Regulation of Adipocyte Lipolysis by TSH and its Role in Macrophage Inflammation

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ABSTRACT

Elevated Thyroid-Stimulating Hormone (TSH) is associated with an increased risk of cardiovascular disease (CVD). We hypothesized that TSH-stimulated FA release from adipocytes contributes to macrophage inflammation. 3T3-L1 and human subcutaneous differentiated adipocytes were treated with TSH for 4 hours under various conditions and lipolysis assessed via glycerol secretion. Optimal conditions were determined and protein expression of ATGL, HSL and perilipin remained stable. TSH-stimulated 3T3-L1 or human adipocyte-conditioned medium (T-ACM) was placed on murine J774 or human THP-1 macrophages, respectively, and macrophage cytokine mRNA levels (IL-1β, IL-6, MCP-1, and TNFα) were measured by real-time RT-PCR. T-ACM did not change cytokine mRNA expression in J774 macrophages or THP-1 macrophages when compared to ACM. Absence of BSA in the medium may have hindered release of FA from differentiated adipocytes into the medium, BSA may be required to permit adequate FA accumulation in the medium to then evaluate the effect of T-ACM on macrophages. Further investigation is required to determine the effect of FA on J774 and THP-1 inflammatory response.

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LIST OF ABBREVIATIONS

ACM	Adipocyte-conditioned media
ALA	α-linolenic acid
AT	Adipose tissue
ATM	Adipose tissue macrophage
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
ASC	Acyl-CoA synthetases
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
bTSH	Bovine TSH
CAD	Coronary artery disease
cAMP	3', 5'-cyclic adenosine monophosphate
ccl 2	CC chemokine ligand 2
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CREB	cAMP response element-binding
CRP	C-reactive protein
CLS	Crown-like structures
CS	Calf serum
CVD	Cardiovascular disease
DAG	Diacylglycerol
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethy sulfoxide
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FA	Fatty acid
FFA	Free fatty acid
FAF-BSA	Fatty acid free bovine serum albumin
FBS	Fetal bovine serum
FRTL-5	Fischer rat thyroid line-5
FSH	Follicle-stimulating hormone
GPCR	G-protein coupled receptor
G3P	Glycerol-3-phosphate, α-glycerophosphate
HBSS	Hanks balanced saline solution
HCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HEK293	Human embryonic kidney cell 293

HPT	Hypothalamic-pituitary-thyroid
HSL	Hormone-sensitive lipase
IBMX	Isobutylmethylxanthine
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IP3	Inositol 3,4,5-trisphosphate
IOD	Integrated optical density
LA	Linoleic acid
LDL	Low density lipoprotein
LD	Lipid droplet
LH	Luteinizing hormone
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAG	Monoacyl glycerol
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MGL	Monoacyl glycerol kinase
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
NaCl	Sodium chloride
NaF	Sodium fluoride
NaPPi	Sodium pyrophosphate
Na_3VO_4	Sodium orthovanadate
NEFA	Non-esterified fatty acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
PIP2	Phosphatidylinositol bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PS	Penicillin/streptomycin
PUFA	Poly-unsaturated fatty acid
rhTSH	Recombinant human TSH
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SAT	Subcutaneous adipose tissue
SDS	Sodium dodecyl sulfate
SFA	Saturated fatty acid
T_3	Triiodothryonine
T_4	Thyroxine
TG	Triglyceride
TLR	Toll-like receptor

TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
T-ACM	TSH-stimulated adipocyte conditioned medium
TSHR	Thyroid stimulating hormone receptor
VAT	Visceral adipose tissue
VCAM-1	Vascular adhesion molecule-1
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue
WHO	World Health Organization

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INTRODUCTION

I. TSH

a. TSH Structure

Thyroid stimulating hormone (TSH), also known as thyrotropin, is a heterodimer glycoprotein originating from the anterior pituitary. It is 28-30 kDa in size and is comprised of two subunits, the alpha and beta subunits, held together by non-covalent bonding. Both subunits contain domains involved in receptor binding but the β subunit is unique to TSH and specifies its biological activity and receptor specificity. The β subunit contains the "Keutmann's loop" (TSHβ 32-50) that interacts with the C-terminal domain of the TSH receptor (TSHR) (Morris, McCormick, & Ryan, 1990; Nez-Miguel et al., 2008). This subunit also contains a "seat-belt" domain (TSHβ 88-105) that interacts with the α subunit of TSH with the N-terminal domain of the TSHR and (Grossmann et al., 1997; Nez-Miguel et al., 2008). The TSH α subunit, on the other hand, is common to several other glycoprotein hormones including follicle-stimulating hormone (FSH), luteinizing hormone (LH) and human chorionic gonadotropin (hCG).

TSH also contains several post-translational modifications. Asparagine-linked glycosylation makes up 15-25% of the molecular weight and can display different carbohydrate branching patterns and chain additions (Szkudlinski, Fremont, Ronin, & Weintraub, 2002). These carbohydrate chains can be terminally sulfated or sialylated on the biantennary ends and can influence hormone structure stability, receptor binding affinity and activity (Szkudlinski et al., 2002).

b. TSH and the Hypothalamic-Pituitary-Thyroid Axis

TSH contributes to metabolic regulation through the hypothalamic-pituitary-thyroid axis (HPT axis). Thyrotropin-releasing hormone (TRH), synthesized and released by the hypothalamus, stimulates production and release of TSH from the anterior pituitary. The physiological range of serum TSH in humans is approximately 0.5 to 4.5 mU/L. TSH, in turn, stimulates the production and secretion of thyroid hormone. TSH is also known to act on the thyroid to control iodine uptake, thyroid hormone synthesis, cell survival and growth (Grossmann et al., 1997). Thyroid hormone, a tyrosine-derived hormone, is secreted predominantly as thyroxine (T_4) and is deiodinated to the bioactive triiodothyronine (T_3).

Within the HPT axis there is a feedback loop whereby thyroid hormone negatively regulates TRH and TSH; TSH is also capable of negatively regulating TRH. This ensures appropriate thyroid hormone levels for proper physiological function.

Thyroid hormone has a myriad of effects within the body. Thyroid hormone is a determinant of metabolic rate through glucose uptake regulation and oxygen consumption (Yen, 2001). It is also involved in bone development and growth, cardiac output, adipocyte development and function, liver metabolic processes, brain development in neonates, as well as the synthesis and secretion of other pituitary hormones (Yen, 2001). These effects are exerted in both a genomic and non-genomic fashion (Zoeller, Tan, & Tyl, 2007).

c. Commercial Preparations of TSH

There are several forms and sources of commercially prepared TSH for use *in vivo* and *in vitro*. Since it is a glycoprotein with post-translational modifications, there can be differences in the structure and function of the protein that must be taken into account.

Purified bovine TSH (bTSH), given its strong receptor affinity, bioactivity and low cost, is commonly used in *in vitro* studies. Other animal purified TSH preparations, such as human and rat, are also available, although the limited availability and high cost make them an impractical option for *in vitro* study. There are three glycosylation sites that remain the same on both bTSH and human TSH (hTSH), however the oligosaccharide size and composition may differ (Green & Baenziger, 1988a). Moreover, there is a noted difference in the oligosaccharide terminal caps as the bTSH are found to be fully sulfated while hTSH is only half sulphated (Green & Baenziger, 1988b; Grossmann et al., 1997). These molecular additions have an impact on binding affinity, bioactivity and half-life length.

With the emergence of recombinant protein engineering, recombinant human TSH (rhTSH) has become another source of TSH. Produced in Chinese hamster ovary (CHO) cells, rhTSH lacks the penultimate N-acetylgalactosamine and terminal sulfate found on human TSH (Szkudlinski, 2004). Instead, it has increased sialylation that results in a longer half-life, thereby making up for the reduced human TSH receptor affinity and weak intrinsic bioactivity when compared to other forms *in vitro* and *in vivo* (Szkudlinski, 2004). These differences in TSH structure may affect signalling pathways and therefore it is important to compare the activity of different TSH sources.

d. TSH Receptor

G protein-coupled receptors (GPCR) are the largest family of receptors with nearly 800 human genes encoding them. They are coupled to intracellular G proteins that are critical for determining signal transduction. These receptors are structurally characterized by an extracellular N-terminal domain, followed by 7 transmembrane α-helices and an

intracellular C-terminal tail. The TSH receptor (TSHR) is a GPCR that is a member of the rhodopsin/β-adrenergic receptor family and glycoprotein hormone receptor subfamily (Urizar et al., 2005). In addition to the 7 transmembrane domains of the TSHR, a large extracellular N-terminal domain containing leucine rich-repeats is also present, making the TSH receptor inherently complex (Kajava, Vassart, & Wodak, 1995). The structure is also complicated by the potential cleavage of the mature receptor into two subunits, an extracellular α- and transmembrane β-subunit, which are held together via disulfide bonds (Davies, Marians, & Latif, 2002). This cleavage removes a 50 amino acid chain and has no impact on signalling function but allows for the potential for α-subunit shedding and subsequent receptor degradation (Chazenbalk, Tanaka, McLachlan, & Rapoport, 1999). The receptor has been described as 'noisy', capable of signal transduction despite ligand absence, and the binding specificity remains to be fully elucidated (Latif, Morshed, Zaidi, & Davies, 2009; Vassart et al., 1995). Thus the existence and function of these structurally different TSH receptors has had confounding effects on the receptor characterization and their physiological relevance. Despite this ambiguity about the receptor structure, an understanding of its intracellular signalling as a GPCR family member has emerged.

e. TSH Signalling

In thyrocytes, the TSH receptor is coupled to different intracellular G-proteins that initiate alternative downstream signalling cascades upon activation (Laugwitz et al., 1996). When TSHR signals through Gαs, adenyl cyclase activity is stimulated, thereby generating cAMP (Zanger, Cohen, Hashimoto, Radovick, & Wondisford, 1999). Cyclic AMP activates a variety of targets, such as PKA, leading to phosphorylation of cAMP response element-

binding (CREB) (Zanger et al., 1999). The PKA pathway is mainly responsible for the secretion of thyroid hormone, differentiation and proliferation of thyrocytes (Dumont, Lamy, Roger, & Maenhaut, 1992; Rivas & Santisteban, 2003). Alternatively, when TSHR signals through Gαq, phospholipase C is activated, leading to the hydrolysis of phosphatidylinositol 4,5, bisphosphate (PIP2) to inositol 3,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Morshed, Latif, & Davies, 2009). This pathway activity is implicated in the production of thyroid hormone (Van Sande et al., 2006).

f. Extra-thyroidal TSH targets

Expression of TSHR outside the thyroid gland is now an accepted fact. While TSHR expression has not been observed in J774 macrophages, its expression has been documented in adipocytes, lymphocytes, retro-orbital fibroblasts, neuronal cells, osteoclasts and astrocytes (Latif et al., 2009; Szkudlinski et al., 2002). Consequently, an altered level of TSH not only has an impact on thyrocytes in the HPT axis, but could potentially alter the function of non-thyroidal targets as well. The role of TSH in non-thyroidal systems is incompletely understood and requires further investigation (Szkudlinski et al., 2002). Subclinical hypothyroidism is one such condition that results in elevated TSH levels.

II. Subclinical Hypothyroidism (SCH)

a. SCH: Pathophysiology and Treatment

Mild thyroid gland failure can result in subclinical hypothyroidism. The initial reduction in thyroid hormone levels as the gland begins to fail is restored by a compensatory increase in TSH levels. The most common cause for thyroid gland failure is chronic autoimmune thyroiditis. SCH is believed to occur in ~11% of the population depending on population demographics and higher prevalence rates of SCH have been found in females versus males as well as with increasing age (Canaris, Manowitz, Mayor, & Ridgway, 2000; Duntas & Wartofsky, 2007; Hak et al., 2000; Jones, May, & Geraci, 2010; Ochs et al., 2008). As time goes by, and if thyroid gland failure worsens, SCH can progress to overt hypothyroidism, with frankly low thyroid hormone values (Tunbridge et al., 1981). SCH is often asymptomatic, but some patients may exhibit symptoms that are mild or non-specific (Jones et al., 2010). Symptoms of SCH can include fatigue, cold intolerance, skin dryness and constipation (Jones et al., 2010). Laboratory findings indicate that SCH is sometimes associated with elevated levels of total cholesterol, low-density lipoprotein-cholesterol (LDL-cholesterol), and serum triglyceride when compared to euthyroid controls (Caraccio et al., 2005; Kvetny, Heldgaard, Bladbjerg, & Gram, 2004; Tremen, Cetinarslan, Sahin, Cantrk, & Tarkun, 2011).

There is no clear consensus on the approach to manage SCH. Some argue that although TSH levels are increased, thyroid hormone levels are within normal limits so that no treatment is warranted. Long-term follow up, every 6 to 12 months, is suggested instead (Surks et al., 2004). Alternatively, others argue that subclinical hypothyroidism is not a

completely euthyroid state, that it may contribute to poor health, and that progression to overt hypothyroidism is a concern. They suggest early treatment of SCH with thyroid hormone, especially when considering several factors including the extent of the rise in TSH (>10 mU/L), the presence of thyroid goiter, presence of autoimmune thyroid antibodies, and other medical conditions (Cooper, 2001; Kaplowitz, 2010; McDermott & Ridgway, 2001).

III. SCH and Cardiovascular Disease

a. Cardiovascular Disease

Cardiovascular disease (CVD) is the general term for all arterial, venous and heart specific diseases and continues to be the largest cause of death in the world (WHO, 2011). Atherosclerosis, one of the main cardiovascular diseases, involves the development of fatty streaks that progress to plaques within the vascular walls, leading to coronary artery disease, cerebrovascular disease, and peripheral vascular disease (G. Hansson, Robertson, & Sderberg-Nauclr, 2006). Underlying determinants and conventional risk factors for CVD include age, sex, dyslipidemia, diabetes, hypertension, obesity, psychological stress, and nutrition (Roifman, Beck, Anderson, Eisenberg, & Genest, 2011). A feature that is common to some of CVD predictors is a state of low-grade inflammation. Adipose tissue, when dysfunctional, can be a prominent source of inflammatory molecules, and this will be discussed in further detail in section IV. Adipocytes are a TSH-responsive extra-thyroidal target, and the elevated TSH values in SCH may contribute to CVD risk.

b. ***SCH and CVD Risk***

SCH is associated with increased arterial stiffness, endothelial dysfunction, mild ECG abnormalities, impaired vasodilation, carotid artery-intima media thickening and a prothrombotic state (Cantrk et al., 2003; Cikim et al., 2004; S. Kim, Kim, Park, Park, & Cho, 2009; Owen et al., 2006; Taddei et al., 2003; Tunbridge et al., 1977). These pathophysiological changes suggest a link between SCH and cardiovascular disease (CVD). The evidence supporting this link has accumulated over several decades.

Many studies have investigated the associated risk of SCH and CVD and they have recently been subjected to a meta-analysis. The individual clinical data of 55287 patients (3450 with subclinical hypothyroidism and 51837 euthyroid controls) was used for the assessment of this risk (Rotondi, Magri, & Chiovato, 2010). The authors concluded that a significant trend of increased risk of CHD events is found with increasingly elevated levels of TSH (Rotondi et al., 2010). This is notable in concentrations greater than 10mU/L, whose hazard ratio was 1.89 (Rotondi et al., 2010). This association was independent of age, gender, or traditional risk factors such as systolic blood pressure, smoking, total cholesterol and diabetes (Rotondi et al., 2010).

The underlying causal basis for this CVD risk association is not known. Some pathophysiological mechanisms have been studied and suggested, but the mode of action remains unclear. Increased levels of TSH in SCH can increase markers of oxidative stress and produce low-grade inflammation through elevation of C reactive protein (CRP), IL-6 and TNFα (Caraccio et al., 2005; Kvetny et al., 2004; Marfella et al., 2011; Tremen et al., 2011). Similarly, rhTSH administration to humans, whose thyroids have been removed due to thyroid cancer, enhances proatherogenic inflammation and reduces endothelium-

dependent vasodilation through oxidative stress (Dardano et al., 2006; Desideri et al., 2009). Understanding the underlying mechanisms responsible for this associated risk could help determine the indications for, and the types of approach to, treatment of this condition. As previously mentioned, adipocytes have functional TSH receptors and could also be involved in this setting of elevated TSH levels.

IV. Adipose Tissue (AT)

a. AT as an Extra-Thyroidal TSH Target

There are two main types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). Brown adipose tissue consumes high amounts of energy and is thermogenic. It is present in the adult human body in a restricted anatomic distribution, located in the paracervical and supraclavicular area (Cypess et al., 2009; Virtanen et al., 2009). WAT, the focus tissue of my thesis, is found throughout the human body with large intra-abdominal and subcutaneous depots (Gesta, Tseng, & Kahn, 2007). White adipose tissue stores excess energy as lipids in the form of triacylglycerol, and elegant control mechanisms enable it to release this stored energy in times of need. The discovery of leptin by Friedman et al in 1994, and further studies demonstrating that adipose cells can produce and secrete a variety of bioactive factors supports a role for adipose tissue as an endocrine organ (Cypess et al., 2009; Vázquez-Vela, Torres, & Tovar, 2008). Adipocytes are capable of releasing cell-signalling factors called cytokines. Cytokines comprise of a large group of proteins and peptides whose intercellular communication help regulate pro- and anti-inflammatory responses.

Adipose tissue consists of a heterogeneous mixture of cells, with adipocytes comprising ~ 50-70% of the cells (Hauner, 2005). Other cell types present include preadipocytes, endothelial cells and macrophages, all of which could potentially interact in an endocrine, paracrine or autocrine fashion to help regulate metabolic functions (Hauner, 2005; Lee, Kehlenbrink, Lee, Hawkins, & Yudkin, 2009; Suganami, Nishida, & Ogawa, 2005). The functional ability of adipose tissue means that it may have a role in the etiology of certain diseases and conditions when improperly regulated.

Adipocytes may be an extra-thyroidal target for TSH as they exhibit functional TSH receptors. In fact, our laboratory has previously demonstrated that thyroidectomized patients (previous thyroid cancer treatment) administered two doses of rhTSH for diagnostic purposes on day 1 and day 2 exhibit elevated levels of serum free fatty acids on day 5 post injections (Gagnon et al., 2010). Others have shown that adipose-specific TSH receptor knockout mice have a 10-fold reduction in TSH stimulated lipolysis, a larger adipocyte size, and a higher level of basal lipolysis (Elgadi, Zemack, Marcus, & Norgren, 2010). In the case of elevated levels of TSH in patients with SCH, adipocytes may become dysfunctional, suggesting a potential pathway that may promote cardiovascular disease. There is evidence to suggest that this route may be through FA and their potential to provoke inflammation within the body.

Obesity is another well documented example of when adipose tissue becomes dysfunctional. It is defined by a body mass index (where weight is in kg/height in m^2) of greater than 30 kg/m^2, and is implicated in cardiovascular disease, insulin resistance, type 2 diabetes mellitus, and some forms of cancer (Haslam & James, 2005). It is important to

understand how adipocytes function to delineate cellular responses involved in pathophysiology.

b. Regional Differences of AT

Adipocytes have marked regional differences in metabolic activity, both hormonal and basal responsiveness, depending on the region of the body from which they originate (Kissebah & Krakower, 1994). White adipose tissue is traditionally subdivided into visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT). Subcutaneous adipose tissue can be further subdivided into upper or lower body fat. Due to metabolic differences in regional fat deposits, a person's weight distribution can determine their potential risk for metabolic syndrome. Metabolic syndrome describes a cluster of metabolic defects that increases the risk of cardiovascular disease and diabetes. The conditions defining metabolic syndrome vary depending on the authors, but, in general, include: hypertension, insulin resistance, abnormal cholesterol levels and abdominal obesity. In obese subjects with a pear-shaped figure, lower body subcutaneous fat accumulation predominates and there is a lower risk of metabolic syndrome or diabetes (Lapidus et al., 1984; Rimm, Hartz, & Fischer, 1988). Obese subjects with an apple-shaped distribution (central) have increased visceral and upper subcutaneous adipose tissue and have a higher risk of diabetes and cardiovascular disease (Lapidus et al., 1984; Ohlson et al., 1985; Rimm et al., 1988). Upper body adipose tissue has been shown to have greater lipolytic activity than lower body SAT (Jensen, 1997). Within the upper body adipose tissues, VAT has been shown to have greater lipolytic activity than abdominal SAT (Ostman, Arner, Engfeldt, & Kager, 1979). However, upper body SAT lipolysis contributes a considerable amount more to systemic FFA in upper body

obesity and may be important to peripheral dysfunction (Jensen, 2006; Koutsari & Jensen, 2006). VAT also secretes more IL-6 than SAT *ex vivo* (Fain, Madan, Hiler, Cheema, & Bahouth, 2004; Fried, Bunkin, & Greenberg, 1998).

c. Adipocyte Cell Models

Two adipocyte cell models, one murine and one human, were used in this study. The murine adipocyte model is the 3T3-L1 cell line, which can undergo adipocyte differentiation from preadipocytes to adipocytes. It is derived from disaggregated Swiss 3T3 embryos; the resulting cells were immortalized through a protocol of seeding at a density of 3×10^5 cells per $20cm^2$ dish and transferred every three days (Todaro & Green, 1963). They efficiently and reproducibly differentiate into mature, lipid-filled adipocytes (90-95% differentiation) upon induction with pharmacological activators and insulin. Figure 1 depicts the 3T3-L1 model and its stages of differentiation It is important to note that these cells are aneuploid and therefore may have characteristics that are unlike primary preadipocyte (Cornelius, MacDougald, & Lane, 1994). Another drawback to the use of this model is that they do not allow for the study of depot-specific differences (Rosen & Spiegelman, 2000). Nonetheless, they display general characteristics of adipocytes and their relative ease of use, reliability and consistency make them a widely accepted mouse model (Sorisky, Antunes, & Gagnon, 2008).

