypothyroidism.

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REFERENCES

Antunes, T.T., Gagnon, A., Bell, A., and Sorisky, A. (2005). Thyroid-stimulating hormone stimulates interleukin-6 release from 3T3-L1 adipocytes through a cAMP-protein kinase A pathway. Obes Res *13*, 2066-2071.

Antunes, T.T., Gagnon, A., Chen, B., Pacini, F., Smith, T.J., and Sorisky, A. (2006). Interleukin-6 release from human abdominal adipose cells is regulated by thyroid-stimulating hormone: effect of adipocyte differentiation and anatomic depot. Am J Physiol Endocrinol Metab *290*, E1140-1144.

Berg, A.H., Lin, Y., Lisanti, M.P., and Scherer, P.E. (2004). Adipocyte differentiation induces dynamic changes in NF-kappaB expression and activity. Am J Physiol Endocrinol Metab *287*, E1178-1188.

Cao, X., Kambe, F., and Seo, H. (2005). Requirement of thyrotropin-dependent complex formation of protein kinase A catalytic subunit with inhibitor of {kappa}B proteins for activation of p65 nuclear factor-{kappa}B by tumor necrosis factor-{alpha}. Endocrinology *146*, 1999-2005.

Chung, S., Lapoint, K., Martinez, K., Kennedy, A., Boysen Sandberg, M., and McIntosh, M.K. (2006). Preadipocytes mediate lipopolysaccharide-induced inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes. Endocrinology *147*, 5340-5351.

Dardano, A., Ghiadoni, L., Plantinga, Y., Caraccio, N., Bemi, A., Duranti, E., Taddei, S., Ferrannini, E., Salvetti, A., and Monzani, F. (2006). Recombinant human thyrotropin reduces endothelium-dependent vasodilation in patients monitored for differentiated thyroid carcinoma. J Clin Endocrinol Metab *91*, 4175-4178.

Davies, T., Marians, R., and Latif, R. (2002). The TSH receptor reveals itself. J Clin Invest *110*, 161-164.

Fisman, E.Z., Benderly, M., Esper, R.J., Behar, S., Boyko, V., Adler, Y., Tanne, D., Matas, Z., and Tenenbaum, A. (2006). Interleukin-6 and the risk of future cardiovascular events in patients with angina pectoris and/or healed myocardial infarction. Am J Cardiol *98*, 14-18.

Hotamisligil, G.S. (2006). Inflammation and metabolic disorders. Nature *444*, 860-867.

Karin, M. (2006). Role for IKK2 in muscle: waste not, want not. J Clin Invest *116*, 2866-2868.

Kimura, T., Van Keymeulen, A., Golstein, J., Fusco, A., Dumont, J.E., and Roger, P.P. (2001). Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models. Endocr Rev *22*, 631-656.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lee, K.W., Hill, J.S., Walley, K.R., and Frohlich, J.J. (2006). Relative value of multiple plasma biomarkers as risk factors for coronary artery disease and death in an angiography cohort. CMAJ *174*, 461-466.

Lobbes, M.B., Lutgens, E., Heeneman, S., Cleutjens, K.B., Kooi, M.E., van Engelshoven, J.M., Daemen, M.J., and Nelemans, P.J. (2006). Is there more than C-reactive protein and fibrinogen? The prognostic value of soluble CD40 ligand, interleukin-6 and oxidized low-density lipoprotein with respect to coronary and cerebral vascular disease. Atherosclerosis *187*, 18-25.

Mohamed-Ali, V., Goodrick, S., Rawesh, A., Katz, D.R., Miles, J.M., Yudkin, J.S., Klein, S., and Coppack, S.W. (1997). Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. J Clin Endocrinol Metab *82*, 4196-4200.

Rodondi, N., Aujesky, D., Vittinghoff, E., Cornuz, J., and Bauer, D.C. (2006). Subclinical hypothyroidism and the risk of coronary heart disease: a meta-analysis. Am J Med *119*, 541-551.

Shoelson, S.E., Lee, J., and Goldfine, A.B. (2006). Inflammation and insulin resistance. J Clin Invest *116*, 1793-1801.

Skurk, T., van Harmelen, V., and Hauner, H. (2004). Angiotensin II stimulates the release of interleukin-6 and interleukin-8 from cultured human adipocytes by activation of NF-kappaB. Arterioscler Thromb Vasc Biol *24*, 1199-1203.

Stork, S., Feelders, R.A., van den Beld, A.W., Steyerberg, E.W., Savelkoul, H.F., Lamberts, S.W., Grobbee, D.E., and Bots, M.L. (2006). Prediction of mortality risk in the elderly. Am J Med *119*, 519-525.

Van Sande, J., Costa, M.J., Massart, C., Swillens, S., Costagliola, S., Orgiazzi, J., and Dumont, J.E. (2003). Kinetics of thyrotropin-stimulating hormone (TSH) and thyroid-stimulating antibody binding and action on the TSH receptor in intact TSH receptor-expressing CHO cells. J Clin Endocrinol Metab *88*, 5366-5374.

Van Sande, J., Dequanter, D., Lothaire, P., Massart, C., Dumont, J.E., and Erneux, C. (2006). Thyrotropin stimulates the generation of inositol 1,4,5-trisphosphate in human thyroid cells. J Clin Endocrinol Metab *91*, 1099-1107.

Ye, R.D. (2001). Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. J Leukoc Biol *70*, 839-848.

5.0 GENERAL DISCUSSION

Stage of differentiation and basal IL-6 release

IL-6 is a protein broadly expressed in a variety of cell types. Besides having a wide scope of action, it is well known for its important role in the mediation of inflammation (Hoene and Weigert, 2008). IL-6 release from adipose cells is a subject that has gained significant attention in the last decade, because obesity is a condition characterized by a chronic low-grade inflammation with elevated systemic IL-6 levels (Trayhurn and Wood, 2004).

I investigated basal IL-6 release from adipose cells. My data show that preadipocytes and adipocytes from 3T3-L1 mouse as well as from human abdominal subcutaneous and omental depots are a source of IL-6. This finding is in agreement with previous studies examining adipose cells. In 3T3-L1, primary mouse and human adipose cells, as well as adipose cells derived from genetically obese mouse, IL-6 gene expression and protein release are seen in both preadipocytes and adipocytes (Gonzales and Orlando, 2008; Harkins et al., 2004; Hugo et al., 2006; Wang et al., 2005).

I also showed that in human adipose cells, basal IL-6 release decreases during the differentiation process. Previous investigations from our laboratory also noted the same pattern for the 3T3-L1 adipose cells (preadipocytes: 19.4 ± 1.7 pg/ml/μg protein versus adipocytes: 6.2 ± 1.1 pg/ml/μg protein; data from Dr. Sorisky's former Ph.D student Andrea Bell). Although I did not investigate IL-6 mRNA expression during differentiation, other groups have also reported that a decrease in IL-6 mRNA and protein release occur during the adipogenic process (Harkins et al., 2004; Hugo et al., 2006). In contrast, a study from

Wang *et al.* on human subcutaneous adipose cells reported that although IL-6 mRNA levels decreased at the beginning of differentiation, IL-6 mRNA returned back to preadipocyte levels by the end of the differentiation period (Wang et al., 2005). Although the reason for this discrepant result is unclear, it should be pointed out that Wang *et al.* did not measure IL-6 protein release, only mRNA expression.