The second *in vitro* adipocyte model that was used is the human subcutaneous abdominal differentiated adipocyte. They are derived from primary preadipocytes isolated from adipose tissue samples obtained from patients undergoing elective abdominal surgery or liposuction. These preadipocytes are isolated by collegenase digestion of the stromal vascular fraction,

followed by centrifugation and size filtration. They can only be passaged a limited number of times prior to inducing differentiation as they will otherwise not differentiate adequately (Avram, Avram, & James, 2007; Hauner, Skurk, & Wabitsch, 2001). The isolation process is tedious and cell growth rate is slow. Donor tissue variability can affect growth rate, differentiation capacity and cellular function in primary cultures (Kirkland, Hollenberg, & Gillon, 1990; van Harmelen et al., 2003). Thus while these primary diploid human cells offer a better representation of *in vivo* adipocyte response and function, they are challenging to use, given the limitations described. Figure 2 depicts the human subcutaneous preadipocyte and differentiated adipocyte stages.

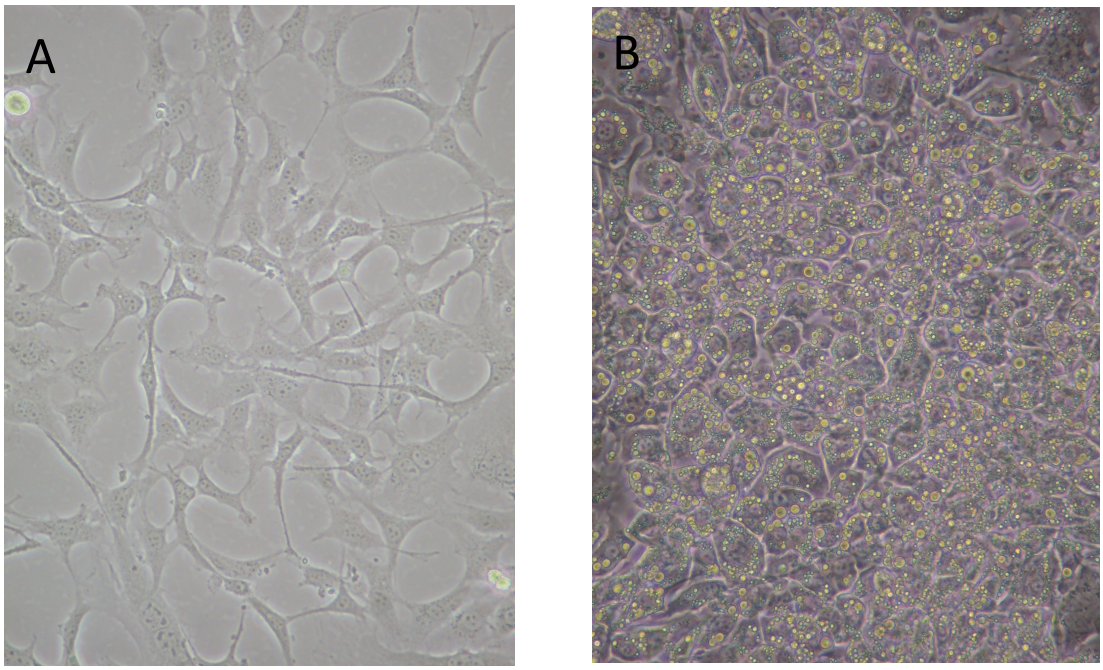

Figure 1. Murine 3T3-L1 preadipocyte and differentiated adipocyte cell models. A) Murine 3T3-L1 preadipocytes maintained in Dulbecco's Modified Eagle Medium (DMEM), 10% calf serum (CS) and antibiotics (100U/ml penicillin, 0.1 mg/ml streptomycin). B) Murine 3T3-L1 adipocytes differentiated in DMEM containing 10% fetal bovine serum (FBS) and antibiotics as described in materials and methods. Cell cultures photographed at a magnification of 200x.

V. AT Lipid Metabolism

a. Lipogenesis

Adipocytes accumulate lipids and store them in a unilocular lipid droplet. The lipid droplet occupies roughly 90% of the cell volume and pushes the organelles and nucleus to the periphery of the adipocyte (Frühbeck, Gómez-Ambrosi, Muruzbal, & Burrell, 2001). Lipid accumulation occurs through two main pathways, TAG synthesis from pre-existing FA and *de novo* FA synthesis. *De novo* lipogenesis in rodents occurs within both the liver and WAT through conversion of carbohydrates to FAs (McDevitt et al., 2001). In humans, TAG synthesis predominantly uses pre-existing FAs for esterification. These FA are found either in the circulation or are derived from circulating TAG present in chylomicrons and very-low-density lipoproteins (VLDL), through the action of lipoprotein lipase (LPL). These TAG-rich particles are too large to pass through the endothelial lining of the AT and are processed within the luminal space where released FA can be taken up by the adipocytes (Lafontan, 2008). The exact mechanism of FA uptake by adipocytes is still unclear, it is currently understood that FA can rapidly traverse the cellular lipid bilayer passively or gain entry through active protein-mediated transport (Wang et al., 2008). Once FAs have entered the cell, they can be incorporated as TAG through esterification for storage until later use.

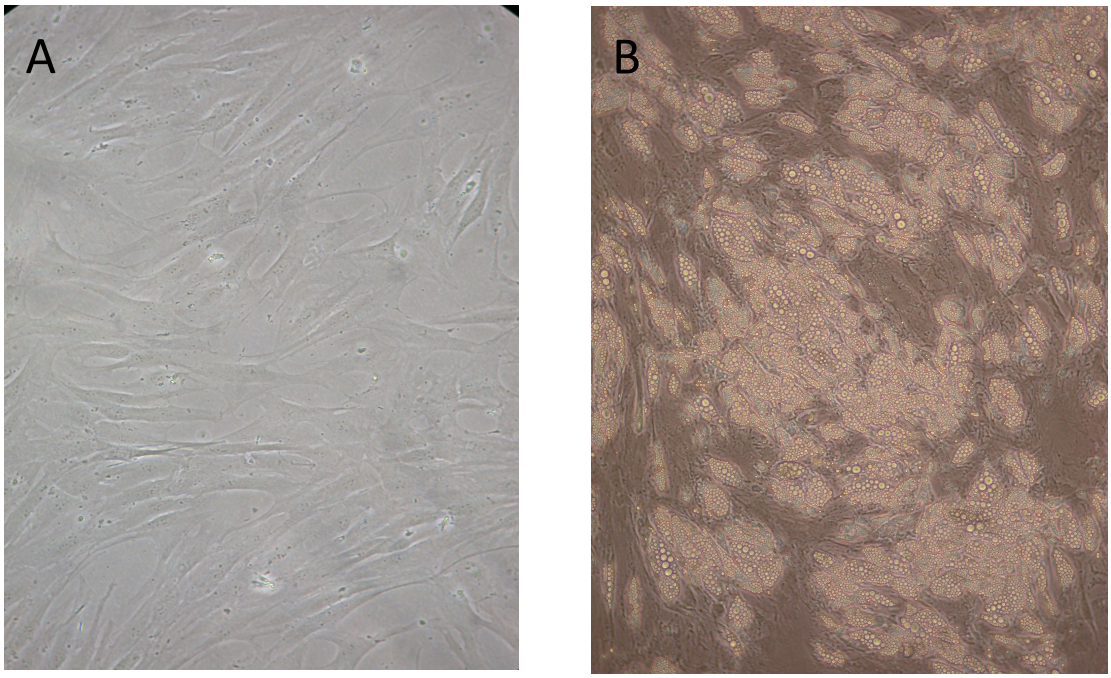

Figure 2. Human subcutaneous adipose tissue preadipocyte and differentiated adipocyte cell models. A) Human subcutaneous preadipocytes maintained in DMEM, 10% calf serum (CS), antibiotics and nystatin. B) Human subcutaneous adipocytes differentiated in DMEM containing 10% FBS, antibiotics and nystatin. Cell cultures photographed at a magnification of 100x.

b. Esterification

The process of esterification requires a 3-carbon backbone in the form of either glycerol-3-phosphate (G3P, α-glycerophosphate), which is generated from glucose metabolism, or monoacylglycerol (Coleman & Lee, 2004). FAs require activation to fatty acyl-CoA via acyl-CoA synthetases prior to their sequential addition to the carbon backbone (Watkins, 1997). Fatty acyl-CoA molecules are enzymatically and sequentially added by three substrate specific acyltransferases to form TAG (Watkins, 1997). G3P acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT) and diglyceride acyltransferase (DGAT) are the enzymes used to respectively add the first, second and third FAs to form TAG. TAGs are then stored in the lipid droplet until they are hydrolytically cleaved through the process of lipolysis.

c. Lipolysis

Lipolysis allows for the release of adipose energy stores in the form of FA when the metabolic demand for energy is increased, such as the fasting state. The complete reaction converts TAG into 3 FA and glycerol, the 3-carbon backbone of triglyceride. FA released by adipocytes are taken up by other tissues and can undergo beta-oxidation to generate ATP.

Lipolysis is classically activated through catecholamine stimulation of the β-adrenergic receptor. There are three lipases currently known to hydrolyze adipocyte TAG. Hormone sensitive lipase (HSL) is capable of hydrolyzing both triacylglycerol (TAG) and diacylglycerol (DAG) through hormonal activation (Langin, Holm, & Lafontan, 1996). In the inactive state, HSL is localized in the cytoplasm with little ability to hydrolyze TAG or DAG (Sztalryd et al., 2003). When β-adrenergic receptor activation occurs, levels of cyclic

AMP rise, PKA is activated, and it phosphorylates HSL. This promotes its translocation to the lipid droplet where it can act on DAG. Concurrently, the lipid droplet coating protein, perilipin, is also phosphorylated by PKA, and that promotes lipid droplet fragmentation as well as providing a required anchoring site for HSL (Sztalryd et al., 2003; H. Wang et al., 2009). HSL knockout mice are not obese and display DAG accumulation (Haemmerle et al., 2002). This is because adipose triglyceride lipase (ATGL, also known as desnutrin and phospholipase-A), a lipid droplet associated protein, is capable of hydrolyzing TAG to DAG (Jenkins et al., 2004; Villena, Roy, Sarkadi Nagy, Kim, & Sul, 2004; Zimmermann et al., 2004). It is not yet clear if this lipase is hormonally regulated, although evidence suggests that ATGL requires a cofactor, ABHD5, for proper enzymatic function and efficiency (Schweiger et al., 2006). After ATGL and HSL cleavage of the first two FAs, MAG is then hydrolyzed by monoacylglycerol lipase (MGL). This lipase is abundant and not hormonally regulated; its final action provides a net result of three non-esterified FAs (NEFA) and one glycerol molecule.

Aside from this canonical pathway, lipolysis can also be regulated through control of lipolytic protein expression levels. Both IL-6 and TNF-α reduce perilipin A expression level which has been inversely correlated with elevated basal lipolysis rate (Bézaire, Mairal, Anesia, Lefort, & Langin, 2009; Kralisch et al., 2005; Petersen et al., 2005; Päth et al., 2001; Souza et al., 2003; Yang, Ju, Zhang, & Yang, 2008). Perilipin acts as a barrier on the lipid droplet and reduces the lipase interaction with the TG substrates (Brasaemle et al., 2000). Lipolytic stimulation is only observed after a longer incubation period, from 6 up to 24 hour depending on the cell culture, as this time is required for the reduction in the perilipin coat on the droplet to occur (Laurencikiene et al., 2007; Päth et al., 2001).

d. Non-Esterified Fatty Acids

A non-esterified FA, also called free fatty acid or simply fatty acid, consists of a carboxylic acid attached to an unbranched carbon chain and is categorized based on the carbon chain length, degree of saturation and type of double bonds present. FAs subdivided by carbon chain length are defined most often as short (<8) medium (8-12), long (13-22) or very long (>22) (The nomenclature of lipids (recommendations 1976) IUPAC-IUB commission on biochemical nomenclature.1978; Harvey et al., 2010; Oram & Bornfeldt, 2004). The degree of saturation divides FA into saturated FA (SFA), monounsaturated FA (MUFA) or polyunsaturated FA (PUFA). Finally, bond types found in the FA, specifically cis- or trans- configuration, are also used to distinguish classes of FAs.

These three physical aspects influence FA functions with respect to the immune system. Generally, saturated FAs are inflammatory whereas monounsaturated FAs (MUFAs) are anti-inflammatory. A Mediterranean diet with high dietary MUFAs in place of SFAs reduces CVD risk (Grundy, 1986; Kris Etherton et al., 1999). The actions of polyunsaturated FA (PUFAs) depend on the placement of the first double bond in the FA chain with the methyl end being designated the first carbon, or n-1. Generally speaking, n-3 PUFAs are anti-inflammatory whereas n-6 PUFAs have a pro-inflammatory effect (Simopoulos, 1999). Fish oils, which are rich in the omega n-3 FAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been well documented as having an anti-inflammatory effect (Calder, 2003). These n-3 FAs replace the n-6 PUFA arachidonic acid (AA) in cell membranes and reduce the amount of pro-inflammatory AA-derived prostaglandins, leukotrienes and inflammatory molecules (Calder, 2003).

Finally, the notorious trans-fats are FA containing one or more trans-isomer double bonds. These FA can be subdivided into their origin of production: industrial or natural. Industrial trans-fats are produced through the hydrogenation of vegetable oils and are considered more inflammatory than their natural counterparts produced in ruminant stomachs through bacterial interaction (Mozaffarian, Katan, Ascherio, Stampfer, & Willett, 2006). Trans fats are not essential for health and increase CVD risk.

e. Re-esterification

FAs newly released from lipolysis may also be re-incorporated into TAG within the adipocyte (re-esterification). Studies have shown that newly cleaved FA in the cytoplasm cannot be re-esterified immediately but instead must cycle through the process of exiting and re-entering the adipocyte before reintroduction into the lipid droplet (Edens, Leibel, & Hirsch, 1990). Little else is known about the process. The glycerol produced from full hydrolysis however is not used in re-esterification and is subsequently released. There is very little glycerokinase present in adipocytes to convert glycerol to G3P, and glycerol is released from the adipocyte for use in other metabolic processes (Margolis & Vaughan, 1962; Vaughan, 1962). The uptake of glycerol by adipocytes is a contentious issue that requires further study (Coppack, Persson, Judd, & Miles, 1999). Most plasma glycerol is used and converted into glucose in the liver, although the kidney and muscle tissue are capable of utilizing glycerol as well (Landau, 1999). Furthermore, a minor amount is oxidized for energy production in the peripheral tissue (Bortz, Paul, Haff, & Holmes, 1972; Burelle et al., 2001; Massicotte et al., 2006).

f. TSH Stimulation of Lipolysis

Evidence supporting TSH-stimulated lipolysis has existed for decades but has not been widely recognized (Benoit, 1967; Marcus, Ehrn, Bolme, & Arner, 1988). As mentioned, several different groups have confirmed the expression of TSHR on adipocytes (Bell et al., 2000; Crisp, Lane, Halliwell, Wynford Thomas, & Ludgate, 1997; Endo, Ohta, Haraguchi, & Onaya, 1995). Our laboratory has shown that TSH activates lipolysis, similarly to catecholamines, through activation of PKA and the phosphorylation of HSL and perilipin (Gagnon et al., 2010). As previously discussed, our laboratory has also shown that rhTSH administration to thyroidectomized patients is also capable of increasing serum FFA, suggesting that a TSH-stimulated adipocyte response may have pathophysiological relevance in SCH (Gagnon et al., 2010). Little else is known about the process or the physiological relevance of TSH-stimulated lipolysis. Understanding the role of adipocytes as extra-thyroidal TSH targets, the process of TSH-stimulated lipolysis and its potential effect on inflammation may provide insight to the role of adipocytes in the CVD risk associated with SCH.

VI. Inflammation

a. Inflammation as a CVD Risk Factor

The understanding of inflammation and its role in CVD has evolved over the years. Whereas the inflammatory response is a fundamental aspect in body repair and infection clearance, a chronic response can be deleterious. Inflammation is believed to be the principal mechanism in the development of CVD, since a number of inflammatory markers such as

IL-6, TNF-α and CRP are consistently associated with an increased CVD risk (Cesari et al., 2003; Roifman et al., 2011). Indeed a recent review suggests there is an increased risk of coronary artery disease (CAD) in patients with chronic inflammatory conditions such as systemic lupus erythematosus, rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis (Cesari et al., 2003; Roifman et al., 2011).

Inflammation in the body is regulated by leukocytes. These specialized cells begin as multipotent hematopoietic stem cells within the bone marrow. These multipotent cells can differentiate into specialized leukocytes subdivided into either adaptive or innate immune cells. Adaptive immune cells are generally classified as lymphocytes and are capable of antigen-specific responses (DeNardo & Coussens, 2007; Schiller, Metze, Luger, Grabbe, & Gunzer, 2006). Adaptive immune cells will not be considered in the context of this study. Innate effector cells include macrophages, granulocytes, mast cells, dendritic cells, and natural killer cells and can provide a general immune defence through immune cell recruitment, adaptive immune cell activation, infection identification and infection clearance (DeNardo & Coussens, 2007; Schiller et al., 2006). Macrophages, differentiated from the monocyte lineage, are phagocytic immune cells that offer general defence to cellular debris and microbial pathogens. Their presence in adipose tissue makes them a potential candidate as a mediator of SCH-induced inflammation.

b. Fatty Acids as a Cardiovascular Risk Factor

Elevated levels of FAs have been associated with an increased risk of arterial hypertension, myocardial dysfunction, and atherosclerosis (Pilz & Mrz, 2008). FAs act through a variety of mechanisms and the importance of FFA in cardiovascular risk is being

increasingly recognized. Within adipose tissue, increased release of FAs by adipocytes was shown to increase Adipose tissue macrophage (ATM) accumulation, AT chemoattractant activity and lipid uptake by ATM (Kosteli et al., 2010). Thus an efflux of FAs stemming from the adipose tissue is involved in the initiation of an inflammatory response involved in the progression of CVD. Elevated FAs released into circulation might also influence macrophage activation peripherally. However, determining the FA effect on peripheral macrophages like those in the arterial wall would be difficult to establish and conclusions cannot be easily drawn through *in vitro* studies. The objective of my thesis will be to examine the potential interaction of FAs released from TSH-stimulated adipocytes and ATMs. TSH may stimulate adipocyte lipolysis, leading to macrophage inflammation and the development of CVD. Understanding this relationship could provide new targets for future therapeutic approach to reduce low-grade inflammation and the CVD risk observed in SCH.