I observed that basal IL-6 release is greater from omental versus subcutaneous abdominal preadipocytes. The same depot-related pattern, although not reaching statistical significance, was seen for differentiated adipocytes. Fried *et al* showed that in massively obese subjects (mean BMI of 52), adipose tissue explants and isolated adipocytes from the omental depot release more IL-6 release basally compared to the abdominal subcutaneous depot (Fried et al., 1998). Fain *et al*, in a study of obese patients (mean BMI of 32), showed that adipose tissue explants, but not isolated adipocytes, from the omental depot showed higher basal IL-6 release compared to the subcutaneous depot (Fain et al., 2004). Since in my studies adipose tissue samples were obtained from patients ranging in BMI from 23 (normal weight) to 33 (obese), it suggests that higher basal IL-6 release from the omental depot is a consistent feature across the spectrum of adiposity. Omental adipose tissue is noteworthy as it directly drains into the portal vein (Jensen, 2006). In fact, a recent *in vivo* study, in which plasma IL-6 levels was 2-fold higher in the portal vein compared to the peripheral circulation, supports the above-mentioned *in vitro* findings (Fontana et al., 2007).

Stage of differentiation and cellular responsiveness to TSH

I investigated IL-6 release upon TSH stimulation from adipose cells. My data indicate that stage of differentiation is an important determinant of TSH responsiveness. The preadipocyte models studied did not increase IL-6 release upon TSH treatment. The lack of IL-6 release by TSH is not due to a generalized limitation of preadipocytes to external stimuli, since other inflammatory triggers, such as LPS and fatty acids, have been shown to stimulate IL-6 release from preadipocytes (Chung et al., 2005; Harkins et al., 2004). Moreover, since both 3T3-L1 and human abdominal subcutaneous preadipocytes activate the PI3K/Akt/p70S6K signaling pathway upon TSH treatment (Bell et al., 2002; Bell et al., 2000), the lack of IL-6 release by TSH cannot be attributed to the unresponsiveness of preadipose cells to TSH stimulation. This could suggest that activation of this specific signaling route by TSH does not modulate IL-6 release from preadipocytes.

Once preadipocytes were differentiated, TSH-induced IL-6 release was seen for the 3T3-L1 and human abdominal subcutaneous, but not the omental adipocytes. The responsiveness of the differentiated adipocytes to TSH can not be attributed to the presence of the differentiation inducers, since differentiation medium was removed 2 (humans) and 3 days (3T3-L1) before TSH treatment occurred. For human differentiated adipocytes, the difference in TSH responsiveness to IL-6 secretion could be on the basis of TSHR protein expression. However, based on a small sample, TSHR protein expression appeared to be equal in preadipocytes and differentiated adipocytes from both depots (Bell et al., 2000). However, I did not directly measure TSHR protein expression in my samples. It would be interesting in the future to correlate TSHR protein and mRNA expression with the effect of TSH on IL-6 release as a function of BMI. With the small number of patients available to

me for my study, such an analysis was not possible. In 3T3-L1 cells, TSHR mRNA expression increases as preadipocytes differentiate (Haraguchi et al., 1996), but a direct comparison of the receptor expression at the protein level during the differentiation process has not yet been performed. Nonetheless, a functional TSHR is present in both preadipocytes and differentiated adipocytes (Bell et al., 2002; Haraguchi et al., 1996).

There are different possibilities that could explain the difference between subcutaneous versus omental differentiated adipocytes in response to TSH in terms of IL-6 release. First, it is possible that the type of G protein that couples with the TSHR in the abdominal subcutaneous adipocytes to induce IL-6 release upon TSH stimulation is not the same as in the omental adipocytes. Second, even if the adipocytes from both depots activate the same G protein upon TSH stimulation, it is possible that activation of downstream signaling components and/or transcription factors differ. Third, it is possible that the refractoriness of the differentiated omental adipocytes to TSH is a specific event of the *in vitro* model system used. To address this question, the effect of TSH on IL-6 release on freshly isolated adipocytes (Fried and Moustaid-Moussa, 2001) from the abdominal subcutaneous and from the omental depot would need to be tested and compared.

The amount of IL-6 released from the differentiated cells, even after TSH stimulation, is lower than that of the unresponsive preadipocytes. However, it is possible that, *in vivo*, the IL-6 released from TSH-stimulated adipocytes, could act in a paracrine manner on neighbouring preadipocytes to trigger further inflammatory processes. Increased IL-6 release from the adipose tissue could also reach the circulation and by affecting distal tissues, may contribute to the development of CVD in subclinical hypothyroidism.

The role of cAMP/PKA on TSH-induced IL-6 release

In thyroid cells, most cellular responses regulated by TSH are accomplished through activation of the $G\alpha_s$ and the cAMP/PKA signaling pathway (Rivas and Santisteban, 2003). In 3T3-L1 and human abdominal subcutaneous preadipocytes, TSHR activation is not coupled with cAMP activation. However, as these cells differentiate, they acquire the ability to increase cAMP levels upon TSH treatment [(Bell et al., 2002) and Bell A, Ph.D thesis]. I therefore postulated that cAMP/PKA was the route used by TSH to increase IL-6 secretion. In 3T3-L1 cells, the first indication that activation of cAMP/PKA played a role in IL-6 release was that the AC activator, forskolin, increased IL-6 release by 20-fold. Fasshauer *et al.* had previously suggested that the cAMP/PKA route was involved in IL-6 expression in 3T3-L1 adipocytes by observing that IL-6 mRNA was upregulated upon β-adrenergic stimulation, activation of $G\alpha_s$ subunit with cholera toxin, AC with forskolin, or PKA with a cAMP analog (Fasshauer et al., 2003).

I evaluated the cAMP/PKA pathway in TSH-induced IL-6 release from 3T3-L1 adipocytes by using H89. H89 occupies the ATP binding site of the catalytic subunit of PKA and inhibits its ability to phosphorylate substrates (Lochner and Moolman, 2006). H89 greatly inhibited TSH-induced IL-6 release. Taken together with the cAMP data mentioned above, I suggest that TSH-induced AC activation, elevation of cAMP, and activation of PKA plays a major role in the upregulation of IL-6 release in 3T3-L1 adipocytes. There are, however, some caveats to be mentioned regarding interpretation of these data. H89, at the concentration used, may also inhibit other kinases, some of which can directly participate in the PKA pathway or be involved in other unrelated pathways (Lochner and Moolman, 2006). It is therefore possible that the inhibition seen by H89 on TSH-induced IL-6 release

is due to other kinases/pathways rather than only cAMP/PKA. LaPensee *et al* reported that H89 blocked insulin-mediated IL-6 mRNA and protein release in human preadipocytes by inhibiting cyclic guanosine monophosphate-dependent protein kinase, rather than PKA. Nonetheless, H89, at the same concentration, blocked forskolin-induced IL-6 release, thus showing that it also effectively inhibits the cAMP/PKA pathway (Lapensee et al., 2008).

It has been suggested that, to validate the findings in which H89 indicates a role for cAMP/PKA, other structurally unrelated inhibitors, such as protein kinase inhibitor peptide, or cAMP antagonists should be tested as well (Lochner and Moolman, 2006; Murray, 2008). Despite these limitations, H89 is still widely used by many leading researchers in the cAMP/PKA field (Dremier et al., 2000; Giuliani et al., 2008; Siddappa et al., 2008).

The inhibition of TSH-induced IL-6 release in 3T3-L1 cells by H89 was not complete. Two interpretations are possible. First, H89 concentration and/or the time of incubation were not sufficient to completely block PKA activation. Second, TSH may act through other pathways, unaffected by H89, that lead to IL-6 release. To investigate the true contribution of PKA, studies using more stringent methodologies to eliminate PKA, such as small interfering RNA (siRNA) could be performed. Once the degree of knockdown is carefully addressed, the role of PKA in this process can be determined. If complete blockade of PKA still does not fully abrogate IL-6 release by TSH, the involvement of alternative pathways would be considered more likely. For example, TSH could use the IKKβ-NF-κB as a parallel pathway to promote IL-6 release.

Downstream effectors of PKA that could lead to TSH-stimulated IL-6 production and release by TSH from 3T3-L1 differentiated adipocytes were not addressed in this study. Several possibilities exist. In the cytosol, PKA could participate in the crosstalk with several

signaling pathways, such as the IKKβ-NF-κB, PKC and the MAPK family (Gao et al., 2008, Chio et al., 2004, Nika et al., 2004). In the nucleus, PKA could phosphorylate transcription factors, such as the CREB family members (CREB, cAMP responsive element modulator and activating transcription factor-1). CREB protein, for example, needs to be phosphorylated by PKA at serine 133 to recruit co-activators and therefore become competent to induce gene expression (Sands and Palmer, 2008).

Elevation of cAMP by TSH treatment of thyroid cells activates Epac, as well as PKA (Dremier et al., 2000; Hochbaum et al., 2008). Furthermore, Epac appears to be involved in IL-6 release in some cell types. For example, in murine macrophages, Epac activation leads to increased IL-6 gene expression (Tan et al., 2007). Epac is thus a candidate target by which TSH may mediate IL-6 release. My data show that treatment with 8-pCPT-2'-O-Me-cAMP, an Epac-specific cAMP analog (Enserink et al., 2002), in 3T3-L1 differentiated adipocytes did not result in IL-6 release, suggesting that sole activation of this molecule is not sufficient to trigger an increase in IL-6 release. Due to the unavailability of an Epac inhibitor, the role of Epac on cell functions can only be tested by using dominant negative or siRNA approaches (Hochbaum et al., 2008; Kwak et al., 2008). These are techniques that could further be tested on adipose cells to investigate whether Epac is required for TSH-induced IL-6 release.

In many cell types, Epac activation leads to p42/44 MAPK phosphorylation through the Rap1/B-Raf protein kinase cascade (Dumaz and Marais, 2005). Alternatively, in human embryonic kidney 293 cells, for example, serotonin-induced Gαs activation leads to p42/44 MAPK phosphorylation without involving Epac (Norum et al., 2007). TSH activates p42/44 MAPK in some, but not all thyrocyte models (Buch et al., 2008; Correze et al., 2000;

Dremier et al., 2000). In human differentiated adipocytes, p42/44 MAPK activation by fatty acids is linked to IL-6 release (Brown et al., 2004; Chung et al., 2005). I thus tested whether TSH-induced IL-6 release required p42/44 MAPK. In 3T3-L1 differentiated adipocytes, the MEK-1 inhibitor PD98059 indicated that either TSH does not activate p42/44 MAPK , or that TSH does not require p42/44 MAPK to increase IL-6 release. Although the concentration and the time of pre-incubation of PD98059 used in my studies are well known to inhibit p42/p44 MAPK activation (Iacovelli et al., 2001; Panka et al., 2001), time course and dose response studies on the inhibitory effect of PD98059 on TSH-induced IL-6 release could be further explored. Furthermore, efforts to clearly demonstrate if TSH activates this pathway in these cells may clarify the interpretation of the PD98059 studies.

NF-κB activation and IL-6 release in response to TSH

IL-6 synthesis is under the control of NF-κB in many cell types (Okamoto et al., 2008). I thus examined whether TSH activates NF-κB to increase IL-6 release from human abdominal subcutaneous differentiated adipocytes. The convergent point of various pro-inflammatory agonists on NF-κB pathway is the activation of the IKK complex (Schmid and Birbach, 2008). Although the IKK complex possesses 2 protein kinases, the β subunit (IKKβ) plays a more active role in pro-inflammatory processes (Hacker and Karin, 2006). I observed that TSH treatment induced IKKβ phosphorylation, indicative of activation (Okamoto et al., 2008). Activated IKKβ phosphorylates IκBα on serine residues, an event that marks IκBα for ubiquitination and rapid proteasomal degradation (Schmid and Birbach, 2008). NF-κB, once free from IκBα, moves from the cytosol to the nucleus and activates transcription of pro-inflammatory genes (Hershko et al., 2002). My data reveal that TSH

induced IκBα degradation in the same time period (30 minutes) as IKKβ phosphorylation. Further, I showed that TSH induced NF-κB (p65 subunit) nuclear translocation and DNA binding using an ELISA-based assay for the NF-κB p65 subunit. Due to limitations of supply of primary preadipocytes I could not immunoprecipitate NF-κB bound to DNA upon TSH stimulation and therefore I was unable to show that NF-κB interacts directly with the IL-6 promoter region upon TSH treatment using the chromatin immunoprecipitation assay technique. Recently, a study conducted in TSHR-expressing cells showed by a luciferase reporter assay that the NF-κB promoter activity is enhanced by TSH (Voigt et al., 2007), supporting the idea that TSH induces NF-κB binding to its promoter site and activation of transcription.

To study whether IL-6 release by TSH involves the NF-κB pathway, specific pharmacological inhibitors, sc-514 and SN50, were used. Sc-514 is a compound that occupies reversibly the ATP binding site of IKKβ and thereby inhibits it from phosphorylating IκBα. This results in a decrease in the amount of nuclear NF-κB and also in a reduction of its transcriptional capacity (Kishore et al., 2003). SN-50 is a peptide that possesses the nuclear localization sequence of the p50 subunit of the NF-κB, and thereby competes with and blocks the entrance of NF-κB to the nucleus. The data obtained with the use of these inhibitors strongly implicate this pathway in TSH-induced IL-6 release. However, complete inhibition of TSH-induced IL-6 release was not achieved with either inhibitor. As for the other pharmacological inhibitors used in my study, the time chosen for the pre-incubation period and/or the concentration used were selected based on the relevant literature. Thus, it is possible that, as for the other pharmacological inhibitors, optimal pre-incubation time and doses for SC-514 and SN50 need to be established in my cell model. It

is also possible that TSH may activate alternative pathways to induce IL-6 release. The use of dominant negative or siRNA approaches would help to clarify whether NF-κB is a unique pathway used by TSH to induce IL-6 release.

The mechanism of NF-κB activation in thyroid cells by TSH seems to differ from what I observed in adipocytes. Kikumori *et al* showed that in rat thyroid cells, TSH does not activate NF-κB on its own, but can potentiate the NF-κB transactivation capacity in combination with TNF-α (Kikumori et al., 2001). Later, it was reported that TSH increases the NF-κB transactivation ability by activating PKA to phosphorylate the NF-κB p65 subunit. PKA-induced p65 phosphorylation in thyroid cells by TSH occurs in a very peculiar manner. Instead of the usual cAMP binding to the PKA-R subunit and displacement of PKA-C subunit, TSH treatment promoted the binding of the PKA-C subunit to IκBα/p50-p65, thus forming a PKA-C/ IκBα/p50-p65 complex, independent of cAMP elevation (Cao et al., 2005).