VII. Macrophages

a. Macrophage Classification

Macrophages are involved in the innate immune response, manage general immune system needs, and can be found dispersed throughout all types of tissue. Macrophages have traditionally been subdivided into two sub-types: the M1 or 'classically activated' and the M2 or 'alternatively activated' macrophages. The classically activated macrophages are involved in bacterial infection clearance, tissue destruction, tumor resistance and adaptive Th1 response through means of pro-inflammatory pathways (Mantovani et al., 2004). These cells were originally characterized based on their response to interferon-γ (IFNγ) and tumor

necrosis factor (Mosser & Edwards, 2008). Alternatively activated macrophages work in tissue remodelling, tumor promotion, angiogenesis, parasite encapsulation and immunoregulation through an anti-inflammatory response (Mantovani, Sica, Allavena, Garlanda, & Locati, 2009). They work to maintain tissue homeostasis through clearance of cellular debris and release of anti-inflammatory cytokines (Gordon & Taylor, 2005; Mantovani, Sozzani, Locati, Allavena, & Sica, 2002). Alternative macrophages were described based on their response to interleukin-4 (IL-4) or IL-13 (Daley, Brancato, Thomay, Reichner, & Albina, 2009). Recent work suggests however that the M1/M2 taxonomic structure must be revised as all non-M1 activated macrophages have been inappropriately labelled as M2 macrophages (Bourlier et al., 2008; Edwards, Zhang, Frauwirth, & Mosser, 2006; Mosser & Edwards, 2008). The system is too simplistic in nature to fully define macrophage phenotypes as just M1 or M2 and further division is required. Nonetheless the traditional M1/M2 phenotyping, or a slight variation thereof, continues to be used to distinguish pro- and anti-inflammatory differences. The focus of this study surrounds the pro-inflammatory response from macrophages, or the M1 type response.

b. Adipose Tissue Macrophage (ATM) Polarization

Macrophages present in adipose tissue can make up 1 to 30 % of the tissue depending on the inflammatory status of the tissue (Hauner, 2005). Macrophages in non-inflamed adipose tissue have a multitude of functions with the overall objective of maintaining tissue homeostasis. In homeostatic tissue, they are found randomly dispersed throughout, in a balance of pro- and anti-inflammatory macrophages clearing cellular debris, bacterial infections and tissue repair

In homeostatic adipose tissue, mostly M2 and very few M1 macrophages are found in the tissue clearing cellular debris, bacterial infections and tissue repair, thereby maintaining proper adipose tissue function and turnover (Bourlier et al., 2008; Cinti et al., 2005; Itoh, Suganami, Hachiya, & Ogawa, 2011; Lumeng, Bodzin, & Saltiel, 2007; Lumeng, DelProposto, Westcott, & Saltiel, 2008). In a transition to chronic inflammation, notably seen in adipose tissue of obese individuals, macrophages are recruited. The ratio of M1/M2 macrophages changes and the population shifts to a more M1 polarized state where classically activated macrophages can be found forming crown-like structures (CLS) around dying adipose tissue (Cinti et al., 2005; Lumeng et al., 2007; Lumeng et al., 2008). This inflammatory state leads to a preponderance of pro-inflammatory M1 macrophages that can raise the risk for insulin resistance, cardiovascular disease and other inflammatory diseases through tissue interaction and subsequent dysfunction (Surmi & Hasty, 2008). Indeed adipocytes and macrophages studied in a co-culture system have been shown to work in a paracrine loop to release free FAs and TNF-α respectively, thereby promoting an inflammatory response that is cyclical in nature (Suganami et al., 2005). Adipocyte-derived free FAs have gained more attention in recent years as their role in macrophage recruitment and function has become more important that originally thought (Li & Renier, 2007; Schaeffler et al., 2009).

c. Macrophage Role in CVD

Inflammation stemming from adipose tissue in obesity contributes to a systemic inflammatory state that promotes CVD progression. Studies have shown that adipose tissue macrophages release high levels of IL-1β, IL-6, TNF-α, MCP-1, macrophage inflammatory

protein 1α (MIP-1α) and migration inhibitory factor (MIF) (Hauner, 2005; Zeyda et al., 2007). The role of these cytokines is wide-ranging; IL-1β and TNF-α activate endothelial cells that in turn can promote macrophage recruitment (Mathew, Tay, & Cusi, 2010). This macrophage recruitment comes through the increased expression of ICAM-1 and VCAM-1 on endothelial cells which increases the firm adhesion of circulating blood monocytes to endothelial cells for migration to occur (Mathew et al., 2010; Nakashima, Raines, Plump, Breslow, & Ross, 1998). MCP-1 also promotes macrophage accumulation through chemotactic recruitment, thereby promoting a chronic inflammatory state in the adipose tissue. IL-6 has been identified as an independent risk factor of CVD and has been shown to promote myocardiocyte injury (Ridker, Rifai, Stampfer, & Hennekens, 2000). Inflammatory macrophages also stimulate adipocyte lipolysis, thereby promoting increased circulation of lipids that have been implicated in atherosclerosis (Hansson, 2005). Thus chronic inflammation in adipose tissue can promote macrophage activity and the further release of inflammatory molecules that can drive the progression of CVD.

Macrophages also have an important role in the arterial wall, clearing oxidized LDL that accumulates. When their capacity is exceeded, macrophages become foam cells and promote further macrophage recruitment (Gosling et al., 1999; Hansson, 2009). Pro-inflammatory cytokines, such as the chemokine MCP-1, released from these foam cells are what recruit additional macrophages that infiltrate these lesions, leading to the formation of plaque and atheromas of atherosclerosis (Gosling et al., 1999; Nelken, Coughlin, Gordon, & Wilcox, 1991). Complications due to thrombus of atheromas in atherosclerosis can lead to myocardial infarction or ischemic stroke (Hansson, 2005).

d. Macrophage Cell Models

Two macrophage cell models, one murine and one human, were used for these studies. The mouse macrophage cell line, a murine reticulum cell sarcoma named J774, is a widely used model that accurately represents murine macrophage characteristics through adherence, morphology and phagocytic properties (Ralph & Nakoinz, 1975). However, as they are derived from cancer cells, their properties may deviate from those expected from primary macrophage cell function. Snyderman et al. observed that the migration capabilities of J774 macrophages to lymphocyte-derived chemotactic factor (LDCF) were unexpectedly absent (Snyderman, Pike, Fischer, & Koren, 1977). Nonetheless, their ease of use in culture and general macrophage characteristics make them an effective macrophage model and are depicted in Figure 3.

The human macrophage cell line is derived from THP-1 monocytes. THP-1 monocytes are a human acute monocytic leukemia cell line that are maintained in suspension and can be induced to differentiate into macrophages using 100nM 12-O-Tetradecanoylphorbol-13-acetate (TPA) over 24 hours (Auwerx, 1991; Tsuchiya et al., 1980). Once differentiated, they adhere to the plate as seen in Figure 3. Compared to other myeloid-derived cell lines, THP-1 cells behave more like native monocyte-derived macrophages (Auwerx, 1991). Similar to the J774A.1 macrophages, THP-1 macrophages are derived from a cancer line and may exhibit unwanted properties or characteristics unlike primary macrophages. However, their general macrophage characteristics and ease of use in culture make them a suitable macrophage model.

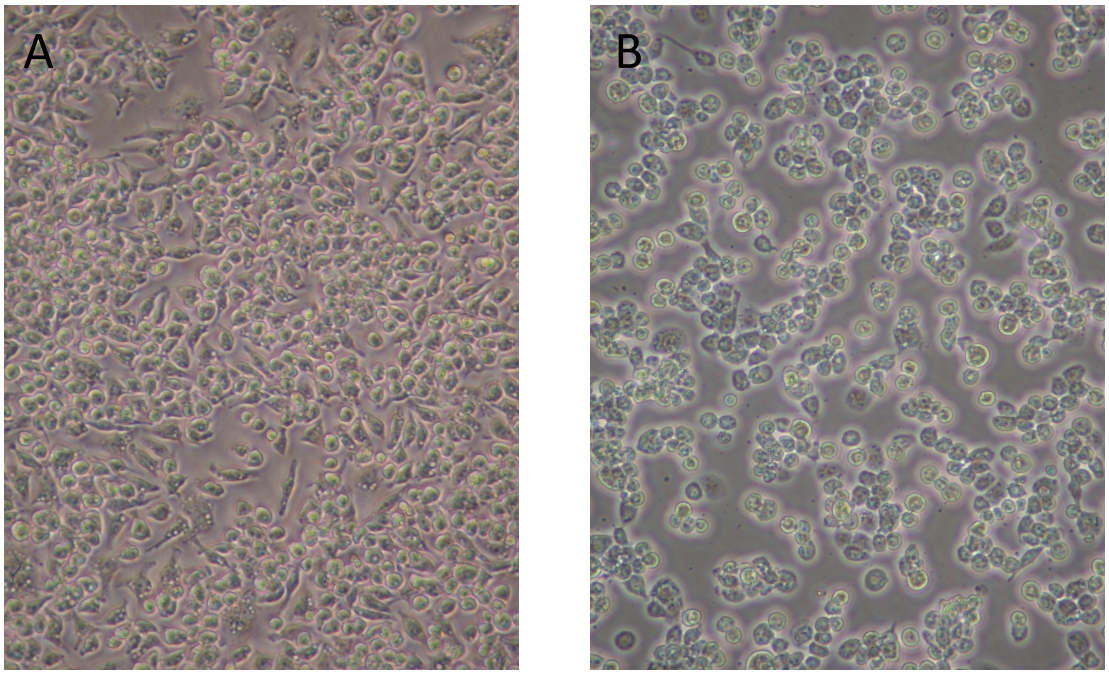

Figure 3. Murine and human macrophage cell models. A) Murine J774A.1 macrophages maintained in DMEM, 10% FBS and antibiotics B) Differentiated human THP-1 macrophages after 24 hour monocyte to macrophage induction using 100nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). Cell cultures photographed at a magnification of 200x.

HYPOTHESIS

TSH-stimulated FAs released from adipocytes contribute to macrophage activation and inflammation.

OBJECTIVES

I. Determine Optimal Conditions for TSH-Stimulated Lipolysis in Differentiated Adipocytes

II. Investigate Effect of TSH-Stimulated Adipocyte-Conditioned Medium on Macrophages

a. Investigate TSH-Stimulated Adipocyte-Conditioned Medium on Macrophages

b. Evaluate Macrophage Response to LPS or FFA

c. Evaluate FFA Release from TSH-Stimulated Adipocytes

III. Evaluate the Effect of Pro-Inflammatory Cytokines on TSH-Stimulated Adipocyte Lipolysis

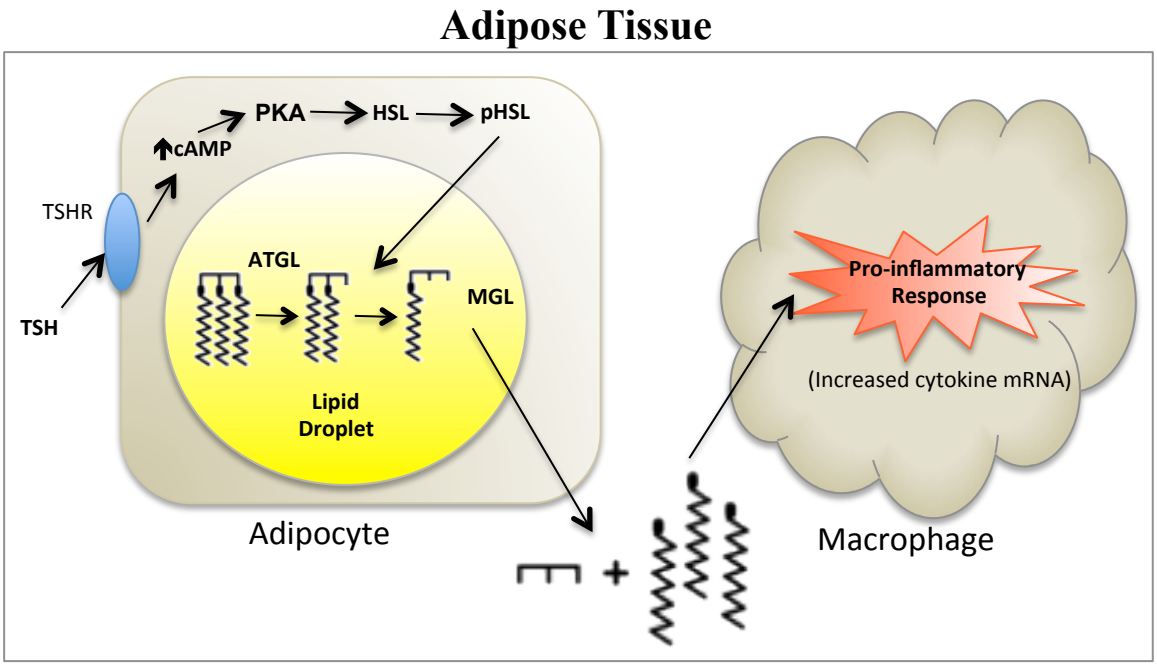

Figure 4. Hypothetical working model for TSH-stimulated adipocyte activation of macrophage inflammation. Elevated levels of TSH from the anterior pituitary interact with TSH receptors (TSHR) on adipocytes to activate adenyl cyclase and increase production of cAMP. PKA phosphorylates hormone sensitive lipase (HSL), causing translocation to the lipid droplet where it hydrolyzes a fatty acid from diglyceride (DG). This is the intermediary step in lipolysis between the hydrolytic activity of adipose triglyceride lipase (ATGL) and monoacylglycerol lipase (MGL) that allows for full hydrolysis of triglyceride (TG) to three fatty acids and glycerol in stimulated lipolysis. Fatty acids have been demonstrated to elevate inflammation in macrophages. We propose that increased fatty acid release from TSH-stimulated adipocytes can activate transcription of pro-inflammatory cytokines, resulting in a greater inflammatory response. This response may contribute to the association between subclinical hypothyroidism (SCH) and the increased risk of cardiovascular disease.

MATERIALS AND METHODS

3T3-L1 Preadipocyte Culture and Differentiation

3T3-L1 preadipocytes (American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum (CS) and antibiotics (100U/mL penicillin, 0.1 mg/mL streptomycin)(growth medium). Subconfluent cultures were passaged no more than 12 times and passaging was done using trypsin-EDTA to detach the preadipocytes from the dish. Cells were reseeded in growth media that was changed the day after passaging and every second day thereafter. Two day post-confluent cells were induced to differentiate using DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (basal medium) with 0.25 μM dexamethasone, (DEX) and 0.5mM IBMX for the first 2 days and 1 μM insulin for the first 4 days. Cells were maintained in basal media for 4 more days, with a fresh media change on the second day. On the eighth day cells were used as indicated.

Human Abdominal Subcutaneous Preadipocyte Isolation, Culture and Differentiation

Human abdominal subcutaneous preadipocytes were obtained during elective abdominal surgery from males and females with a mean age (±SD) of 52 (±9) years old with a mean BMI (±SD) of 27.6 (±7.2) kg/m^2. The Ottawa Hospital Research Ethics Board has approved the use of this human tissue for experimental purposes. The isolation of stromal-vascular preadipocytes has been previously described (Hauner et al., 2001) and is established in our laboratory (Gagnon et al., 2010). Connective tissue and blood vessels were carefully removed from the adipose tissue and the tissue was then subjected to collagenase CLS type 1

(600 U/g tissue) digestion at 37°C for 1 hour on a rotator. Following digestion, cells were passed through size filtration using a 200 micron sterile nylon filter. After centrifugation (20 minutes at 200g) of disaggregated cells, the supernatant containing mature adipocytes was discarded and the pellet, containing the stromal preadipocytes, was resuspended in 10% FBS and subjected to further size filtration (100, 50, 25 micron sterile nylon filter). The supernatant was further centrifuged for 20 minutes at 200g to separate out any remaining mature adipocytes. The pellet was then resuspended in erythrocyte-lysing buffer containing 155 mM ammonium chloride, 5.7 mM potassium phosphate and 0.1 mM EDTA at a pH of 7.3 and incubated for 5 minutes. Cell suspension was centrifuged for 5 minutes at 200g and the pellet, now considered to be principally composed of isolated preadipocytes, was resuspended in growth medium consisting of DMEM supplemented with 10%FBS, antibiotics (100U/mL penicillin, 0.1 mg/mL streptomycin) and 50U/mL nystatin. Preadipocytes were seeded at a density of 6 x 10^3 cells/cm^2 and grown to 80-90% confluence prior to cryopreservation of unused portions. Cells were suspended in growth media containing 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until further use. To recommence cell growth, cryopreserved cells were removed from the liquid nitrogen storage and placed in a 37°C water bath until thawed. Cells were then slowly resuspended in 15 mL of growth media and centrifuged for 5 minutes at 200g with no brake action. Supernatant was aspirated and cells resuspended in growth media. The growth medium was changed every 3 days and the cells grown to ~90% confluence prior to passaging. Preadipocytes were limited to 3 passages prior to induction in adipocyte differentiation (Adams et al., 1997; Hutley et al., 2003). Cryopreservation under these conditions has been shown to have no effect on the differentiation capacity of preadipocytes (Ort et al., 2005).

Confluent human abdominal subcutaneous preadipocytes were induced to differentiate using growth media supplemented with 5μg/mL insulin, 100 μM indomethacin, 0.5 μM dexamethasone and 0.25 mM isobutylmethylxanthine (IBMX) for 13-15 days. On the final day of differentiation, cells were washed once with growth medium prior to being maintained in regular growth medium for 3 days. On the third day cells were used as indicated.

Conditioning of 3T3-L1 and Human Subcutaneous Differentiated Adipocyte Media

For conditioning of 3T3-L1 and human subcutaneous differentiated adipocyte media for use on J774A.1 and THP-1 macrophages respectively, 3T3-L1 or human subcutaneous differentiated adipocytes were placed in DMEM with 1%CS and antibiotics overnight prior to stimulation. Cells were then washed 2x with phenol red-free DMEM and stimulated for 4 hours with or without 5 mU/mL TSH (Sigma) in phenol-red free DMEM containing 1%CS and antibiotics. Control media, free from cell exposure, were also subjected to incubator conditions for 4 hours. After the incubation period, media was carefully removed and centrifuged for 5 minutes at 200g with no brake action. The media were then transferred to new tubes and placed at -20°C until use for macrophage studies.

J774A.1 Macrophage Culture

J774A.1 macrophages (ATCC) were maintained at 80% density in growth medium consisting of DMEM supplemented with 10% FBS and antibiotics (100U/mL penicillin, 0.1 mg/mL streptomycin). J774A.1 macrophages were passaged by gentle scraping of the cells off the dish and reseeded at a ratio of 1:6. Growth medium was replaced the following day

and changed every 2 days thereafter. For mRNA extraction studies, cells were seeded at a density of 5x 10^5 cells per 35 mm plate, which allowed them to become 90% confluent in 48 hours. The cells were used as indicated.

THP-1 Monocyte Culture and Macrophage Differentiation

THP-1 monocytes (ACTT) were grown in growth medium consisting of Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate ($NaHCO_3$), 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 10% FBS, antibiotics (100U/mL penicillin, 0.1 mg/mL streptomycin) and 0.05 mM β-mercaptoethanol. Cells were maintained in suspension at a density of 0.25-1 x 10^6 cells per mL with a complete medium change every 7 days. Medium change was performed through centrifugation of the cell suspension for 5 minutes at 200g with no brake. Cells were subsequently transferred to growth media adjusted to incubator conditions. Cells were counted 2-4 times using a Neubauer hemocytometer.

To induce differentiation of THP-1 monocytes to macrophages, cells were seeded at a concentration of 1 x 10^6 cells per mL, 2 mL per 35 mm plate, and exposed to 100nM phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) for 24 hours. Approximately 90% adherence was observed after this period, cells were then used as indicated.

Palmitic Acid Treatment

To assess the effect of FA exposure on macrophage cytokine mRNA expression profiles, palmitic acid (PA) was added to phenol red-free DMEM containing 1%CS and antibiotics. To do this, 100mM PA in ethanol was transferred to 15 mL falcon tubes and

dried using a Meyer N–EVAP Analytical Evaporator. Phenol red-free DMEM containing 1%CS and antibiotics was added to the dried PA and these solutions were placed in a sonicating bath for 15 minutes. Solutions were then warmed to 37°C in a water bath and placed on J774A.1 or THP-1 macrophages. The final solutions were verified with NEFA quantification to confirm the concentrations.

Glycerol Quantification

3T3-L1 and human abdominal subcutaneous differentiated adipocytes were serum reduced overnight (1% CS) prior to stimulation with the exception of one study that investigated the effect of using their respective regular growth media in place of overnight serum reduction. The differentiated adipocytes were then stimulated for 4 hours with 5 or 50 mU/mL TSH (Sigma; Calbiochem, San Diego, CA, USA; Alextrend; NIH, Bethesda, MD, USA; Seradyn, Indianapolis, IN, USA; or Trophogen, Rockville, MD, USA as indicated) in Krebs/Ringer/Hepes (KRH) buffer or phenol red free DMEM containing 1%BSA, or 1%CS, or both as indicated. Conditioned media was then removed, centrifuged for 5 minutes at 200g without braking to remove any cellular debris and transferred to new tubes. Glycerol was quantified using a Glycerol Detection Kit (Zen-Bio; Research Triangle Park, NC, USA) according to manufacturer's protocol and expressed as fold over control or μmol glycerol per mg protein. Briefly, 50 μl of conditioned media (diluted if necessary) was mixed with 50 μl of Zen-Bio's glycerol reagent A, incubated for 15 minutes at room temperature and the absorbance was read at 540 nm. The observed colorimetric change occurs when a quinoeimine dye is produced by the coupling of 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl)m-anisidine catalyzed by hydrogen peroxide. The hydrogen peroxide comes

from the phosphorylation of glycerol to glycerol-1-phosphate (G-1-P) by glycerol kinase, which is then oxidized to dihydroxyacetone phosphate and hydrogen peroxide by glycerol phosphate oxidase.