Although my study indicates that in human abdominal subcutaneous differentiated adipocytes, TSH does not require PKA to activate NF-κB (as shown by the lack of effect of H89 on TSH-induced IKKβ phosphorylation and IκBα degradation), I did not test NF-κB p65 subunit phosphorylation by TSH nor the involvement of PKA on this process. This point could further be addressed by immunoblot analysis using antibodies that recognize NF-κB p65 subunit phosphorylation by PKA (serine 276) (Perkins, 2007). Also, the possibility that PKA is activated by TSH to induce IL-6 release without involving activation of IKKβ-NF-κB needs to be addressed in the future.

In my studies, upstream signaling proteins responsible for IKKβ phosphorylation were not investigated. Most studies have used TNF-α and LPS to investigate this event

(Hacker and Karin, 2006). Recently, investigations on GPCR–stimulated upstream signaling leading to IKK activation have been reported. It appears that the intermediates that link GPCR to NF-κB activation can be numerous. In cardiomyocytes, angiotensin II, endothelin-1 and phenylephrine-induced NF-κB activation through intracellular ROS elevation (Hirotani et al., 2002). In Hela cells transfected with the B2-type bradykinin receptor, bradykinin treatment resulted in NF-κB activation by coupling to Gαq and inducing activation of PI3K (Xie et al., 2000). In human bronchial epithelial cells, lysophosphatidic acid (LPA) induced IκB phosphorylation, NF-κB activation and IL-8 secretion by activating the novel PKCδ isoform (Cummings et al., 2004). In mouse embryonic fibroblasts (MEFs), endothelin-1 and LPA use the B cell chronic lymphocytic lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue lymphoma translocation gene 1 (MALT1) adapter proteins to bridge GPCR with the IKK complex (Wang et al., 2007; Wegener and Krappmann, 2007). Interestingly, MEFs deficient in Bcl10 showed, besides impaired ability to activate IKK/NF-κB, blunted release of IL-6 in response to LPA, suggesting that Bcl10-MALT1 could be upstream signaling proteins for IL-6 release by a GPCR agonist (Klemm et al., 2007). It remains to be determined whether any of the above mentioned intermediates for NF-κB activation by GPCR stimulation are used by TSH to promote IL-6 release in human adipose cells.

Requirement of transcription for TSH to stimulate IL-6 protein release

The fact that TSH activates a transcription factor (i.e.NF-κB) to induce IL-6 release together with the existence of a 4-hour lag period between TSH treatment and IL-6 secretion suggests that there is a need for gene transcription prior to protein release. This was

investigated using the transcriptional inhibitor actinomycin D and semiquantitative reverse transcription-PCR measurements in experiments using the 3T3-L1 adipocytes. Actinomycin D, which binds to DNA and inhibits RNA synthesis (Sobell, 1985), potently blocked TSH-induced IL-6 release. Further, I used semiquantitative reverse transcription-PCR to show that TSH increases IL-6 mRNA expression in these cells.

Several studies in a variety of cells, including adipose cells, demonstrated a requirement for transcription-dependent protein release. In 3T3-L1 and human adipose cells, insulin, isoproterenol, and TNF-α induced both IL-6 mRNA expression and protein release (Gonzales and Orlando, 2008; Lapensee et al., 2008; Path et al., 2001). Alternatively, IL-6 protein release could depend on post-transcriptional events, such as mRNA stabilization, increased translation or release from preformed pools of protein-containing vesicles (Persson et al., 2004). In rat thyroid cells, IL-1β-induced increase in IL-6 release required enhanced transcription and stabilization of IL-6 mRNA (Szabo-Fresnais et al., 2008). In a human preadipose cell line, IL-6 release by insulin appears to involve multiple factors such as increased gene transcription, augmented protein translation and recruitment of preformed IL-6-containing vesicles (Lapensee et al., 2008). In adipocytes, the release of IL-6 from preformed vesicles has yet to be investigated.

For 3T3-L1 adipocytes, I conclude that increased transcription is an important mechanism by which TSH induces IL-6 release, since actinomycin D completely blocked this action. In human abdominal subcutaneous differentiated adipocytes, the importance of transcription for TSH to stimulate IL-6 release would require further investigation, such as use of actinomycin D and reverse transcription-PCR.

Through a time-course study on 3T3-L1 adipocytes, I showed that IL-6 release by TSH occurs at 4 hours, and is absent at 24. A more complete time course would be needed to determine the earliest time point that TSH can promote IL-6 release, and to more precisely identify when the peak of IL-6 release by TSH occurs. The reason for the unresponsiveness at 24h is unknown. It is possible that by 24h TSH has been degraded in the culture medium, or that TSHR has been desensititized.

As was done for all studies on 3T3-L1 adipose cells, the data describing the IL-6 release by TSH in this experimental setting was normalized to the total protein cell content. Cell protein content did not change throughout the time-course experiment (up to 24 hours), or by the addition of pharmacological activators or inhibitors.

TSH increases IL-6 levels in vivo

I demonstrated, in collaboration with Drs. Pacini and Smith, that rhTSH increases IL-6 serum levels in thyroidectomized patients. Our study is in agreement with the study performed by Dardano *et al*, which also reported that TSH increased IL-6 levels, as well as TNF-α; there was also a doubling in CRP levels that did not reach significance (Dardano et al., 2006). One advantage of this experimental model of rhTSH administration is that since T4 levels are maintained in the normal reference range during rhTSH administration, the outcomes of this study can be attributed solely to TSH effects. Furthermore, since patients have no remaining thyroid gland, which can produce IL-6 upon TSH stimulation (Weetman et al., 1990), the increase in circulating IL-6 levels in thyroidectomized patients by rhTSH has to be from other tissues. Whether adipose tissue is among the extrathyroidal effects of TSH on IL-6 circulating levels *in vivo* has yet to be determined. Subclinical hypothyroid

patients have increased circulating IL-6 levels (Taddei et al., 2006), and it is possible that the IL-6 is secreted from adipocytes in response to the elevated TSH levels. Increased IL-6 levels in subclinical hypothyroid patients may be (one of) the underlying reason that these patients are at a higher risk of developing cardiovascular disease.

In summary, I show that adipose cell differentiation plays an important role for secretion of IL-6 in response to TSH in the 3T3-L1 and human abdominal subcutaneous depot. The differentiation status of adipose cells from the omental depot, on the other hand, do not allow these cells to respond to TSH and secrete IL-6. In 3T3-L1, activation of PKA plays a major role in TSH-induced IL-6 secretion. In human abdominal subcutaneous differentiated adipocytes, the IKKβ-NF-κB signaling pathway is critical for TSH-induced IL-6 release. The observation that rhTSH increases IL-6 levels in thyroidectomized patients raises the possibility that the extra-thyroidal action of TSH could be on abdominal subcutaneous adipose cells.

My findings suggest that TSH may be an important regulator of IL-6 production from adipose cells in vitro and can lead to elevated circulating IL-6 levels in vivo. This may be of significance to CVD risk in subclinical hypothyroidism, given that IL-6 is an established CVD risk factor.

Proposed model of the signaling pathways employed by TSH to induce IL-6 release from adipose cells

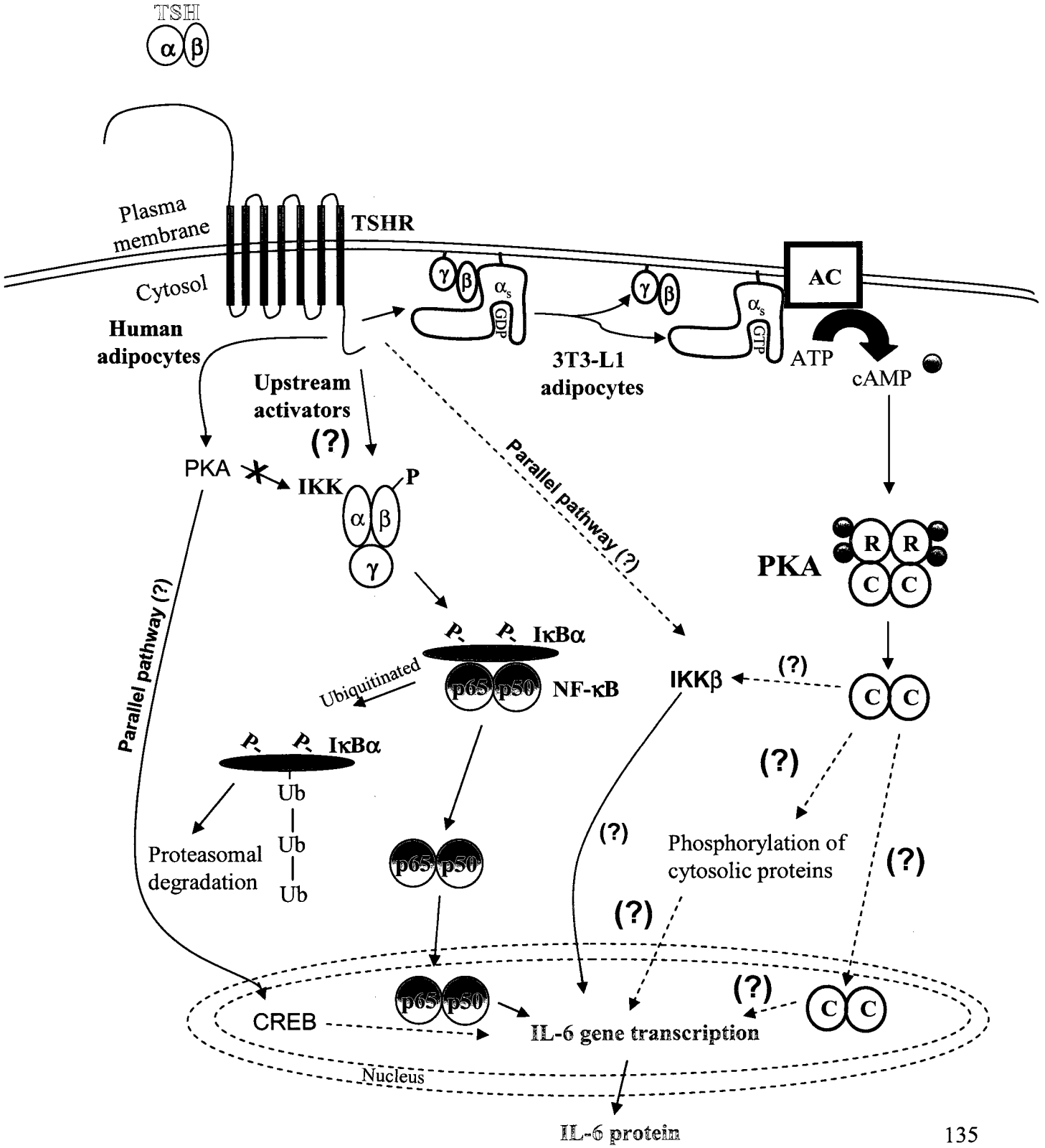
Figure 5.1 depicts my proposed model of the signaling mechanisms activated by TSH to induce IL-6 release from adipocytes. In 3T3-L1differentiated adipocytes, I propose that TSH, by binding to the TSHR, activates the $G\alpha_s$ subunit and subsequently AC. AC

promotes cAMP synthesis, an event that leads to PKA activation. At present, downstream targets of PKA to activate IL-6 release are not known. Activated PKA could either phosphorylate target proteins on the cytosol such as IKKβ, or in the nucleus to regulate IL-6 gene transcription and protein release. The possibility that TSH uses parallels pathways, such as IKKβ-NF-κB, to induce IL-6 release, could be further speculated.

In human abdominal subcutaneous differentiated adipocytes, the upstream steps that result in IKKβ activation upon TSH treatment are currently not known. As mentioned in the General Introduction, TSHR activation by TSH can couple to a variety of G proteins in thyroid cells. In human adipocytes, it remains to be determined which G protein(s) couple to TSHR upon TSH stimulation to induce IL-6 release. Furthermore, I did not investigate the steps that follow G protein activation to induced IKKβ activation by TSH in adipocytes. As mentioned in the General Discussion, activation of the NF-κB pathway by GPCR agonists involves elevation of intracellular ROS, activation of PI3K or PKCδ, or recruitment of the Bcl10-MALT1 adaptor proteins. These are possible pathways that TSH could use in human differentiated adipocytes to induce IL-6 release. Despite the fact that PKA is not the kinase that leads to IKKβ activation in human adipocytes, it is still possible that PKA activation by TSH contributes to IL-6 release. This needs further investigations.

I thus propose that in human abdominal subcutaneous differentiated adipocytes TSH induces IKKβ activation, a process that leads to IκBα degradation and release of NF-κB. Free NF-κB moves to the nucleus where it binds to the promoter region of the IL-6 gene, induces IL-6 transcription and promotes IL-6 release.

Figure 5.1: Proposed model of the signaling pathways employed by TSH to induce IL-6 release from adipose cells: See text (page 129) for details.


TSH
α β
Plasma membrane
TSHR
Cytosol
Human adipocytes
γ β αs GDP
3T3-L1 adipocytes
γ β αs GTP
AC
ATP
cAMP
Upstream activators
(?)
PKA
IKK
P
α β γ
Parallel pathway (?)
Parallel pathway (?)
PKA
R R C C
P P IκBα
Ubiquitinated
p65 p50 NF-κB
IKKβ
(?)
C C
P P IκBα
Ub
Ub
Ub
Proteasomal degradation
p65 p50
(?)
Phosphorylation of cytosolic proteins
(?)
(?)
p65 p50
(?)
C C
CREB
IL-6 gene transcription
Nucleus
IL-6 protein

REFERENCES

Bell, A., Gagnon, A., Dods, P., Papineau, D., Tiberi, M., and Sorisky, A. (2002). TSH signaling and cell survival in 3T3-L1 preadipocytes. Am J Physiol Cell Physiol *283*, C1056-1064.

Bell, A., Gagnon, A., Grunder, L., Parikh, S.J., Smith, T.J., and Sorisky, A. (2000). Functional TSH receptor in human abdominal preadipocytes and orbital fibroblasts. Am J Physiol Cell Physiol *279*, C335-340.

Bell, A. (2003) *TSH Signaling and Cellular Responses in Preadipocytes and Adipocytes.* Ph.D. thesis. University of Ottawa, Canada.