NEFA Quantification

3T3-L1 and human abdominal subcutaneous differentiated adipocytes were place in serum reduced media (DMEM, 1%CS and antibiotics) overnight prior to stimulation. Cells were washed twice with phenol red-free DMEM then stimulated for 4 hours with 5 mU/mL TSH (Sigma) in phenol red-free DMEM containing 1%CS, 1% BSA and antibiotics. Conditioned media was then removed, centrifuged for 5 minutes at 200g without braking to remove any cellular debris and transferred to new tubes. NEFA was quantified using Wako's HR Series NEFA-HR-(2) kit (Wako, Osaka, Japan) with a modified manufacturer's protocol and expressed as μmol NEFA per mg protein. Briefly, 225 μL of reagent A (0.53 U/mL acyl-coenzyme A synthase (ASC), 0.31 mM coenzyme A (CoA), 4.3 mM adenosine triphosphate (ATP), 1.5 mM 4-aminoanitipyrine, 2.6 U/mL ascorbate oxidase and 0.062% sodium azide) was incubated with 7 μL of conditioned media at 37°C for 10 minutes. 75 μL of reagent B (50 mM phosphate buffer, pH 7.0, 0.05% Sodium azide) was then added and incubated for a further 10 minutes. Samples were left to cool at room temperature before the absorbance was read at 560 nm.

Triglyceride Quantification

3T3-L1 and human abdominal subcutaneous differentiated adipocytes were washed twice with ice-cold phosphate buffered solution (PBS). Triglycerides were the extracted with

1mL isopropanol:heptane (2:3 ratio; v/v) for 30 minutes at room temperature. A second room temperature incubation of 15 minutes with 0.5 mL of the isopropanol:heptane mixture was done and pooled with the first lipid extract. The pooled lipid extracts were dried down using either a Savant Speed Vac Plus SC110A or Meyer N –EVAP Analytical Evaporator and stored at -20°C. The cellular remains in each well were solubilized according to Cellular Protein Preparation and Quantification. The TG assays were performed as previously described by Neri *et al.* with Triolein as the standard (Neri & Frings, 1973).

Cellular Protein Preparation and Quantification

For immunoblotting and protein quantification for other studies, cells were lysed in Laemmli buffer supplemented with 5% β-mercaptoethanol, 1mM sodium orthovanadate (Na_3VO_4), 5 mM ethylene glycol tetracetic acid (EGTA), and 50 mM sodium floride (NaF) (Laemmli, 1970). Cell lysates were scraped off the plates, passed through a 26½ gauge syringe three times and then boiled for 5 minutes. Cell lysates were then centrifuged for 5 minutes at 500g and the supernatant was transferred to new 1.5 mL eppendorf tubes, leaving behind the lipid layer. These samples were stored at -20°C until further use. For experiments requiring TG extraction prior to cell lysis, the centrifugation and supernatant transfer were not required, samples were placed at -20°C after 5 minutes of boiling. Protein quantification was performed using the Lowry assay and bovine serum albumin (BSA) (Bio-Rad, Hercules, CA) as a standard.

Western Analysis

Equal amounts of solubilized protein (15-40 μg protein, depending on the experiment) were resolved using a 10% or 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membrane. Non-specific binding sites were blocked using PBS with 0.1% Tween20 and 5% w/v skim milk powder for 1 hour at room temperature. The nitrocellulose membranes were probed overnight at 4°C using antibodies for ATGL (1:2000; monoclonal, rabbit) (Epitomics, Burlingame, CA, USA), HSL (1:2000; monoclonal, rabbit) (Cell Signaling, Danvers, MA, USA), perilipin (1:2000; polyclonal, guinea pig) (Progen Biotechnik, Heidelberg, Germany) or ERK1/2 (0.25 μg/mL; monoclonal, rabbit) (Millipore Corp., Billerica, MA, USA) in PBS containing 3%BSA, and 0.02% sodium azide (NaN_3). The membranes were then incubated for 1 hour at room temperature with appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) before being detected through enhanced chemiluminescence (ECL)(Millipore Corp., Billerica, MA, USA). Membranes were exposed to Bioflex Econo Film (Clonex Corp., Markham, ON, CA) and processed using a Konica Minolta Medical Graphic Inc. SRX-101A processor. Relative band intensity was determined using AlphaEaseFC Software (Alpha Innotec Co., San Leandro, CA, USA) and expressed as integrated optical density (IOD).

RNA Isolation

Treated J774A.1 and THP-1 macrophages were lysed using TRI-Reagent (Ambion, Austin, Tx, USA) or QIAzol (Qiagen) and RNA extracted according to manufacturer's protocol. Isolated RNA was treated with DNase I (Ambion) according to protocol. RNA

was quantified using Quant-iT Ribogreen RNA assay kit (Invitrogen) according to manufacturer's protocol and 0.5 μg of RNA from each sample was run on a 1% agarose gel containing 3% formaldehyde. RNA integrity was visualized via ethidium bromide staining of 18S and 28S rRNA using a Gene Genius Bio Imaging System and GeneSnap (version 6.05, SynGene, Cambridge England). Samples were stored at -80°C until further use.

Real Time-PCR

0.5 μg of isolated RNA was combined with 1.25 μg of random primers and denatured at 85°C for 3 minutes. The RNA was them reverse transcribed for 1 hour at 43°C in the presence of 10 U RNAse Out (Ambion), 10x RT Buffer, 10 nM dNTPs, and 100 U of Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen). The reaction was stopped after an incubation of 92°C for 10 minutes. Cytokine cDNA was then amplified using Quantitect SYBR Green (Qiagen) chemistry with 900 nM product primers. The following primers were used for real time PCR detection in murine J774A.1 cells: IL-1β 5'- GCAACTGTTCCTGAACTCAACT-3' (forward) 5'-ATCTTTTGGGGTCCGTCAACT-3'(reverse); IL-6 5'-TAGTCCTTCCTACCCCAATTTCC-3' (forward) 5'-TTGGTCCTTAGCCACTCCTTC-3'(reverse); MCP-1; 5'-AGGTCCCTGTCATGCTTCTGG-3' (forward) 5'-CTGCTGCTGGTGATCCTCTTG-3'(reverse); TNF-α 5'-ACGGCATGGATCTCAAAGAC-3' (forward) 5'-AGATAGCAAATCGGCTGACG-3'(reverse). The following primers were used for real time PCR detection in human THP-1 cells: IL-1β 5'-GATGAAGTGCTCCTTCCAGGACCT-3' (forward) 5'-TGCTGTGAGTCCCGGAGCGT-3'(reverse); IL-6 5'-CCACACAGACAGCCACTCACCTC-3' (forward) 5'-

CTGGCTTGTTCCTCACTACTCTC-3'(reverse); MCP-1; 5'-CAGCCAGATGCAATCAATGC-3' (forward) 5'-GTGGTCCATGGAATCCTGAA-3'(reverse); TNF-α 5'-GCCCCCAGAGGGAAGAGTTCCC-3' (forward) 5'-CAGCTCCACGCCATTGGCCA-3'(reverse). Taqman chemistry (Applied Biosystems) was used for 18S amplification. Real-time PCR analysis occurred for target cytokine genes and 18S over 45 cycles using a Roche LightCycler and LightCycler Data Analysis software (version 3.528) to quantify products. Denaturing, annealing and elongation steps were set at 90°C for 15 seconds, 60°C for 30 seconds and 72°C degree for 30 seconds respectively. Reverse transcription (RT) null samples and melting curves were used to establish purity and specificity of the products. PCR products of interest were normalized to 18S and quantified through the comparative CT method.

Statistical Analysis

Data were analyzed using either paired t-test, one way analysis of variance followed by Newman-Keuls post-hoc test or two-way analysis of variance followed by a Tukey post -hoc test as described in figures. All data with $p < 0.05$ were considered significant.

RESULTS

I. Characterize and Evaluate Optimal Conditions for TSH-Stimulated Lipolysis in Differentiated Adipocytes

a. Comparison of commercial TSH reagents with respect to TSH-stimulated glycerol release in 3T3-L1 and human subcutaneous differentiated adipocytes.

As previously shown by our laboratory, TSH is capable of significantly stimulating glycerol release in both 3T3-L1 and human subcutaneous differentiated adipocytes at 4 hours with 5 or 50 mU/mL (Gagnon et al., 2010). To ensure that the preparation of TSH does not cause any significant difference in glycerol release from 3T3-L1 differentiated adipocytes, 4 different commercial TSH preparations were used for 4 hours at either 5 or 50 mU/mL. TSH resulted in a 1.7 to 2.4-fold increase in glycerol release over basal, with no significant differences between the individual TSH preparations (Figure 5, n=3). All responses reached significance when compared to the basal level ($p<0.05$ or $p<0.01$, n=3). The glycerol response from Trophogen TSH, a recombinant human TSH analogue, was not different from the three bovine TSH reagents used. Although a ten fold increase in TSH concentration (5 vs 50 mU/mL) did suggest a trend to a greater glycerol release, these increases were minimal. Sigma TSH (5 mU/mL) increased glycerol release by 1.7 fold of basal ($p<0.05$, n=3). Sigma TSH is commonly used in the literature for thyrocyte studies and is a suitable, cost-effective preparation for subsequent *in vitro* experiments using 3T3-L1 differentiated adipocytes.

Human subcutaneous preadipocytes are more limited in availability, display donor variation, and are difficult to propagate compared to the mouse 3T3-L1 preadipocyte cell line. Therefore, I evaluated only a single concentration (50 mU/mL) of 5 different commercial TSH preparations. Sigma and Trophogen TSH preparations significantly increased glycerol release, with Sigma TSH providing the greatest overall fold of basal release at 5.5 ($p<0.05$, n=3, Figure 6). The glycerol responses to Calbiochem, NIH and Seradyn TSH reagents did not reach significance. Given the effectiveness of Sigma TSH in both the 3T3-L1 and human subcutaneous differentiated adipocytes, it was deemed suitable for use in both adipocyte models and was employed throughout the course of this project.

b. Optimization of medium conditions for TSH-stimulated lipolysis in 3T3-L1 and human subcutaneous differentiated adipocytes.

Several variables were considered when investigating conditions for TSH-stimulated lipolysis. The formulation of the medium was considered. The night before stimulation, the medium was changed to either fresh growth medium or serum-reduced medium (1%CS). During stimulation, the following variables were examined: Krebs-Ringer-Hepes (KRH) medium versus phenol red-free DMEM; 1% CS versus 1% BSA; and vehicle versus TSH stimulation. All prior studies relating to TSH-stimulated lipolysis assessed by glycerol release from adipocytes were performed using Krebs-Ringer-Hepes (KRH) or a complete assay buffer from Zen-Bio with a proprietary composition. These media could not be used for adipocyte conditioning and eventual addition to macrophages since they do not support macrophage cell culture for extended lengths of time. To plan for use of TSH stimulated adipocyte-conditioned medium on macrophages, it was necessary to evaluate lipolysis using

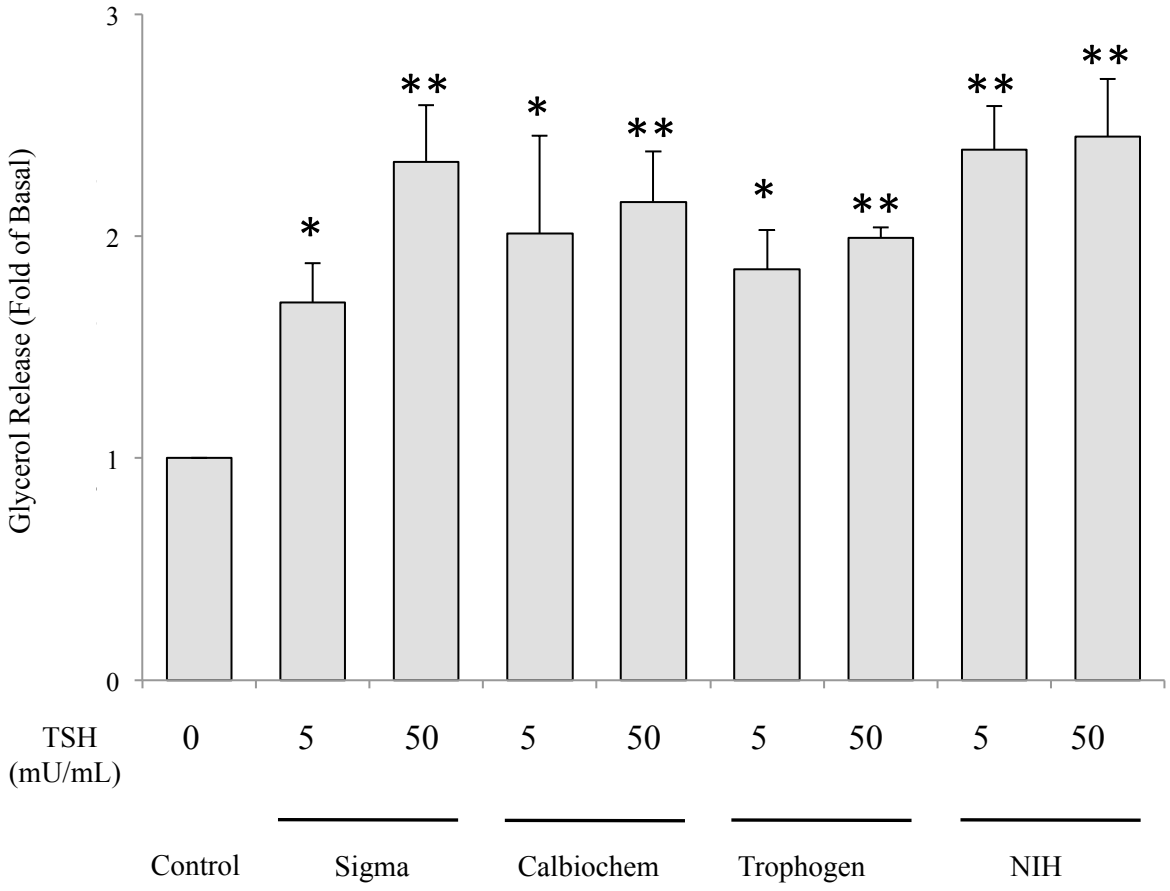

Figure 5: 3T3-L1 differentiated adipocyte glycerol release comparing various commercial TSH sources. Differentiated 3T3-L1 adipocytes were stimulated with 5 or 50 mU/ml of different TSH sources (bovine TSH: Calbiochem, Sigma and NIH; Recombinant human TSH: Trophogen) in assay buffer with 1% CS and antibiotics. After 4 hours, media was collected and assessed for glycerol release and expressed as fold glycerol release. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was determined by a one-way ANOVA, followed by Student-Newman-Keuls post-hoc for comparisons of means. *$p<0.05$, **$p<0.01$ vs. control.

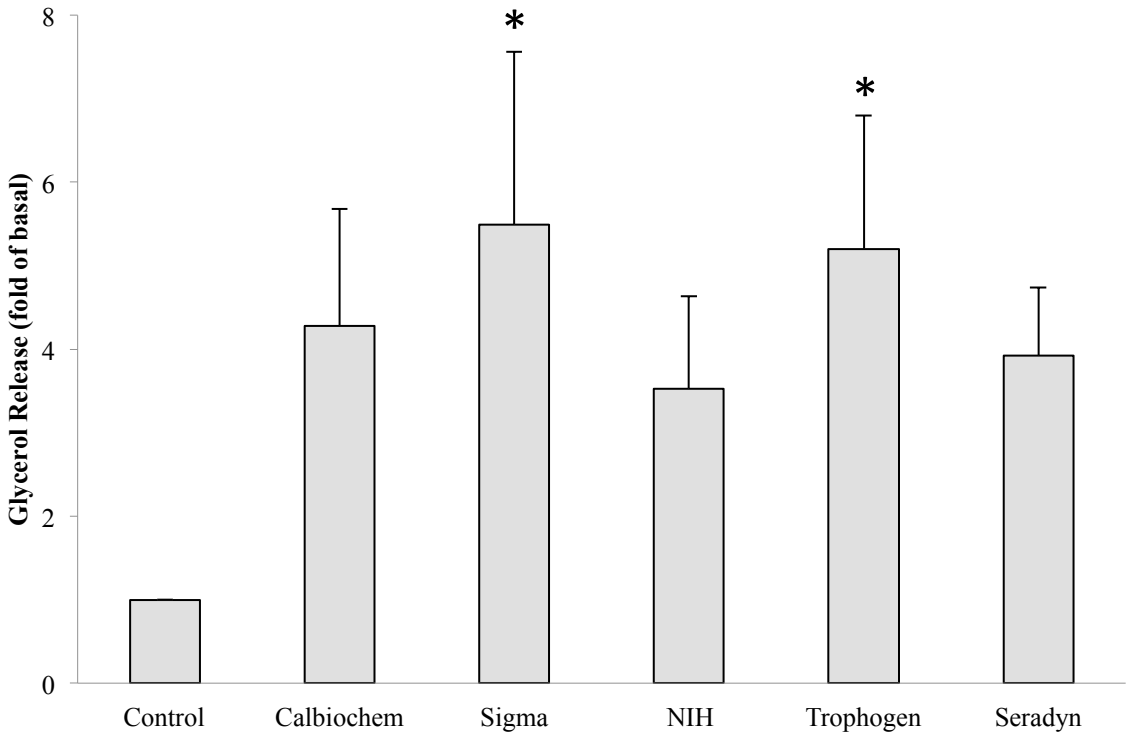

Figure 6. Human subcutaneous differentiated adipocyte glycerol release comparing various commercial TSH sources. Differentiated human subcutaneous adipocytes were stimulated with 50 mU/ml of TSH (bovine TSH: Calbiochem, Sigma and NIH; recombinant human TSH: Trophogen and Seradyn) in assay buffer containing 1% CS and antibiotics. After 4 hours, media was collected and assessed for glycerol release and expressed as fold of glycerol release. Results represent the mean +/- SEM of 4 independent experiments, each performed in duplicates. Statistical significance was determined by a one-way ANOVA, followed by Student-Newman-Keuls for post-hoc comparisons of means. *$p<0.05$ vs. control.

phenol red-free DMEM (prf-DMEM). Phenol red, a pH indicator in DMEM, interferes in the colorimetric range used in the assays that detect glycerol and FA. DMEM is also supplemented with a variety of vitamins, amino acids, glucose and other factors to support cell growth. This comparative study ensured that no factors interfere with TSH-stimulated lipolysis. A review of the literature revealed that lipolysis was assayed under a large variety of conditions, thus the other variables listed above were examined.

Of the conditions used on 3T3-L1 differentiated adipocytes, overnight serum reduction and stimulation in prf-DMEM using 1% CS supplementation provided the greatest fold of basal lipolysis response as assessed through glycerol release (Figure 7). When considering only the prf-DMEM conditions, this set of conditions provided the strongest stimulated response. Conditions using KRH were uniformly observed to have greater glycerol release in both basal and stimulated states when compared to prf-DMEM, but these elevations did not impact fold of basal levels. Furthermore, the KRH conditions were not suitable for creation of adipocyte conditioned medium and use on macrophages and were not considered for this purpose. The DMEM conditions do not show any trends when serum reduction is introduced overnight. Owing to experimental variability, the responses did not reach significance in these studies.

Using human subcutaneous differentiated adipocytes, overnight serum reduction and TSH-stimulation in prf-DMEM using 1% CS supplementation also provided the greatest fold of basal lipolysis response as assessed through glycerol release (Figure 8). It also provided one of the strongest stimulated responses.

The trend towards an increase in glycerol release response of KRH conditions versus DMEM conditions noted in the 3T3-L1 adipocytes was not observed for human

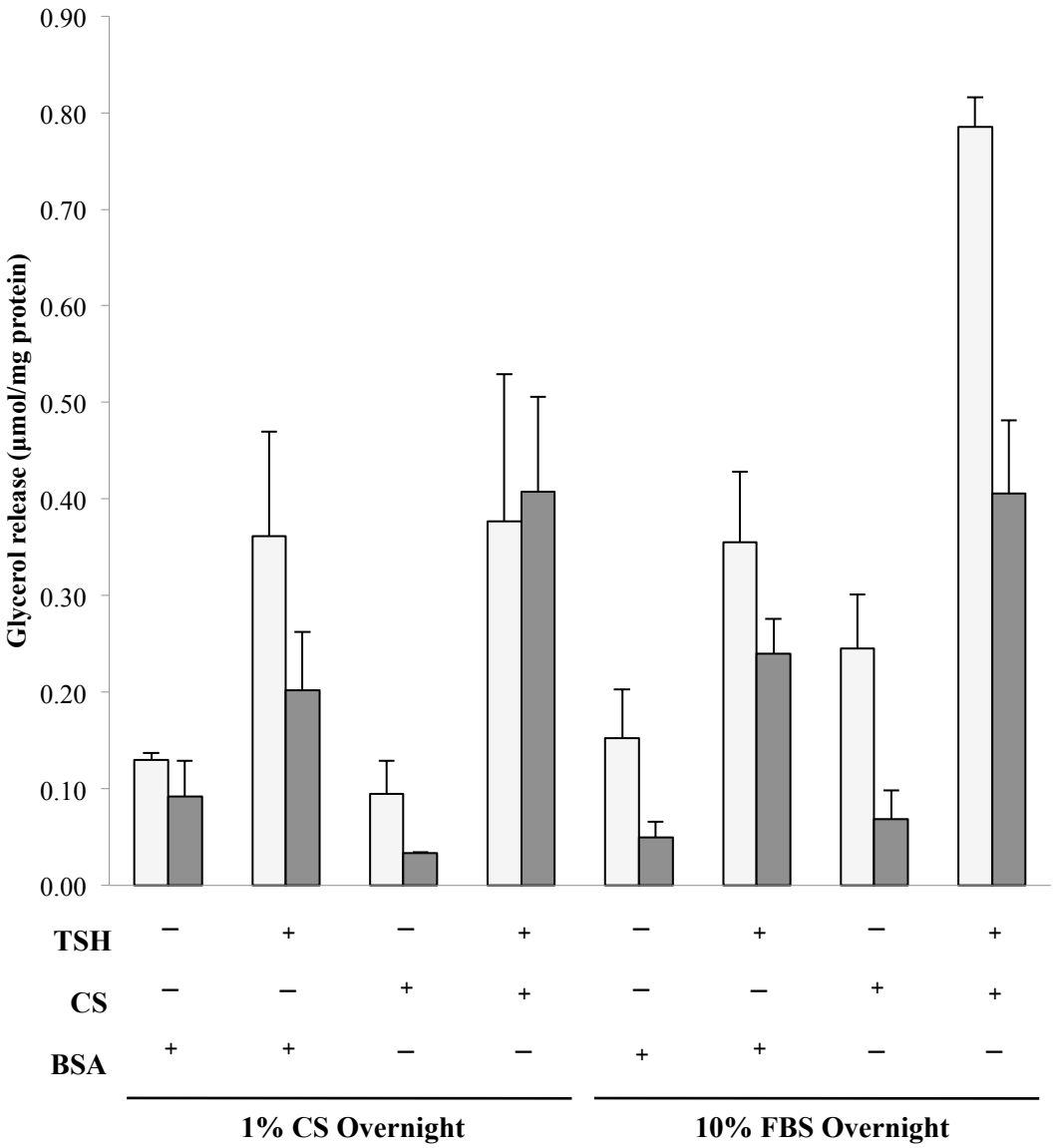

Figure 7. Comparative analysis of 3T3-L1 differentiated adipocyte TSH-stimulated lipolysis using multiple culture conditions. Differentiated 3T3-L1 adipocytes were either serum reduced to 1% CS media supplementation or left in regular growth medium overnight prior to stimulation. Cells were then exposed to vehicle (water) or 5 mU/ml TSH in KRH (□) or phenol red-free DMEM (■) supplemented with 1% CS or 1% BSA. After 4 hours, media was collected and assessed for glycerol release and expressed as uM of glycerol per mg of protein. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was assessed by a two-way ANOVA with no significance observed.

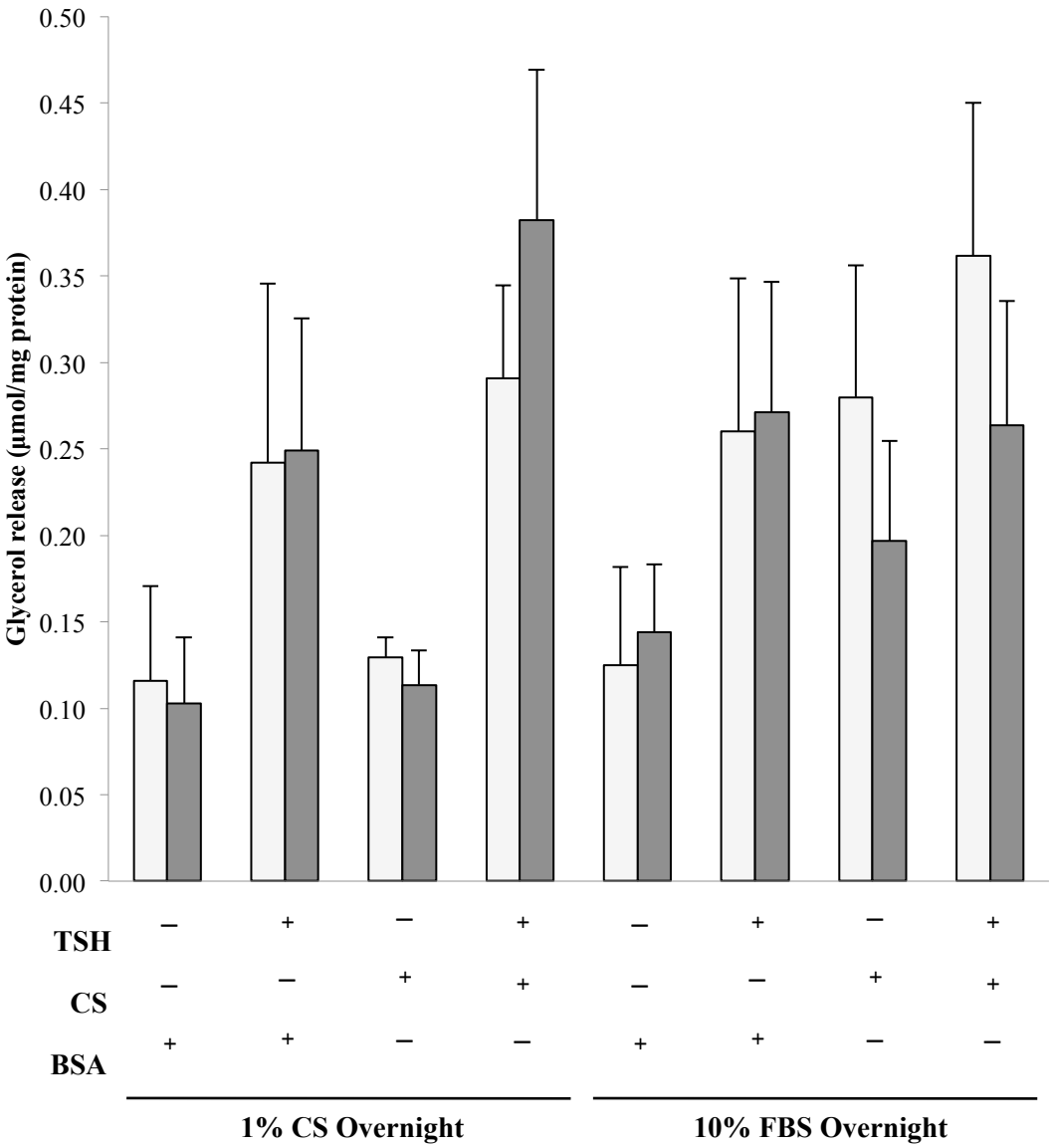

Figure 8. Comparative analysis of human subcutaneous differentiated adipocyte TSH-stimulated lipolysis using multiple culture conditions. Differentiated human subcutaneous adipocytes were either serum reduced to 1% CS media supplementation or left in regular growth medium overnight prior to stimulation. Cells were then exposed to vehicle (water) or 5 mU/ml TSH in KRH (□) or phenol red-free DMEM (■) supplemented with 1% CS or 1% BSA. After 4 hours, media was collected and assessed for glycerol release and expressed as umol of glycerol per mg of protein. Results represent the mean +/- SEM of 4 independent experiments, each performed in duplicates. Statistical significance was assessed by a two-way ANOVA with no significance observed.

subcutaneous differentiated adipocytes. There were no apparent differences comparing BSA to CS. Overnight serum reduction prior to stimulation appears to permit a greater fold of basal difference versus cells maintained in regular growth media overnight by reducing basal glycerol release. This difference however did not reach statistical significance.

The experimental variation in both 3T3-L1 and human subcutaneous differentiated adipocyte studies were such that statistical differences were not obtained. Acknowledging this limitation, overnight serum reduction and the prf-DMEM condition containing 1% CS yielded the greatest observed difference between the basal and stimulated states for both models. Consequently, it was decided to use these conditions in the preparation of adipocyte-conditioned medium for use with macrophages.

c. TSH does not affect protein expression of ATGL, HSL, perilipin or MAPK in 3T3-L1 or human subcutaneous differentiated adipocytes.

Little is known about the effect of TSH on the protein expression of lipolysis-involved proteins. To further characterize how TSH regulates lipolysis, time course studies were performed for 3T3-L1 and human subcutaneous differentiated adipocytes to assess protein levels of ATGL, HSL and perilipin. MAPK was used as a loading control to ensure equivalent protein levels were evaluated in figures 9 and 10 (Prusty, Park, Davis, & Farmer, 2002).

3T3-L1 differentiated adipocytes were stimulated for 30 minutes, 1 hour, 2 hours, 6 hours or 16 hours and the proteins were detected using immunoblot analysis. Over the 16 hour time course, TSH did not affect the expression level of ATGL, HSL or perilipin (Figure 9, n=4). TNF-α was used as a positive control as it has been shown to down-regulate ATGL,

HSL and perilipin (Kim, Tillison, Lee, Rearick, & Smas, 2006; Laurencikiene et al., 2007; Ranjit et al., 2011). As expected, TNF-α downregulated perilipin protein levels compared to its 16 hour time-paired control ($p<0.05$, $n=4$). TNF-α also down regulated HSL when compared to the time-paired control ($p<0.01$, $n=4$). However ATGL protein levels were not reduced. No significant changes were observed in MAPK protein across the conditions.

Human subcutaneous differentiated adipocytes were also stimulated to assess protein expression of ATGL, HSL, perilipin and MAPK. The stimulation was repeated with an additional 24 hour time point (Figure 10, $n=3$). Similar to 3T3-L1 differentiated adipocytes, TSH did not cause any significant changes in the protein expression of ATGL, HSL or perilipin. TNF-α did not cause a significant reduction in any of the proteins evaluated. TNF-α at higher concentrations and longer exposure time has been shown to reduce HSL and perilipin levels in human adipocytes (Laurencikiene et al., 2007). A suggested decrease in protein levels over time was observed for ATGL and HSL in all conditions. MAPK protein levels did not show any significant change.

Overall, TSH had no impact on the protein expression of several key proteins that are involved in lipolysis.

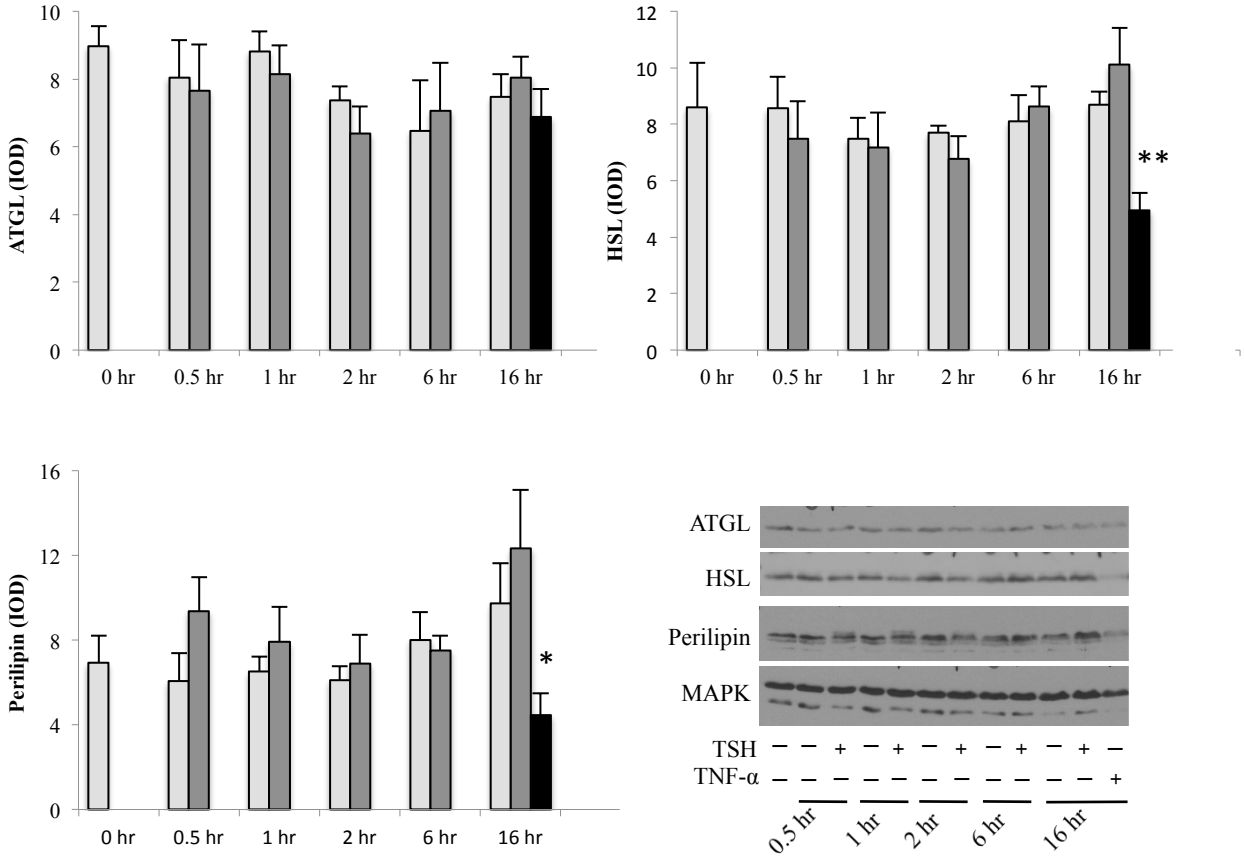

Figure 9. Protein expression of lipolytic proteins in 3T3-L1 differentiated adipocytes. Cells differentiated according to protocol and serum reduced to 1% CS overnight prior to stimulation. Cells stimulated in DMEM (phenol red-free) 1% CS and 1% PS with water (□), 5 mU/ml TSH (▣) or recombinant TNFα (■) and lysed at indicated times. Lysates run on western and immunoblotted for ATGL, HSL, perilipin or MAPK and expressed as integrated optical density. Results represent the mean +/- SEM of 4 independent experiments, two performed in duplicates and two performed as single samples. Statistical significance was determined by a two-way ANOVA, followed by Tukey post-hoc for comparisons of means. *$p<0.05$, and **$p<0.01$ vs. 16 hr control.

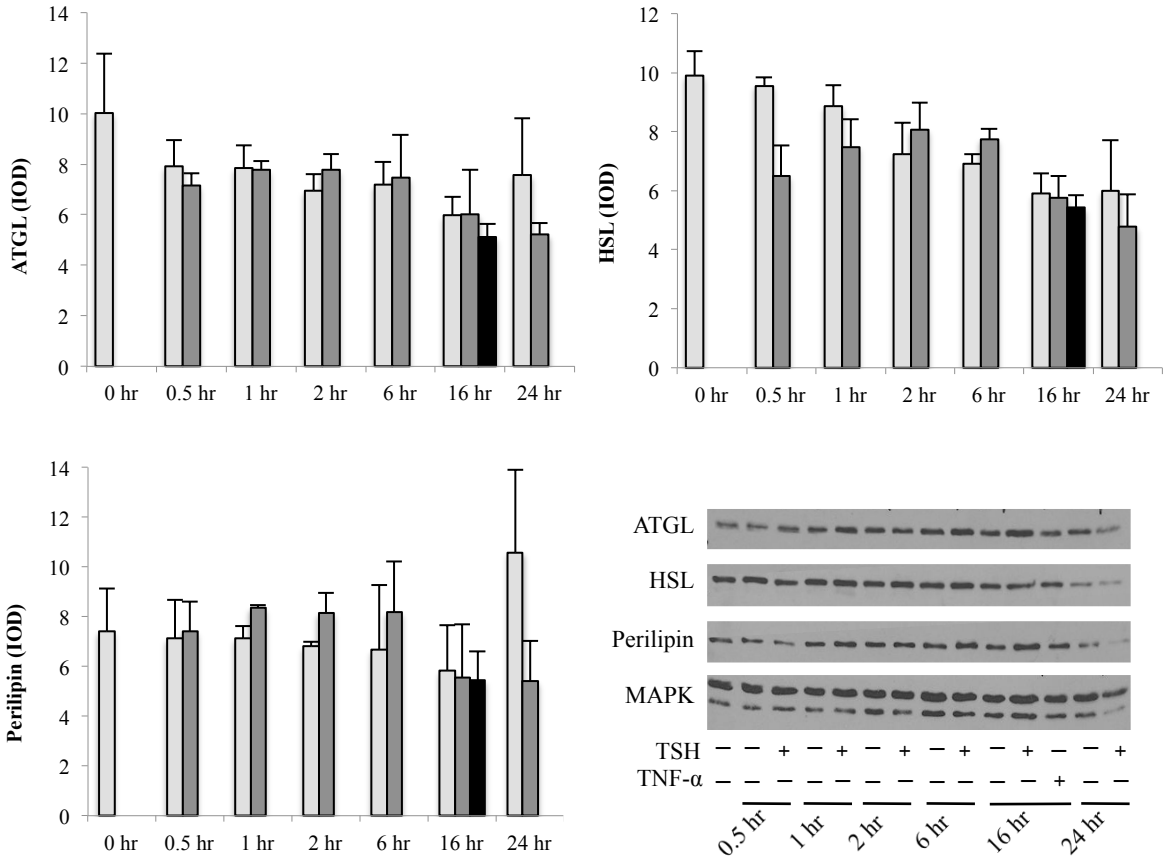

Figure 10. Protein expression of lipolytic proteins in human subcutaneous differentiated adipocytes. Cells differentiated according to protocol and serum reduced to 1% CS overnight prior to stimulation. Cells stimulated in DMEM (phenol red-free) 1% CS and 1% PS with water (□), 5 mU/ml TSH (▩) or recombinant TNFα (■) and lysed at indicated times. Lysates run of western and immunoblotted for ATGL, HSL, perilipin or MAPK and expressed as integrated optical density. Results represent the mean +/- SEM of 3 independent experiments, two performed in duplicates and one performed with single samples. Statistical significance was assessed by a two-way ANOVA with no significance observed.

II. Investigate Inflammatory Effect of TSH-Stimulated Adipocyte-Conditioned Medium on Macrophages

a. Investigate TSH-Stimulated Adipocyte-Conditioned Medium on Macrophages

i. TSH-stimulated 3T3-L1 differentiated adipocyte-conditioned medium does not alter J774 macrophage cytokine response

Control and 3T3-L1 differentiated adipocyte-conditioned medium were generated with or without TSH as described in Materials and Methods. Control medium (C), control medium with TSH (T-C), and adipocyte conditioned medium (ACM) were used as controls to assess TSH-stimulated adipocyte conditioned medium (T-ACM). J774 macrophages were incubated with these conditioned media for 2, 6 or 24 hours before cell lysis and RNA extraction for real time PCR. The four pro-inflammatory cytokines investigated for potential differences in mRNA expression levels were IL-1β, IL-6, MCP-1 and TNF-α.

For IL-1β mRNA, no change was observed after 2 and 6 hour incubations with T-ACM (Figure 11). There was an increase in IL-1β mRNA levels after a 24 hour incubation with T-ACM, however the error present in the 24 hour samples does not allow for significance to be attained. MCP-1 mRNA levels were also relatively unchanged for all 4 conditions after 2 and 6 hour incubations whereas the 24 hour incubation increased MCP-1 mRNA levels in T-C, ACM and T-ACM. The variation observed in the 24 hour samples caused a high level of error, and significance was not obtained. IL-6 mRNA remained relatively unchanged after 2, 6 and 24 hour incubations with all 4 conditions. TNF-α mRNA levels also remained unchanged in all conditions and at all time points investigated. Overall, no significant

changes were observed in the mRNA expression of these four pro-inflammatory cytokines after exposure to T-ACM.