Brown, J.M., Boysen, M.S., Chung, S., Fabiyi, O., Morrison, R.F., Mandrup, S., and McIntosh, M.K. (2004). Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. J Biol Chem *279*, 26735-26747.

Buch, T.R., Biebermann, H., Kalwa, H., Pinkenburg, O., Hager, D., Barth, H., Aktories, K., Breit, A., and Gudermann, T. (2008). G13-dependent Activation of MAPK by Thyrotropin. J Biol Chem *283*, 20330-20341.

Cao, X., Kambe, F., and Seo, H. (2005). Requirement of thyrotropin-dependent complex formation of protein kinase A catalytic subunit with inhibitor of {kappa}B proteins for activation of p65 nuclear factor-{kappa}B by tumor necrosis factor-{alpha}. Endocrinology *146*, 1999-2005.

Chio, C. C., Chang, Y. H., Hsu, Y. W., Chi, K. H., and Lin, W. W. (2004). PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl cAMP. Cell Signal *16*, 565-75.

Chung, S., Brown, J.M., Provo, J.N., Hopkins, R., and McIntosh, M.K. (2005). Conjugated linoleic acid promotes human adipocyte insulin resistance through NFkappaB-dependent cytokine production. J Biol Chem *280*, 38445-38456.

Correze, C., Blondeau, J.P., and Pomerance, M. (2000). The thyrotropin receptor is not involved in the activation of p42/p44 mitogen-activated protein kinases by thyrotropin preparations in Chinese hamster ovary cells expressing the human thyrotropin receptor. Thyroid *10*, 747-752.

Cummings, R., Zhao, Y., Jacoby, D., Spannhake, E.W., Ohba, M., Garcia, J.G., Watkins, T., He, D., Saatian, B., and Natarajan, V. (2004). Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. J Biol Chem *279*, 41085-41094.

Dardano, A., Ghiadoni, L., Plantinga, Y., Caraccio, N., Bemi, A., Duranti, E., Taddei, S., Ferrannini, E., Salvetti, A., and Monzani, F. (2006). Recombinant human thyrotropin reduces endothelium-dependent vasodilation in patients monitored for differentiated thyroid carcinoma. J Clin Endocrinol Metab *91*, 4175-4178.

Dremier, S., Vandeput, F., Zwartkruis, F.J., Bos, J.L., Dumont, J.E., and Maenhaut, C. (2000). Activation of the small G protein Rap1 in dog thyroid cells by both cAMP-dependent and -independent pathways. Biochem Biophys Res Commun *267*, 7-11.

Dumaz, N., and Marais, R. (2005). Integrating signals between cAMP and the RAS/RAF/MEK/ERK signalling pathways. Based on the anniversary prize of the Gesellschaft fur Biochemie und Molekularbiologie Lecture delivered on 5 July 2003 at the Special FEBS Meeting in Brussels. FEBS J *272*, 3491-3504.

Enserink, J.M., Christensen, A.E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H.G., Doskeland, S.O., Blank, J.L., and Bos, J.L. (2002). A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol *4*, 901-906.

Fain, J.N., Madan, A.K., Hiler, M.L., Cheema, P., and Bahouth, S.W. (2004). Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology *145*, 2273-2282.

Fasshauer, M., Klein, J., Lossner, U., and Paschke, R. (2003). Interleukin (IL)-6 mRNA expression is stimulated by insulin, isoproterenol, tumour necrosis factor alpha, growth hormone, and IL-6 in 3T3-L1 adipocytes. Horm Metab Res *35*, 147-152.

Fontana, L., Eagon, J.C., Trujillo, M.E., Scherer, P.E., and Klein, S. (2007). Visceral fat adipokine secretion is associated with systemic inflammation in obese humans. Diabetes *56*, 1010-1013.

Fried, S.K., Bunkin, D.A., and Greenberg, A.S. (1998). Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab *83*, 847-850.

Fried, S.K., and Moustaid-Moussa, N. (2001). Culture of adipose tissue and isolated adipocytes. Methods Mol Biol *155*, 197-212.

Gao, N., Asamitsu, K., Hibi, Y., Ueno, T., and Okamoto, T. (2008). AKIP1 enhances NF-kappaB-dependent gene expression by promoting the nuclear retention and phosphorylation of p65. J Biol Chem *283*, 7834-7843.

Giuliani, C., Noguchi, Y., Harii, N., Napolitano, G., Tatone, D., Bucci, I., Piantelli, M., Monaco, F., and Kohn, L.D. (2008). The flavonoid quercetin regulates growth and gene expression in rat FRTL-5 thyroid cells. Endocrinology *149*, 84-92.

Gonzales, A.M., and Orlando, R.A. (2008). Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes. Nutr Metab (Lond) *5*, 17.

Hacker, H., and Karin, M. (2006). Regulation and function of IKK and IKK-related kinases. Sci STKE *2006*, re13.

Haraguchi, K., Shimura, H., Lin, L., Saito, T., Endo, T., and Onaya, T. (1996). Functional expression of thyrotropin receptor in differentiated 3T3-L1 cells: a possible model cell line of extrathyroidal expression of thyrotropin receptor. Biochem Biophys Res Commun *223*, 193-198.

Harkins, J.M., Moustaid-Moussa, N., Chung, Y.J., Penner, K.M., Pestka, J.J., North, C.M., and Claycombe, K.J. (2004). Expression of interleukin-6 is greater in preadipocytes than in adipocytes of 3T3-L1 cells and C57BL/6J and ob/ob mice. J Nutr *134*, 2673-2677.

Hershko, D.D., Robb, B.W., Luo, G., and Hasselgren, P.O. (2002). Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells. Am J Physiol Regul Integr Comp Physiol *283*, R1140-1148.

Hirotani, S., Otsu, K., Nishida, K., Higuchi, Y., Morita, T., Nakayama, H., Yamaguchi, O., Mano, T., Matsumura, Y., Ueno, H., *et al.* (2002). Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. Circulation *105*, 509-515.

Hochbaum, D., Hong, K., Barila, G., Ribeiro-Neto, F., and Altschuler, D.L. (2008). Epac, in synergy with cAMP-dependent protein kinase (PKA), is required for cAMP-mediated mitogenesis. J Biol Chem *283*, 4464-4468.

Hoene, M., and Weigert, C. (2008). The role of interleukin-6 in insulin resistance, body fat distribution and energy balance. Obes Rev *9*, 20-29.

Hugo, E.R., Brandebourg, T.D., Comstock, C.E., Gersin, K.S., Sussman, J.J., and Ben-Jonathan, N. (2006). LS14: a novel human adipocyte cell line that produces prolactin. Endocrinology *147*, 306-313.

Iacovelli, L., Capobianco, L., Salvatore, L., Sallese, M., D'Ancona, G.M., and De Blasi, A. (2001). Thyrotropin activates mitogen-activated protein kinase pathway in FRTL-5 by a cAMP-dependent protein kinase A-independent mechanism. Mol Pharmacol *60*, 924-933.

Jensen, M.D. (2006). Is visceral fat involved in the pathogenesis of the metabolic syndrome? Human model. Obesity (Silver Spring) *14 Suppl 1*, 20S-24S.

Kikumori, T., Kambe, F., Nagaya, T., Funahashi, H., and Seo, H. (2001). Thyrotropin modifies activation of nuclear factor kappaB by tumour necrosis factor alpha in rat thyroid cell line. Biochem J *354*, 573-579.