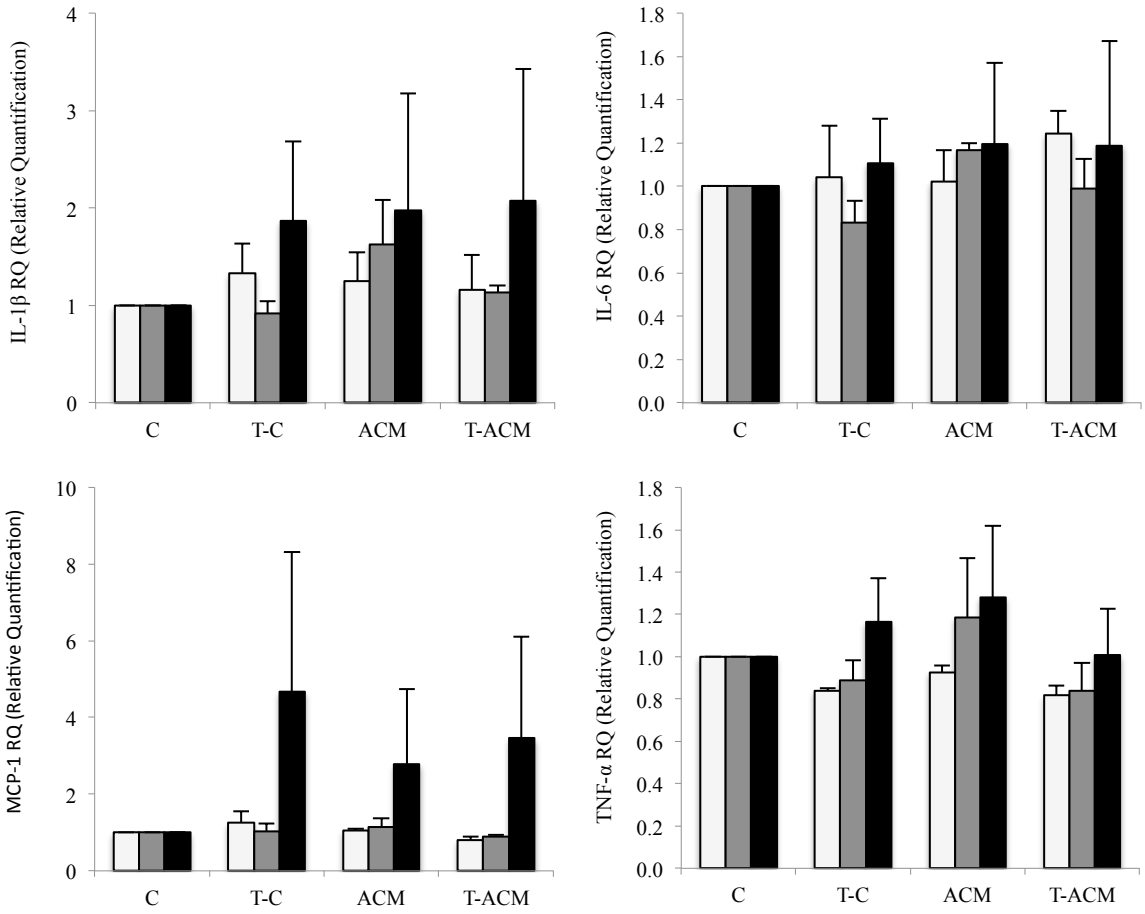

Figure 11. Inflammatory cytokine mRNA expression of J774 macrophages exposed to TSH-stimulated 3T3-L1 differentiated adipocyte media. J774 macrophages were exposed either to control media with vehicle (C) or 5 mU/mL TSH (T-C), or 3T3-L1 adipocyte conditioned media with vehicle (ACM) or stimulated with 5 mU/mL TSH (T-ACM). Each media contained phenol red-free DMEM, 1% CS and antibiotics and were conditioned for 4 hours prior to J774 macrophage exposure. These conditioned media were placed on J774 macrophage for 2 (□), 6 (▩) or 24 hours (■). Isolated RNA was reverse transcribed and probed for IL-1β, IL-6, MCP-1 or TNFα. mRNA quantified via real time PCR, normalized to 18S and expressed as a fold of time paired control media. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was assessed by a two-way ANOVA with no significance observed.

ii. TSH-stimulated human subcutaneous differentiated adipocyte-conditioned medium does not alter THP-1 macrophage cytokine response

THP-1 macrophages were incubated with control or human subcutaneous adipocyte-conditioned media for 2, 6 and 24 hours. IL-6 mRNA levels were too low to be reliably quantified in any of the conditions or incubation times. This observation agrees with Chellat *et al.* who demonstrated that secretory levels of IL-6 in basal THP-1 macrophages were undetectable at 1, 6 and 24 hours (Chellat et al., 2005).

IL-1β mRNA did not change in any of the conditions after the 2 hour incubation (Figure 12). IL-1β mRNA increased slightly in the ACM and T-ACM conditions after the 6 hour incubation, whereas the increase was much larger for ACM and T-ACM after the 24 hour incubation. These increases, however, were not significant.

Similar observations were noted in the MCP-1 mRNA levels. No change was observed in THP-1 macrophage MCP-1 mRNA levels after a 2 hour incubation with C, T-C, ACM or T-ACM. There were suggested elevations in the MCP-1 mRNA levels in THP-1 macrophages exposed to ACM and T-ACM after 6 hour incubation and even greater increases in these conditions after 24 hour incubation. These increases were not found to be significant either. TNF-α mRNA levels were found to be significantly elevated in THP-1 macrophages conditioned with ACM and T-ACM for 24 hours ($p<0.01$, $n=3$), but were not different from each other. Both the 2 and 6 hour incubations had minor potential increases when exposed to ACM and T-ACM although they were not significant. In all conditions and incubated time points, TSH in control media had no direct effect on the macrophage inflammatory status.

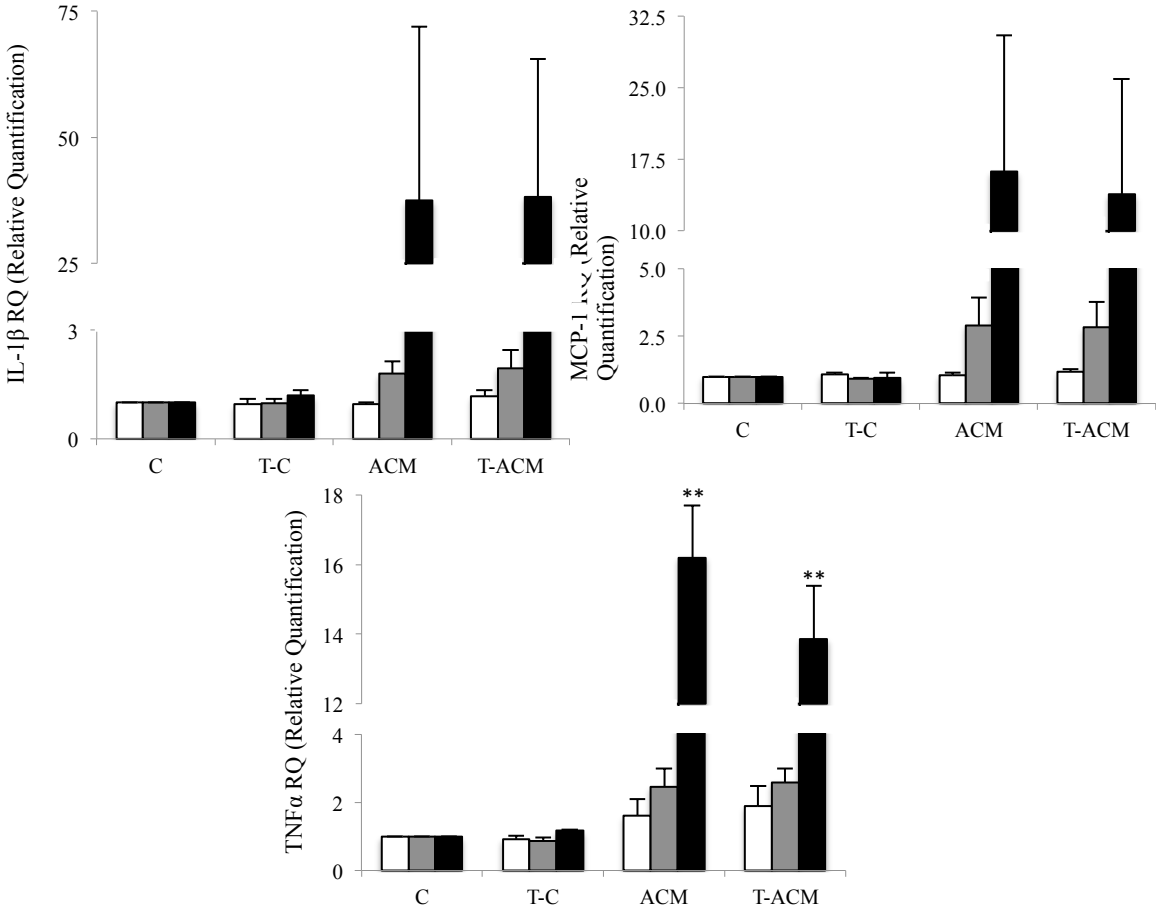

Figure 12. Inflammatory cytokine mRNA expression of THP-1 macrophages exposed to TSH-stimulated human subcutaneous differentiated adipocyte media. THP-1 macrophages were exposed either to control media with vehicle (C) or 5 mU/mL TSH (T-C), or human subcutaneous differentiated adipocyte conditioned media with vehicle (ACM) or stimulated with 5 mU/mL TSH (T-ACM). Each media contained phenol red-free DMEM, 1% CS and antibiotics and were conditioned for 4 hours prior to THP-1 macrophage exposure. These conditioned medias were placed on THP-1 macrophage for 2 (□), 6 (▩) or 24 (■) hours. Isolated RNA was reverse transcribed and probed for IL-1β, MCP-1 or TNFα. mRNA quantified via real time PCR, normalized to 18S and expressed as a fold of time-paired control. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was determined by a two-way ANOVA, followed by Tukey post-hoc for comparisons of means. **$p<0.01$ vs. time-paired control (C).

b. Evaluate Macrophage Response to LPS or FFA

Failure to detect changes in cytokine mRNA expression in either the J774 or THP-1 macrophages led me to consider an investigation of the macrophages to evaluate their responsiveness to a known stimulator of inflammatory cytokine gene expression.

i. Lipopolysaccharide induces J774 and THP-1 macrophage mRNA cytokine expression of IL-1β, IL-6, MCP-1 or TNF-α over after 2, 6 or 24 hour exposure.

LPS, a known pro-inflammatory endotoxin, was used to evaluate mRNA levels of IL-1β, IL-6, MCP-1 or TNF-α at 2, 6 and 24 hours under the same conditions used with the adipocyte-conditioned medium studies. In J774 macrophages, the LPS response was present, but highly variable. Significance was not attained due to the high variability, although the observed increases suggest that these cells are responsive to LPS in a pro-inflammatory nature (Figure 13, n=3).

The THP-1 macrophages were treated in the same manner as the J774 macrophages, employing LPS as the pro-inflammatory stimuli to investigate the inflammatory responses of these cells. Much like the J774 macrophages, LPS appeared to provoke a strong pro-inflammatory response in the THP-1 macrophages throughout the 2, 6 and 24 hour time points (Figure 14, n=3). The increase in MCP-1 and TNF-α mRNA expression at the 2 hour time point was significant ($p<0.05$, $p<0.01$ respectively). Both the murine and human macrophage cell lines appear to up-regulate pro-inflammatory cytokine mRNA expression in response to LPS under the conditions used to assess ACM and T-ACM. The next consideration to address was why there was no macrophage response to ACM and T-ACM.

It was necessary to investigate the pro-inflammatory responsiveness of the macrophage models to FAs.

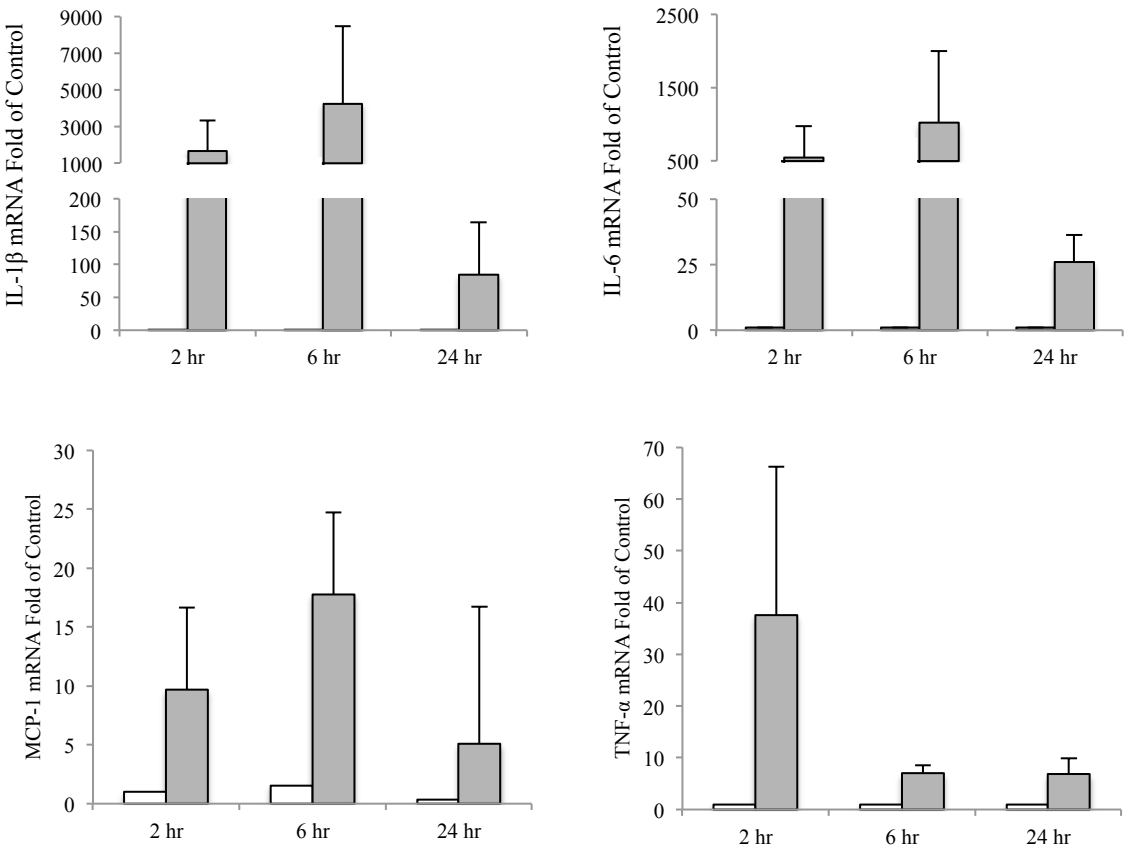

Figure 13. Inflammatory cytokine mRNA expression of J774 macrophages exposed to LPS. J774 macrophages exposed to the vehicle (□) or 10 ng/mL LPS (■) for 2,6 or 24 hours. Cells lysed and mRNA extracted for real time RT-PCR quantification. Values normalized to 18S mRNA and expressed as fold of time-paired controls. Results represent the mean +/- SEM of 3 independent experiments. Statistical significance was assessed by a paired t-test for comparison of means for each individual time point with no significance observed.

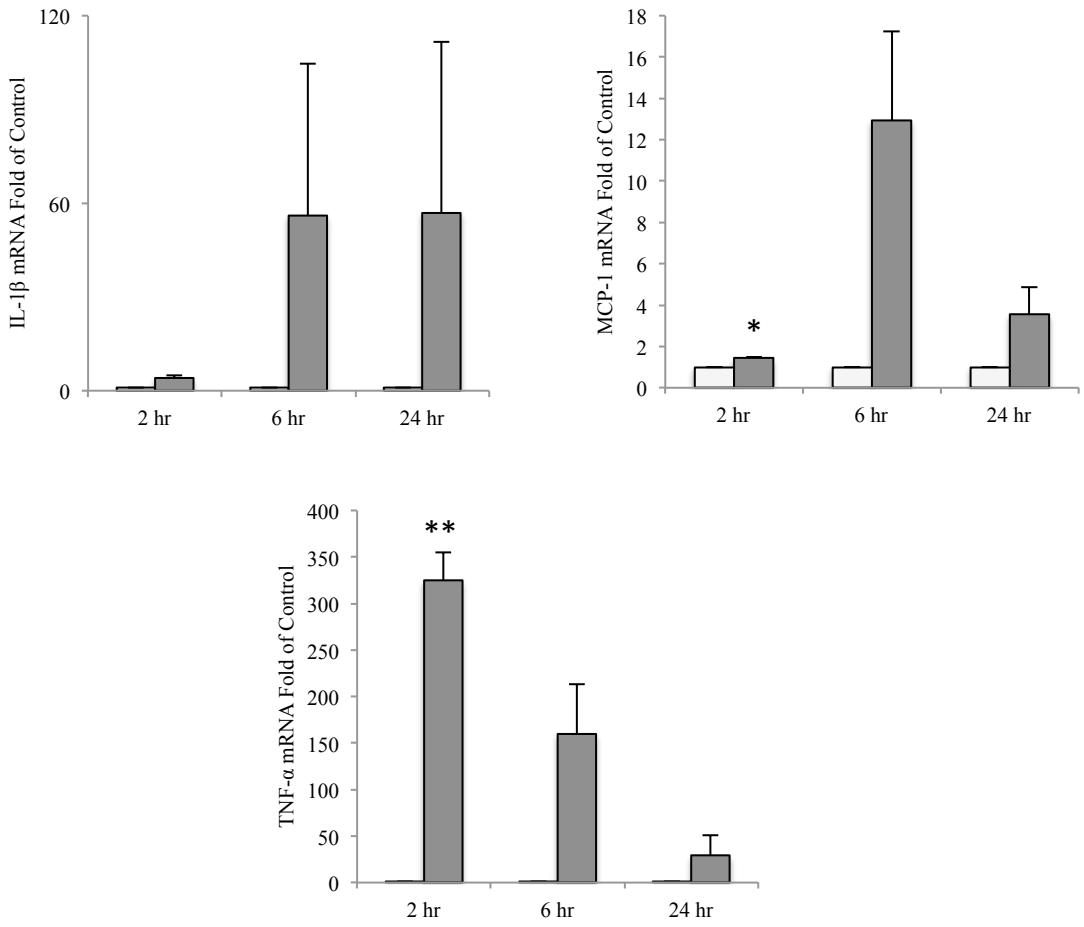

Figure 14. Inflammatory cytokine mRNA expression of THP-1 macrophages exposed to LPS. THP-1 macrophages exposed to the vehicle (□) or 10 ng/mL LPS (■) for 2,6 or 24 hours. Cells lysed and mRNA extracted for real time RT-PCR quantification. Values normalized to 18S mRNA and expressed as fold over time-paired controls. Results represent the mean +/- SEM of 3 independent experiments. Statistical significance was determined by a paired t-test for comparison of means for each individual time point. *$p<0.05$, **$p<0.01$ vs control.

ii. Palmitic acid does not increase macrophage mRNA cytokine expression of IL-1β, IL-6, MCP-1 or TNF-α in J774 macrophages after 1 hour exposure.

To determine the pro-inflammatory capabilities of FAs on macrophages under conditions used throughout the course of this investigation, palmitic acid was employed. PA is a 16-carbon saturated FA ($C_{16:0}$) and one of the most common saturated FA in plants and animals. It has previously been used to assess the pro-inflammatory responses of J774 and THP-1 macrophages. In J774 macrophages, de Lima-Salgado *et al.* reported that TNF-α mRNA increased 1.75 fold of basal after 1 hour in response to 50 μM PA (de Lima-Salgado, Alba Loureiro, do Nascimento, Nunes, & Curi, 2011). Concentrations of 50 μM and 100 μM of PA have previously been used on macrophages to assess their pro-inflammatory response (de Lima-Salgado et al., 2011).

After a 1 hour exposure in prf-DMEM supplemented with 1% CS, neither 50 μM nor 100 μM PA increased TNF-α mRNA transcription levels relative to the control in J774 macrophages (Figure 15, n=3). The mRNA expression level of MCP-1 also remained unchanged after a 1 hour exposure to 50 μM and 100 μM PA. The levels of IL-1β mRNA also showed minimal deviation from the control. Finally, IL-6 mRNA levels displayed slight, albeit non-significant, elevations.

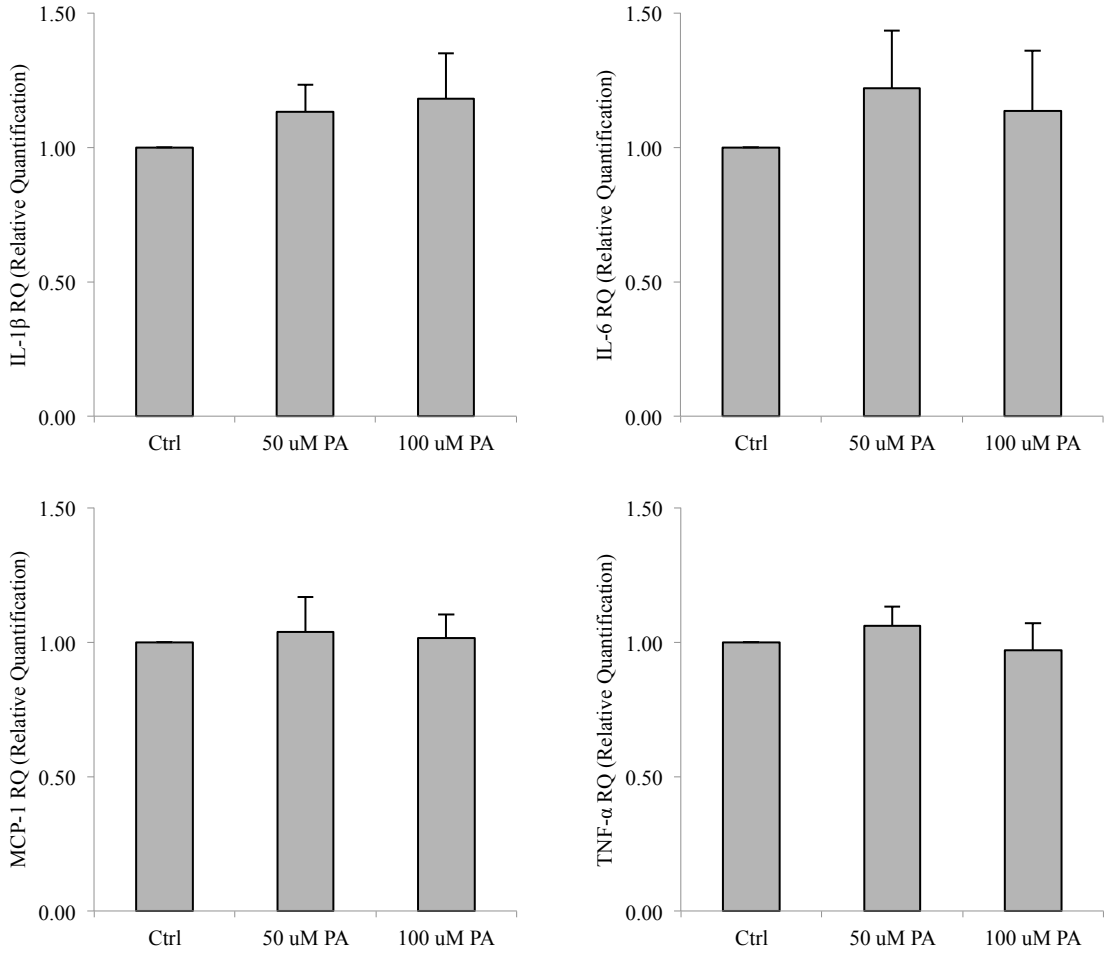

Figure 15. Inflammatory cytokine mRNA expression of J774 macrophages exposed to palmitic acid. J774 macrophages exposed to 50 μM or 100 μM palmitic acid (PA) for 1 hour in phenol red-free DMEM, 1% CS and antibiotics. Cells lysed and mRNA extracted for real time RT-PCR quantification. Values normalized to 18S mRNA and expressed as fold over control. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was assessed by a one-way ANOVA with no significance observed.

iii. Palmitic acid does not increase macrophage mRNA cytokine expression of IL-1β, MCP-1 or TNF-α in THP-1 macrophages.

Similar to the J774 macrophages, the THP-1 pro-inflammatory response was assessed under conditions containing prf-DMEM, 1%CS and antibiotics. THP-1 macrophages were also exposed to 50 μM and 100 μM PA, this time for 24 hours. PA was previously shown to increase both IL-1β and TNF-α mRNA levels in a dose dependent manner after 24 hour exposure (Subbaramaiah et al., 2011).

TNF-α mRNA levels were slightly reduced after 24 hour exposure to 50 μM or 100 μM PA although they did not reach significance (Figure 16). IL-1β mRNA levels on the other hand, were significantly reduced to 0.60 and 0.71 RQ after 24 hour exposure to 50 μM or 100 μM of PA respectively ($p<0.05$, $n=3$). MCP-1 mRNA levels did not significantly change after exposure to the two concentrations of PA.

J774 and THP-1 macrophages failed to produce a pro-inflammatory response to PA, a known pro-inflammatory FA. Failure to see a response using PA suggests that any FA present in ACM and T-ACM may not have been able to produce a macrophage pro-inflammatory response.

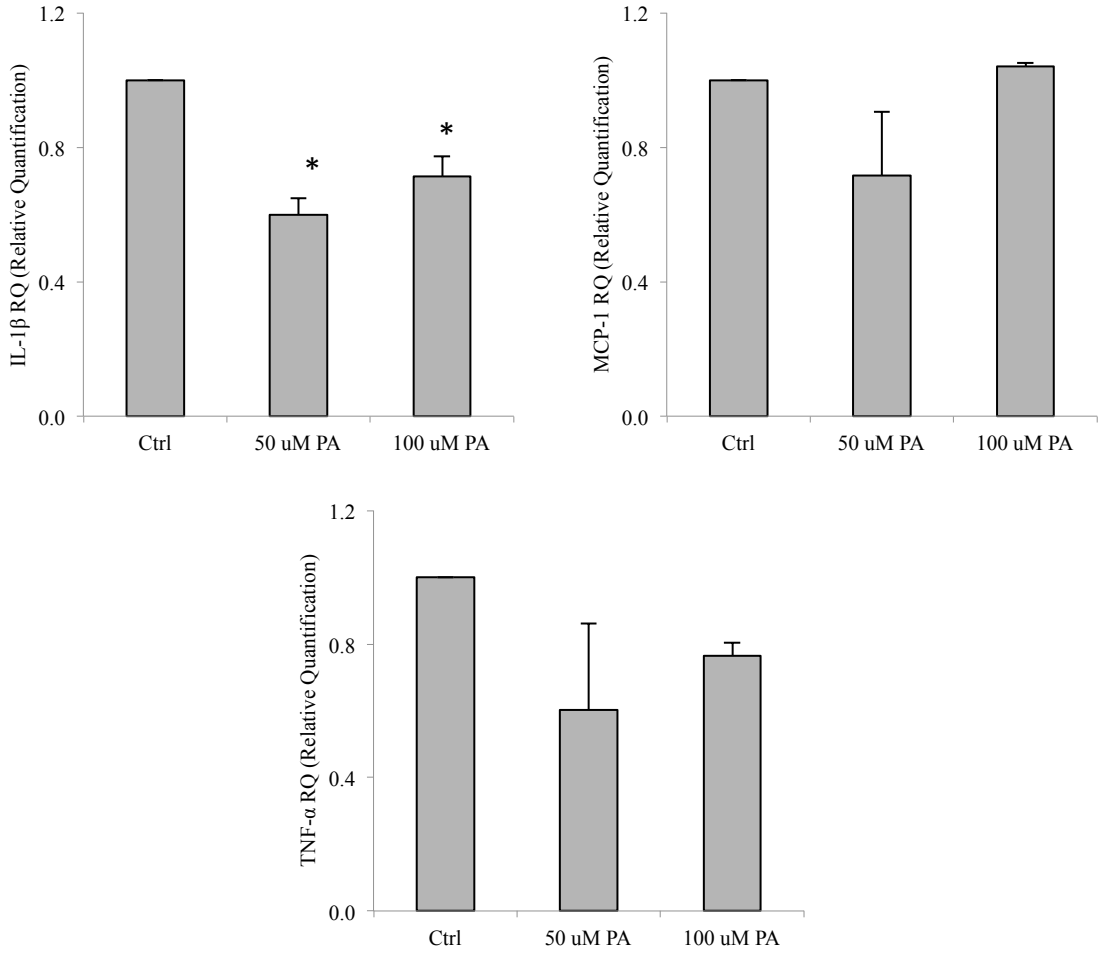

Figure 16. Inflammatory cytokine mRNA expression of THP-1 macrophages exposed to palmitic acid. THP-1 macrophages exposed to 50 µM or 100 µM palmitic acid (PA) for 24 hours in phenol red-free DMEM 1%CS and 1%PS. Cells lysed and mRNA extracted for real time RT-PCR quantification. Values normalized to 18S mRNA and expressed as fold over control. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was determined by a one-way ANOVA, followed by Student-Newman-Keuls post-hoc for comparisons of means. *$p<0.05$ vs control.

c. Evaluate FA Release from TSH-Stimulated Adipocytes

i. TSH-stimulated 3T3-L1 differentiated adipocytes do not increase FA release over 4 hour stimulation.

Prior experiments assessed lipolysis by measuring glycerol release. To verify FA were also present in the 1% CS-supplemented medium, conditioned medium was assayed for FAs. We could not reliably detect any FA in media from 3T3-L1 differentiated adipocytes in DMEM containing only 1% CS (data not shown). The absence of FA led to the suspicion that there was insufficient carrier protein, such as BSA, capable of binding to and stabilizing FA in the 1% CS-supplemented media (Vaughan, 1962). TSH-stimulated lipolysis was thus re-investigated using both 1% CS and 1% BSA supplementation together. There was still no increase in FA observed in 3T3-L1 differentiated adipocytes stimulated with TSH, despite the addition of BSA to the media (Figure 17). The level of glycerol, although insignificant, went from to 0.05 to 0.15 μmol/mg protein, which suggests the rate of lipolysis may have increased over the 4 hour stimulation period. Further experimentation would be required however to confirm this observation.

ii. TSH-stimulated human subcutaneous differentiated adipocytes may require BSA supplementation for increased FA release over 4 hour stimulation.

Similar to the 3T3-L1 differentiated adipocytes, we could not measure FA in DMEM with 1% CS supplementation from TSH-stimulated human subcutaneous differentiated adipocytes (data not shown). The human subcutaneous differentiated adipocytes were reassessed using DMEM containing both 1% CS and 1% BSA medium. An increase in FA was observed upon TSH stimulation as basal FA levels were 0.24 μmol/mg protein and increased to 0.69 μmol/mg protein upon TSH stimulation (Figure 18). This complemented a glycerol increased from 0.14 μmol/mg protein to 0.60 μmol/mg protein. Owing to experimental variation, these increases in FA and glycerol however were not found to be statistically significant. Despite the lack of significance, the addition of BSA allowed for quantifiable levels of FA and suggested a need when investigating FA release.

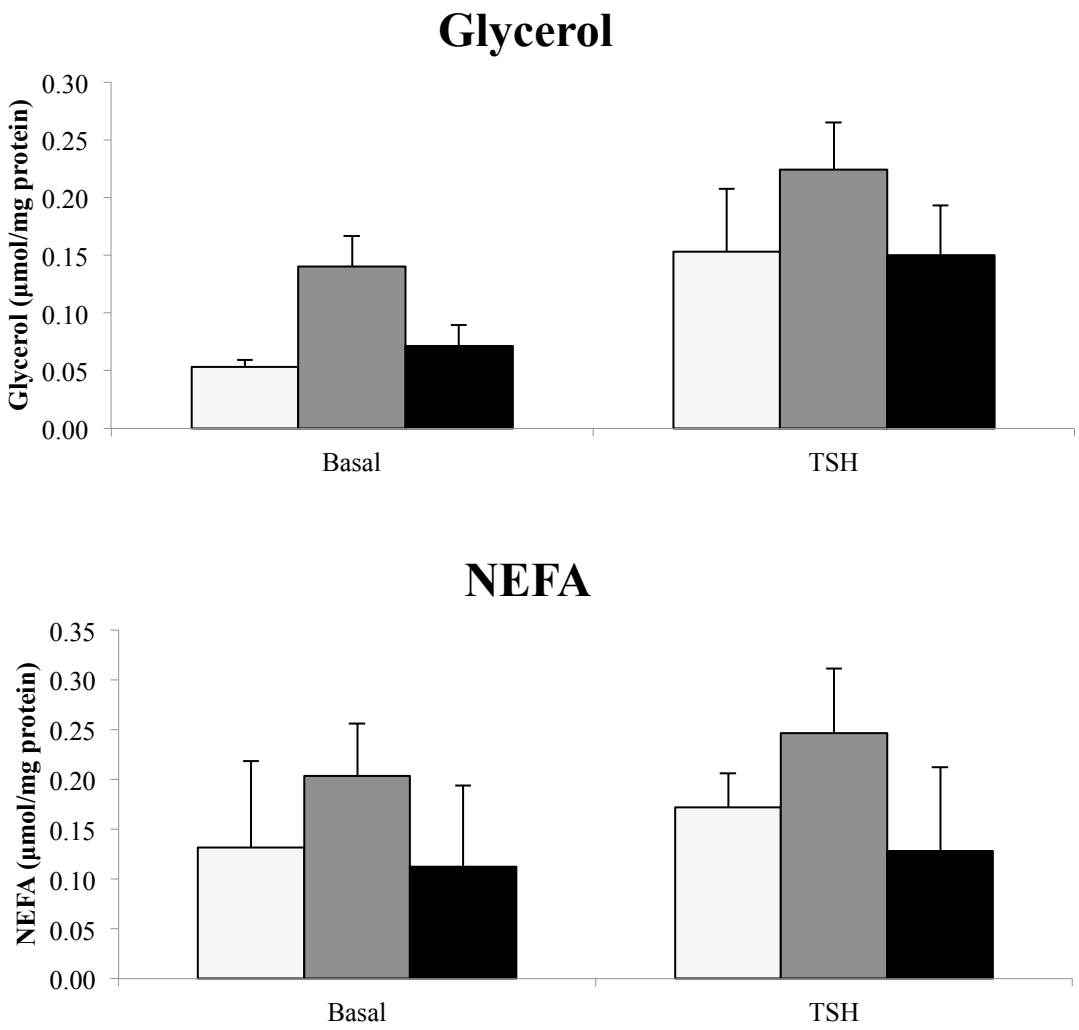

Figure 17. TSH-stimulated lipolysis in 3T3-L1 differentiated adipocytes post cytokine exposure. 3T3-L1 adipocytes differentiated as per protocol and exposed to 10ng/ml of recombinant mouse TNF-α (▩), IL-6 (■) or with vehicle (□) (PBS, 0.1% BSA) for 24 hours. Cells washed and stimulated with water (vehicle) or 5 mU/ml TSH for 4 hours. Media collected and assessed for glycerol and NEFA and expressed as µmol/mg protein. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was assessed by a two-way ANOVA with no significance observed. TG extracted and cells lysed for protein analysis.

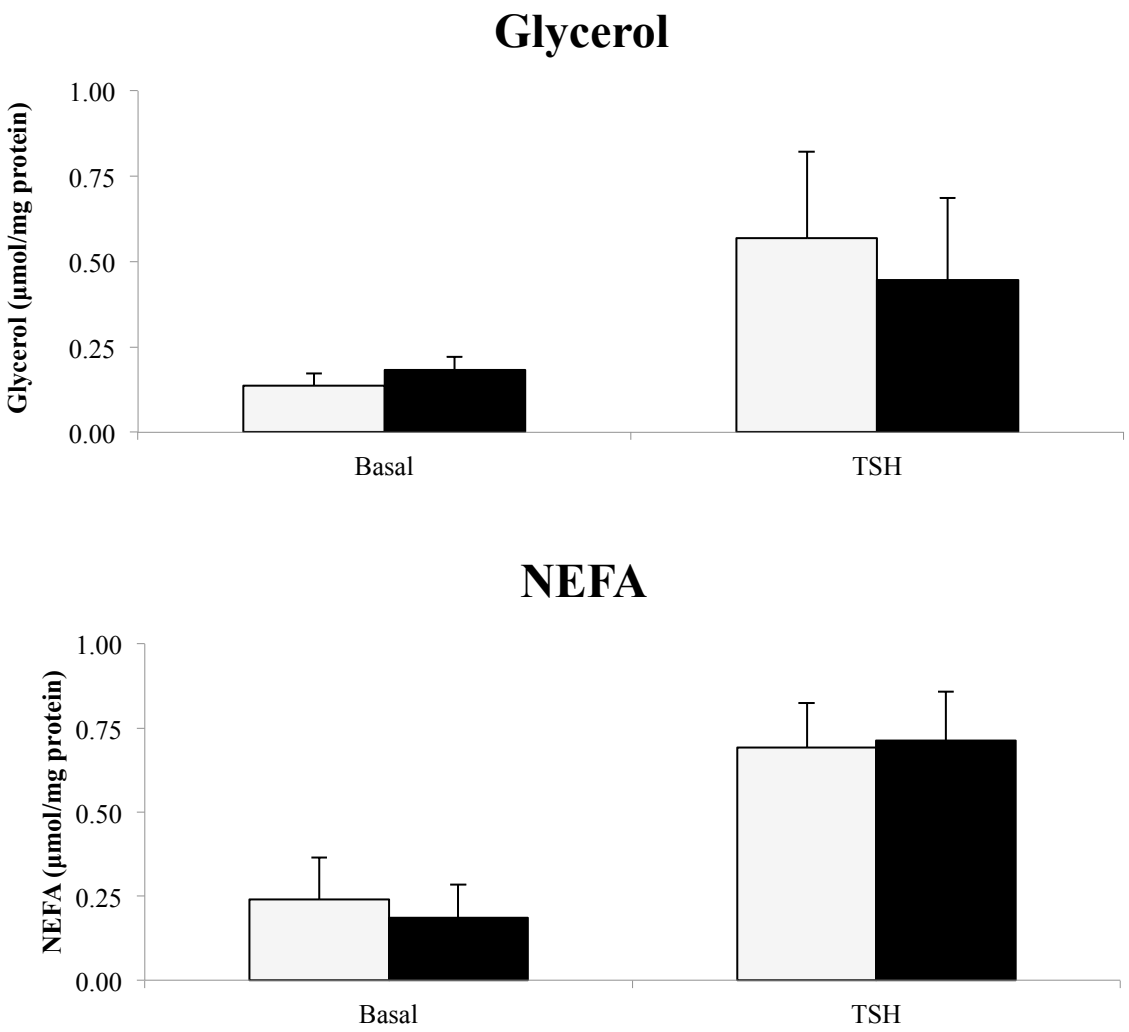

Figure 18. TSH-stimulated lipolysis in human subcutaneous differentiated adipocytes post cytokine exposure. Human subcutaneous adipocytes differentiated as per protocol and exposed to 10ng/ml of recombinant mouse IL-6 (■) or with vehicle (□) (PBS1x, 0.1% BSA) for 24 hours in growth media. Cells washed and stimulated with water (vehicle) or 5 mU/ml TSH with 1% CS, 1% BSA supplementation for 4 hours. Media collected and assessed for glycerol and NEFA and expressed as µmol/mg protein. Results represent the mean +/- SEM of 3 independent experiments, each performed in duplicates. Statistical significance was assessed by a two-way ANOVA with no significance observed. TG extracted and cells lysed for protein analysis.

III. Evaluate the Effect of Cytokines on TSH-Stimulated Adipocyte Lipolysis

a. TSH-stimulated lipolysis in 3T3-L1 and human subcutaneous differentiated adipocytes following cytokine treatment.

IL-6 and TNF-α increase lipolysis through reduction of perilipin protein expression (Bézaire et al., 2009; Kralisch et al., 2005; Petersen et al., 2005; Pä th et al., 2001; Souza et al., 2003; Yang et al., 2008). Elevated levels of these cytokines have also been documented in patients with increased levels of TSH in the context of subclinical hypothyroidism (Tremen et al., 2011). To assess the potential effect of pro-inflammatory cytokines on TSH stimulated lipolysis, 3T3-L1 differentiated adipocytes were pre-incubated with IL-6 or TNF-α, each at 10 ng/mL, for 24 hours, before changing the medium and stimulating with or without 5 mU/mL of TSH for 4 hours. The medium was then analyzed for FA and glycerol. Basal glycerol level of 3T3-L1 differentiated adipocytes without cytokine exposure was 0.05 μmol/mg protein after 4 hours of incubation (Figure 17). Glycerol levels post exposure to IL-6 or TNF-α for 24 hours were 0.07μmol/mg protein and 0.14 μmol/mg protein respectively. These elevations were not found to be significant. A suggested elevation in FA was observed post TNF-α exposure without TSH stimulation when compared to the medium that was free of both cytokines and TSH, however the elevation was also insignificant. Stimulation with TSH increased FA levels in all conditions when compared to their respective basal controls, but no significance was attained.