Kishore, N., Sommers, C., Mathialagan, S., Guzova, J., Yao, M., Hauser, S., Huynh, K., Bonar, S., Mielke, C., Albee, L., *et al.* (2003). A selective IKK-2 inhibitor blocks NF-kappa B-dependent gene expression in interleukin-1 beta-stimulated synovial fibroblasts. J Biol Chem *278*, 32861-32871.

Klemm, S., Zimmermann, S., Peschel, C., Mak, T.W., and Ruland, J. (2007). Bcl10 and Malt1 control lysophosphatidic acid-induced NF-kappaB activation and cytokine production. Proc Natl Acad Sci U S A *104*, 134-138.

Kwak, H.J., Park, K.M., Choi, H.E., Chung, K.S., Lim, H.J., and Park, H.Y. (2008). PDE4 inhibitor, roflumilast protects cardiomyocytes against NO-induced apoptosis via activation of PKA and Epac dual pathways. Cell Signal *20*, 803-814.

Lapensee, C.R., Hugo, E.R., and Ben-Jonathan, N. (2008). Insulin Stimulates Interleukin-6 Expression and Release in LS14 Human Adipocytes through Multiple Signaling Pathways. Endocrinology.

Lochner, A., and Moolman, J.A. (2006). The many faces of H89: a review. Cardiovasc Drug Rev *24*, 261-274.

Murray, A.J. (2008). Pharmacological PKA inhibition: all may not be what it seems. Sci Signal *1*, re4.

Nika, K., Hyunh, H., Williams, S., Paul, S., Bottini, N., Tasken, K., Lombroso, P. J., Mustelin, T. (2004). Haematopoietic protein tyrosine phosphatase (HePTP) phosphorylation by cAMP-dependent protein kinase in T-cells: dynamics and subcellular location. Biochem J *378*, 335-42.

Norum, J.H., Dawood, H., Mattingly, R.R., Sandnes, D., and Levy, F.O. (2007). Epac- and Rap- independent ERK1/2 phosphorylation induced by Gs-coupled receptor stimulation in HEK293 cells. FEBS Lett *581*, 15-20.

Okamoto, H., Cujec, T.P., Yamanaka, H., and Kamatani, N. (2008). Molecular aspects of rheumatoid arthritis: role of transcription factors. FEBS J *275*, 4463-4470.

Panka, D.J., Mano, T., Suhara, T., Walsh, K., and Mier, J.W. (2001). Phosphatidylinositol 3-kinase/Akt activity regulates c-FLIP expression in tumor cells. J Biol Chem *276*, 6893-6896.

Path, G., Bornstein, S.R., Gurniak, M., Chrousos, G.P., Scherbaum, W.A., and Hauner, H. (2001). Human breast adipocytes express interleukin-6 (IL-6) and its receptor system: increased IL-6 production by beta-adrenergic activation and effects of IL-6 on adipocyte function. J Clin Endocrinol Metab *86*, 2281-2288.

Perkins, N.D. (2007). Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol *8*, 49-62.

Persson, P.B., Skalweit, A., and Thiele, B.J. (2004). Controlling the release and production of renin. Acta Physiol Scand *181*, 375-381.

Rivas, M., and Santisteban, P. (2003). TSH-activated signaling pathways in thyroid tumorigenesis. Mol Cell Endocrinol *213*, 31-45.

Sands, W.A., and Palmer, T.M. (2008). Regulating gene transcription in response to cyclic AMP elevation. Cell Signal *20*, 460-466.

Schmid, J.A., and Birbach, A. (2008). IkappaB kinase beta (IKKbeta/IKK2/IKBKB)--a key molecule in signaling to the transcription factor NF-kappaB. Cytokine Growth Factor Rev *19*, 157-165.

Siddappa, R., Martens, A., Doorn, J., Leusink, A., Olivo, C., Licht, R., van Rijn, L., Gaspar, C., Fodde, R., Janssen, F., *et al.* (2008). cAMP/PKA pathway activation in human mesenchymal stem cells in vitro results in robust bone formation in vivo. Proc Natl Acad Sci U S A *105*, 7281-7286.

Sobell, H.M. (1985). Actinomycin and DNA transcription. Proc Natl Acad Sci U S A *82*, 5328-5331.

Szabo-Fresnais, N., Blondeau, J.P., and Pomerance, M. (2008). Activation of the cAMP pathway synergistically increases IL-1-induced IL-6 gene expression in FRTL-5 thyroid cells: involvement of AP-1 transcription factors. Mol Cell Endocrinol *284*, 28-37.

Taddei, S., Caraccio, N., Virdis, A., Dardano, A., Versari, D., Ghiadoni, L., Ferrannini, E., Salvetti, A., and Monzani, F. (2006). Low-grade systemic inflammation causes endothelial dysfunction in patients with Hashimoto's thyroiditis. J Clin Endocrinol Metab *91*, 5076-5082.

Tan, K.S., Nackley, A.G., Satterfield, K., Maixner, W., Diatchenko, L., and Flood, P.M. (2007). Beta2 adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA- and NF-kappaB-independent mechanisms. Cell Signal *19*, 251-260.

Trayhurn, P., and Wood, I.S. (2004). Adipokines: inflammation and the pleiotropic role of white adipose tissue. Br J Nutr *92*, 347-355.

Voigt, C., Prodinger, C., and Paschke, R. (2007). The TSH receptor is linked with AP1 and NFkappaB signaling in COS7 cells. Exp Clin Endocrinol Diabetes *115*, 590-593.

Wang, B., Jenkins, J.R., and Trayhurn, P. (2005). Expression and secretion of inflammation-related adipokines by human adipocytes differentiated in culture: integrated response to TNF-alpha. Am J Physiol Endocrinol Metab *288*, E731-740.

Wang, D., You, Y., Lin, P.C., Xue, L., Morris, S.W., Zeng, H., Wen, R., and Lin, X. (2007). Bcl10 plays a critical role in NF-kappaB activation induced by G protein-coupled receptors. Proc Natl Acad Sci U S A *104*, 145-150.

Weetman, A.P., Bright-Thomas, R., and Freeman, M. (1990). Regulation of interleukin-6 release by human thyrocytes. J Endocrinol *127*, 357-361.

Wegener, E., and Krappmann, D. (2007). CARD-Bcl10-Malt1 signalosomes: missing link to NF-kappaB. Sci STKE *2007*, pe21.

Xie, P., Browning, D.D., Hay, N., Mackman, N., and Ye, R.D. (2000). Activation of NF-kappa B by bradykinin through a Galpha(q)- and Gbeta gamma-dependent pathway that involves phosphoinositide 3-kinase and Akt. J Biol Chem *275*, 24907-24914.

6.0 CURRICULUM VITAE

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EDUCATION

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University of Ottawa, Biochemistry Program. September 2003 - Present
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Bachelor of Science
Clinical Analysis and Biochemistry May 1999 - June 2000
Universidade Federal de Santa Catarina - UFSC, Florianópolis - SC - Brazil

Bachelor of Pharmacy August 1995 - May 1999
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PUBLICATIONS

Gagnon A, **Antunes TT**, Ly T, Pongsuwan P, Gavin C, Lochnan H, Sorisky A. TSH stimulates lipolysis in adipocytes in culture and raises serum free fatty acid levels in vivo. Am J Physiol Endocrinol Metab. (submitted June, 2009).

Langille ML, Gagnon A, **Antunes TT,** Sorisky A. TSH Stimulates MCP-1 Production in Abdominal Differentiated Human Adipocytes. Arterioscler Thromb Vasc Biol. (submitted June, 2009).

Antunes TT, Gagnon A, Langille ML, Sorisky A. Thyroid Stimulating Hormone Induces Interleukin-6 Release from Human Adipocytes Through Activation of the NF-{kappa}B Pathway. Endocrinology. 2008;149(6):3062-6.

Sorisky A, **Antunes TT**, Gagnon A. The Adipocyte as a Novel TSH Target. Mini Rev Med Chem. 2008;8(1):91-6.

Antunes TT, Gagnon A, Chen B, Pacini F, Smith TJ, Sorisky A. IL-6 release from human abdominal adipose cells is regulated by TSH: the effect of adipocyte differentiation and anatomic depot. Am J Physiol Endocrinol Metab. 2006;290(6):E1 140-4.

Antunes TT, Gagnon A, Bell A, Sorisky A. TSH stimulates interleukin 6 release from 3T3-L1 adipocytes via a cAMP-protein kinase A pathway. Obes Res. 2005;13(12):2066-71.

Sorisky A, Gagnon A, Bell A, **Antunes TT**. Extra-thyroidal effects of TSH on adipose cells. Recent Res Devel Physiol. 2004; 2: 25-34.

Terluk M, Cunha Da Silva E, **Antunes T**, Assreuy J. The presence of the endothelial layer reduces nitric oxide-induced hyporesponsiveness to phenylephrine in rat aorta. Endothelium. 2004;11(3-4):181-7.

Tanphaichitr N, Haebe J, Leader A, Carmona E, Harris J,Silva S, **Antunes T**, Chakrabandhu K, and Léveille MC. Towards a More Precise Assay of Sperm Function in Egg Binding: Which ART is Best Suited for Treating Male Infertility. J Obstet Gynaecol. 2003;25(6):461-70.

POSTER PRESENTATIONS

Gagnon A, **Antunes TT**, Ly T, Pongsuwan P, Gavin C, Lochnan H and Sorisky A. June 2009. Thyroid- Stimulating Hormone Induces Lipolysis in 3T3-L1 and Human Subcutaneous Abdominal Adipocytes. Endocrine Society's 91st Annual Meeting, Washington, DC, USA.

Langille ML, **Antunes TT**, Gagnon A and Sorisky A. June 2009. Pro-inflammatory Actions of TSH on Human Abdominal Differentiated Adipocytes. Endocrine Society's 91st Annual Meeting, Washington, DC, USA.

Antunes TT, Langille ML, Gagnon A and Sorisky A. June 2008. Thyroid Stimulating Hormone Activates the NF-κB pathway to Induce Interleukin-6 Release From Human Adipocytes. Endocrine Society's 90th Annual Meeting, San Francisco, California, USA.

Antunes TT, Gagnon A, Bell A and Sorisky A. June 2007. Thyroid Stimulating Hormone-Induced IL-6 Release from Human Adipocytes Is Independent of cAMP-PKA Signaling. Endocrine Society's 89th Annual Meeting, Toronto, Ontario, Canada.

Antunes TT, Gagnon A, Chen B, Pacini F, Smith TJ, and Sorisky A. June 2006. Effect of Stage of Differentiation, Anatomic Depot, and TSH Treatment on Interleukin 6 release from Abdominal Adipose cells. American Diabetes Association 66th Scientific Sessions, Washington, DC, USA.

Antunes TT, Gagnon A, Bell A and Sorisky A. November 2004. Thyroid Stimulating Hormone Stimulates IL-6 Secretion in 3T3-L1 Adipocytes. 4th Annual Ottawa Health Research Institute Research Day, Ottawa, Ontario, Canada.

Silva S, **Antunes T,** Chakrabandhu K, Harris J, Carmona E and Tanphaichitr N. September, 2002. Binding of Human Sperm to Immobilized Recombinant Human ZP3 (rec hZP3). Attempts to Establish an Alternative Method for Sperm Quality Analysis. 48th Annual Meeting- Canadian Fertility and Andrology Society, Charlevoix, Quebec, Canada.

Antunes TT, Terluk MR and Assreuy J. September 2000. The Endothelium Decreases in Response to Phenylepinephrine Induced by Nitric Oxide in Rat Aorta. XVI Latin-American Congress of Pharmacology, Águas de Lindoia, São Paulo, Brazil.

ORAL PRESENTATIONS

Antunes TT, Gagnon A, Bell A, Sorisky A. October 2005. Thyroid Stimulating Hormone and Interleukin-6 production: the effect of differentiation and anatomic depot. 5th Annual Ottawa Health Research Institute Research Day, Ottawa, ON, Canada.

Antunes TT, Gagnon A, Bell A, Sorisky A. June 2005. Interleukin 6 secretion by 3T3-L1 adipocytes is stimulated by thyroid stimulating hormone via a cAMP-PKA dependent pathway. Endocrine Society's 87th Annual Meeting, San Diego, CA, U.S.A.

Antunes TT, Gagnon A, Bell A and Sorisky A. October 2004. Thyroid Stimulating Hormone Stimulates IL-6 Secretion in 3T3-L1 Adipocytes. 8th Annual Canadian Diabetes Association Professional Conference and Annual Meetings, Quebec City, Quebec, Canada.

ACADEMIC AWARDS

Heart and Stroke Foundation of Canada Doctoral Research Award
June 2006 – December 2009

Oral presentation (first prize):
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Abstract Travel Award
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PROFESSIONAL EXPERIENCES

Research Trainee
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Pharmacist
Phama 55 Pharmacy, Itajai, Brazil August 2000 - February 2001
Dispensing medication, psychotropic drugs control, health orientation.

Technical Assistant March 2000 - July 2000
Clinical Analysis Laboratory, UFSC, Brazil.
Laboratory techniques: Parasitology, Development of Lutz Method, Faust Method and Rugai Method.

Research Trainee
Department of Pharmacology, UFSC, Brazil. October 1999 - July 2000
Project: Study on the Involvement in the Septic Shock, in the Inflammation and in Citotoxicity on Tumor Cells.

Teaching Assistant,
Pharmacotechnique, UFSC, Brazil. February 1999 - July 1999
Teaching the students during the classes.
Laboratory techniques: Manipulation of formulas such shampoo, conditioner, sun block, lipstick and make up.

Teaching Assistant
Toxicological Information Centre, UFSC, Brazil. September 1998 - December 1998
Giving information to the hospitals about poisoning and its treatment.

Pharmacist Trainee,
SESI Pharmacy, UFSC, Brazil. March 1998 – July 1999
Dispensing medication, allopathic, homeopathic medication and cosmetic manipulation.

LABORATORY SKILLS

Adipocyte isolation
Adipose organ culture
Cell culture
Chromatin Immunoprecipitation (ChIP)
DNA extraction
ELISA
Enzymology
Light and Fluorescent microscopy
Northern Blot
Protein purification (size exclusion chromatography, ion-exchange)

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Reverse Transcription (RT) Polymerase Chain Reaction (PCR)
Real Time PCR
SDS-PAGE
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