In human subcutaneous differentiated adipocytes, IL-6 did not have an effect on either basal or TSH stimulated responses versus control conditions (Figure 18, n=3). The basal glycerol response was 0.14 μmol/mg protein, post exposure to IL-6 the value was 0.18

μmol/mg protein. When the cells were stimulated with TSH the glycerol level was found to be 0.57 μmol/mg protein and stimulation with TSH after 24 hour exposure to IL-6 gave a response of 0.45 μmol/mg protein. The FA levels were found to be 0.24 μmol/mg protein basally and 0.19 μmol/mg protein after 24 hour exposure to IL-6. The FA levels after TSH stimulation was found to be 0.69 μmol/mg protein and 0.71 μmol/mg protein after TSH stimulation following 24 hour exposure to IL-6.

DISCUSSION

The study set out to test the hypothesis that TSH-stimulated FA release from adipocytes contributes to macrophage activation and inflammation. Initial preparatory studies were conducted to establish optimal conditions for producing potent TSH-stimulated adipocyte-conditioned medium. The medium, from either mouse 3T3-L1 adipocytes or human differentiated adipocytes, was added to either murine or human macrophages, respectively, and cellular pro-inflammatory mRNA cytokine expression was assessed. No changes in macrophage cytokine mRNA expression were noted, therefore I subsequently aimed to evaluate the integrity of the macrophage models by assessing their responses to known pro-inflammatory stimuli, namely LPS and PA. Furthermore, I re-evaluated the medium conditions with respect to their FA content.

Part 1

a. Different TSH sources

In this thesis, my studies confirm the concept that TSH stimulates lipolysis using both bovine and recombinant human TSH reagents from multiple sources. The TSH source was a question to be considered as comparative studies using multiple TSH sources indentified differences in *in vivo* and *in vitro* responses in other tissues (East Palmer, Szkudlinski, Lee, Thotakura, & Weintraub, 1995; M. W. Szkudlinski et al., 1993). Our studies show that there are no differences in 3T3-L1 or human subcutaneous differentiated adipocyte stimulation between these commercial preparations over a 4 hour period (Figures

5 and 6 respectively). This observation comes despite variation in sialylation and glycosylation of TSH from the different sources. It is possible that differences in their responses might arise over longer incubations, as their posttranslational modifications can increase TSH half-life and binding abilities. For the purpose of these studies however, I concluded that using bovine TSH from Sigma was appropriate. In the 3T3-L1 adipocytes, an increase was observed upon using 50 mU/mL over 5 mU/mL. However, this was not significant and 5mU/mL was considered to be sufficient for use in 3T3-L1 adipocytes. In human subcutaneous differentiated adipocytes, our lab has previously shown that 5mU/mL of TSH was sufficient to generate a significant glycerol response (Gagnon et al., 2010).

b. Lipolysis Conditions

i. DMEM vs KRH

Prior to these studies, TSH-stimulated adipocyte lipolysis had been untested in DMEM. Through experimenting with multiple conditions in 3T3-L1 differentiated adipocytes, I found that there was a consistent trend in reduction of glycerol levels in DMEM compared to KRH levels, no matter what other conditions were present (Figure 7). Niacinamide is capable of inhibiting lipolysis in epididymal adipose tissue of Sprague-Dawley rats at a concentration similar to that found in DMEM (Perpeira & Holland, 1966). Interestingly, this pattern of reduced responses in DMEM compared to KRH was not observed in the experiments using human subcutaneous differentiated adipocytes (Figure 8). Thus, if this trend is due to niacinamide, the lack of any difference may be the result of

decreased potency in human cells. Studies would be required to confirm this possibility about niacinamide.

ii. Overnight Serum Reduction vs Regular Growth Medium

Overnight serum reduction of cells prior to TSH stimulation was compared to cells maintained in regular growth medium. Growth medium for 3T3-L1 adipocytes and human subcutaneous differentiated adipocytes is slightly different, containing either 10% CS or 10% FBS respectively. FBS and CS are highly variable and contain a myriad of bioactive factors that can affect lipolysis and cell function *in vitro*.

In 3T3-L1 differentiated adipocytes, overnight serum reduction appeared to reduce both the basal and stimulated levels of glycerol in KRH. The TSH response, expressed as fold of basal, did not change however. There were no differences observed in DMEM conditions using overnight serum reduction when compared to their respective conditions that used regular medium prior to stimulation. These observations suggest that overnight serum reduction does not have a major impact on TSH-stimulated lipolysis in 3T3-L1 adipocytes.

In human subcutaneous differentiated adipocytes, overnight serum reduction appears to lower the level of glycerol release in nearly all of the basal conditions, thereby providing a greater fold of basal response with TSH treatment. This decrease could be due to reduced lipolytic activity resulting directly from the reduced serum. Unlike the 3T3-L1 adipocytes, the impact of overnight serum reduction in human subcutaneous differentiated adipocytes does not increase the TSH response, expressed as glycerol release. This difference could be related to the difference in serum supplementation used in regular growth medium for the

respective cell lines. As mentioned, both CS and FBS contain a variety of growth factors, including insulin, growth hormone, β-adrenergic receptor agonists and cytokines, all of which can impact lipolysis.

FBS contains a high level of hormones that can also impact cell growth and function. FBS also contains a lower amount of antibodies than CS, thereby reducing immune interactions with cells in culture. In comparison, CS contains lower hormone level, but a higher number of antibodies. These differences could affect cell responses and explain the differences observed between these cell culture models. Regardless of the impact that regular growth medium has on lipolysis, overnight serum reduction prior to stimulation appeared to allow a better response in the human subcutaneous differentiated adipocytes. It was also decided to use serum reduction in the preparation of 3T3-L1 adipocyte-conditioned medium.

iii. CS vs BSA

BSA supplementation can increase cytokine release from human adipocytes and may alter adipocyte function (Schlesinger, van Harmelen, Alberti Huber, & Hauner, 2006). CS can be highly variable and also contribute to altered cell responses. Therefore, I evaluated their effect on TSH-stimulated glycerol release.

In 3T3-L1 differentiated adipocytes, CS seemed to consistently provide a greater difference between the basal and TSH-stimulated conditions when compared to the conditions using BSA. The reason for this is unknown at present.

In the human subcutaneous differentiated adipocytes, CS during stimulation and after incubation overnight in regular growth medium resulted in a decrease in the difference between the basal and TSH-stimulated states when compared to the BSA counterparts. Due

to the differences between the two adipocyte models and the multiple conditions considered, it is difficult to determine the effect each variable had. Interestingly however, this analysis did result in a common set of conditions optimal for both the 3T3-L1 and human subcutaneous differentiated adipocytes. The use of overnight serum reduction and stimulation in prf-DMEM containing 1% CS offered both a low basal level and a strong TSH stimulation.

c. Expression of proteins involved in lipolysis

Regulation of TSH-stimulated lipolysis and the signalling events of TSH on adipocytes are still being clarified. Recent work in our laboratory showed that signalling events for TSH-stimulated lipolysis are similar to those of catecholamine stimulation (Gagnon et al., 2010). Isoproterenol, a catecholamine analogue, has been shown to reduce 3T3-L1 adipocyte mRNA levels of HSL and ATGL in as little as 1 hour at a concentration of 10 nM (Kralisch et al., 2005). Studies were undertaken to investigate the possibility of TSH regulation of lipolytic proteins. Results revealed that TSH does not alter the protein expression of ATGL, HSL or perilipin in 3T3-L1 adipocytes within the 16 hours investigated or within 24 hours in human subcutaneous differentiated adipocytes. Despite the lack of changes in the proteins investigated, this does not discount the potential for other lipolytic proteins to be regulated through TSH stimulation. Fat specific protein 27 (FSP27), a lipid droplet associated protein thought to prohibit lipases from reaching TG, is found to be paradoxically upregulated by isoproterenol (Ranjit et al., 2011). Unfortunately, commercial antibodies currently available are of poor quality, and I could not reliably measure endogenous FSP27 expression.

d. T-ACM on Macrophages

We initially set out to determine if TSH-stimulated adipocyte-conditioned medium was capable of inducing a pro-inflammatory response in macrophages. Experiments were conducted to ensure that the method for conditioning the medium offered the greatest lipolytic response in adipocytes. We expected the increased release of FA from TSH stimulation would promote a pro-inflammatory response within macrophages. The use of TSH-stimulated adipocyte-conditioned medium on macrophages has not been assessed to date. Within the literature, basal adipocyte conditioned medium has been tested on macrophages with contrasting observations. While Liu *et al.* demonstrated IL-1β and TNF-α are increased in RAW264.7 cells after exposure to isolated primary mouse ACM, Kohlstedt *et al*. demonstrated that mature human adipocyte-conditioned medium actually reduces IL-6, MCP-1 and TNF-α mRNA levels in human blood-derived macrophages (Kohlstedt, Trouvain, Namgaladze, & Fleming, 2011; Liu et al., 2010). Moreover, others have shown that basal 3T3-L1 ACM does not induce any secretion of IL-6 or TNF-α in the J774 macrophages (Li & Renier, 2007). Differences in conditions, exposure times and cell lines used could contribute to these responses and demonstrate that the inflammatory response of macrophages exposed to ACM can vary greatly.

In the studies investigating J774 macrophage exposure to various adipocyte-conditioned media, there were no changes in IL-1β, IL-6, MCP-1 or TNF-α mRNA levels after 2 or 6 hour exposure to T-ACM. There were also no significant changes in ACM exposure and this is consistent with the lack of effect observed by Li *et al.* as previously described (Li & Renier, 2007). There was large variation in the mRNA levels of IL-1β and MCP-1 after 24 hour exposure to T-C, ACM and T-ACM and this was due to an outlier

observed in one of the experiment. No significance was obtained in the 24 hour conditions results and there is no indication that T-ACM had a greater pro-inflammatory effect over any of the other conditions. Thus it was concluded from this set of experiments that TSH stimulation of 3T3-L1 differentiated adipocytes did not provoke a greater pro-inflammatory response in J774 macrophages over basal ACM.

Investigating THP-1 macrophage exposure to various adipocyte-conditioned media showed that there was a significant increase in TNF-α mRNA levels after 24 hour exposure to either ACM or T-ACM. There was no difference observed between ACM and T-ACM. This suggested that human subcutaneous differentiated adipocytes are capable of basally secreting pro-inflammatory molecules, such as FA and cytokines, and that there was little change in medium pro-inflammatory capabilities upon stimulation of adipocytes with TSH. The mRNA levels of IL-1β and MCP-1 were also elevated after 24 hour exposure to ACM and T-ACM, but experimental variation was such that significance was not obtained. I concluded from this set of experiments that adipocyte-conditioned medium is capable of provoking a pro-inflammatory response in THP-1 macrophages but T-ACM does not provoke a greater inflammatory response over ACM.

As previously mentioned, the response to ACM from macrophages can vary from anti- to pro-inflammatory. Our initial hypothesis that a pro-inflammatory response would be observed was disproven in both the J774 and THP-1 macrophages as there was a failure by T-ACM to cause an increase in pro-inflammatory cytokine mRNA level over ACM. Efforts were then methodically redirected to either confirm these results as true or provide evidence that the models and conditions employed were defective in some capacity, yielding negative results due to an artifact.

Part 2

a. FA inflammatory profile

The lack of macrophage response could have been due to a variety of factors. One possibility was that the profile of the adipocyte-secreted FAs could have been net-neutral or net-negative in inflammatory status. The types of FAs released via lipolysis are tightly regulated, are subjected to environmental influences, and do not necessarily reflect the composition of those found in TG lipid droplets (Raclot & Oudart, 2000). This differential regulation of FA mobilization favors short and unsaturated FAs over the rest (Raclot & Oudart, 2000). Therefore, the FAs present after TSH-stimulation of adipocytes may have not initiated a pro-inflammatory response in macrophages. Moreover, lipid droplet formation in differentiated adipocytes *in vitro* is dependent on the composition of lipids in the supplemental serum used in the differentiation medium. Independent researchers characterizing FBS showed that docosahexaenoic acid (DHA, 22:6), a well documented anti-inflammatory FA, was found to be 8 times greater in FBS when compared to CS (Stoll & Spector, 1984). The level of PUFA in FBS and CS was found to make up 27.8 % and 52.0% respectively of the FA present (Stoll & Spector, 1984). Commercial suppliers of CS and FBS do not test for the FA composition, thus we do not know the FA profile of CS and FBS used within the lab during these experiments. Nevertheless, despite these theoretical considerations, differentiated adipocyte-derived FAs in a co-culture system with macrophages have been shown to activate macrophage inflammation through increased cytokine secretion (Suganami et al., 2005). In turn, macrophage secreted TNF-α stimulated adipocyte lipolysis to secrete more FA, thereby promoting a positive paracrine interaction.

Consequently, other avenues were investigated prior to considering the labour intensive undertaking of serum lipid characterization.

b. Macrophage inflammatory responsiveness

To ensure that the J774 and THP-1 macrophages were responsive to inflammatory stimuli under the conditions that I used, the macrophages were treated with LPS or PA. These molecules are known to increase cytokine mRNA levels in macrophages (de Lima-Salgado et al., 2011; Subbaramaiah et al., 2011; Yeop Han et al., 2010; Zhao et al., 2005). Whereas LPS produced a pro-inflammatory response of the macrophages, PA failed to induce a response. A response to PA should have been seen, however my conditions were not the same as in the literature. My conditions contained only CS while other studies used BSA. This suggests that PA signalling may require specific conditions to induce inflammation. Cultured spleen cells administered 50 or 100 μM palmitate in RPMI with 10% CS and varying glucose concentrations saw, in some conditions, reductions in secreted TNFα (Thorvaldson, Stlhammar, & Sandler, 2008). This is reminiscent of the reduction in IL-1β I observed in THP-1 macrophages exposed to PA. The culture used but Thorvaldson et al. had been depleted of only erythrocytes and contains mostly B and T lymphocytes. The authors consider the possibility of cell models, culture conditions and glucose concentration as possible causes of their results. They also suggested that the ethanol used to dissolve the palmitate could be the cause of the cytokine decrease. In our PA studies, the ethanol was evaporated using inert nitrogen gas prior to adding the medium. Therefore, it is likely not responsible for the negative results. Interestingly, Thorvaldson *et al*. conditions used 10% CS with no addition of BSA and suggests the idea that BSA is required. FA signalling in

macrophages is not entirely clear and ongoing work in the field has recently implicated endotoxin contamination of BSA in FA-induced inflammation *in vitro*. The presence of BSA may be needed to promote pro-inflammatory signalling.

c. Macrophage Inflammatory Signalling

Toll-like receptors (TLRs) were identified as a target for FA that mediates the activation of macrophages (Shi et al., 2006). However, recent data show that saturated FAs in medium containing contaminant-free BSA failed to activate TLR in HEK293 cells and did not provoke an inflammatory response in macrophages (Erridge & Samani, 2009). These conflicting results suggest the role of FAs in macrophage inflammation is complex and somewhat controversial. Endotoxin contamination, specifically LPS and lipopeptide contaminants, in FA-free BSA (FAF-BSA) has been identified as the potential source of the inflammatory response from FAs and other previously identified TLR ligands such as heat shock protein 60 and 70 (HSP60, HSP70) (Bausinger et al., 2002; Erridge & Samani, 2009; Gao & Tsan, 2003). Further studies suggest that SFAs actually do modulate TLR4 activity by influencing receptor dimerization and recruitment into lipid rafts (Wong et al., 2009). Others have shown that the LPS inflammatory response in increasing cytokines IL-6 and IL-8 depends on the metabolism of SFAs to ceramide in THP-1 monocytes with similar responses in both THP-1 macrophages and human primary monocytes (Schwartz et al., 2010). Yet reports indicate that pro-inflammatory cytokine production through TLR4 activation is independent of ceramide production in murine RAW264.7 macrophages (Holland et al., 2011). The inconsistency in the literature surrounding the issue of whether FAs activate macrophages may relate to differences in the cell models or experimental

conditions used by the various investigators. However, these studies suggest that in the absence of BSA, it is unlikely that a PA response would be seen as endotoxin contaminants would not be present. Since the literature suggests that the presence of endotoxins promotes FA-induced inflammation, the addition of LPS to future FA signalling studies may help facilitate a response. BSA has another important role in binding with FAs. BSA is needed to trap and stabilize FA in a medium that is naturally hydrophilic.

d. Investigate FA in Medium

ACM and T-ACM were tested for the presence of FAs. This was conducted because all prior studies investigated glycerol release and not FA release as an indicator of lipolysis. Glycerol as a measure of lipolysis is often used by investigators in this field but does not measure FA directly, the focus of our investigation. I found that no elevation of FA occurred in either the TSH-stimulated 3T3-L1 or human subcutaneous differentiated adipocyte medium in the presence of just CS. FAs require binding proteins to "trap" them in the medium because of their hydrophobic instability in solution. The initial lipolytic studies using glycerol release as an indicator of lipolysis led us to think that 1% CS contained adequate FA binding proteins. Although the exact amount of protein that could support and stabilize FAs in medium is unknown, estimations suggested that the BSA present in CS may have been insufficient to support and sustain continuous FA release into the medium. Each BSA molecule can interact with multiple FAs and the binding affinity increases as the chain length increases (Brown, J. R., and Schockley, P., 1982). Various studies suggest that albumin has 4 to 6 high affinity binding sites and dozens of weak binding sites (Goodman, 1958; Spector, John, & Fletcher, 1969). More recent data suggest binding site capacity of

BSA decreases as FA chain length increases such that BSA can reliably bind up to 9 $C_{14:0}$ FA but only 4-5 $C_{20:0}$ FA (Choi et al., 2002; Parks, Cistola, Small, & Hamilton, 1983; Teresi & Luck, 1952). With endogenously bound ligands already present in the CS added to the medium, 1% CS supplementation appears to not have been adequate to bind the FAs released from adipocytes. Without FA binding proteins, it is possible that re-esterification was increased. Indeed, Vaughan *et al.* showed that in the absence of albumin, the amount of glycerol release from excised rat fat pads was largely unaffected while the amount of FAs was essentially absent in the medium (Vaughan, 1962). Sorrentino *et al.* demonstrated that the uptake of FA is determined by the oleate/albumin concentration (Sorrentino, Robinson, Kiang, & Berk, 1989). They observed a saturation of oleate uptake by adipocytes upon increasing the oleate/albumin concentration, thereby suggesting that having a greater ratio of FAs over albumin in our studies would favour FA re-uptake and not medium accumulation (Sorrentino et al., 1989). Differences in medium glycerol and FA may then provide insight to the metabolic fate of these lipolytic products and the effect of the conditions presented. In this case, it can be considered that the quantified glycerol becomes indicative of the rate of lipolysis over a period of time while quantified FA suggests a re-esterification rate based on the calculated deficit. Of course this comes on the presumption that little or no glycerol is used by the adipocyte and that all cleaved FA are released into medium, however the fact remains that FAs were absent in the medium of my studies. Therefore these follow-up studies indicated that FA were not elevated in the T-ACM used on macrophages.

To investigate the requirement of BSA for FA release, subsequent studies were conducted with 1% CS and 1% BSA supplementation. A 1% addition of BSA should provide sufficient binding sites for any adipocyte-released FAs. As was observed, this

additional supplementation was sufficient to show an increase of FA in TSH-stimulated human subcutaneous differentiated adipocytes, although the difference fell short of reaching significance. This, along with data from the literature, suggests that BSA is required for the release of FA into medium of human subcutaneous differentiated adipocytes stimulated with TSH. However, this was not the case in the 3T3-L1 differentiated adipocytes, where FA levels remained relatively unchanged despite the addition of BSA. Whereas 1% BSA was added to medium to support FA release and was thought to be sufficient, others have shown that stimulated lipolysis occurs in a BSA dose-dependent manner from concentrations of 0% up to 4% BSA (Allen, 1979; Getty Kaushik, Richard, & Corkey, 2005). It has been suggested that the presence of albumin promotes lipolysis by preventing a negative feedback effect of FAs on the production of cAMP (Allen, 1979). Consequently, even the addition of 1% BSA may diminish the effect of TSH stimulated lipolysis and the release of FAs in 3T3-L1 adipocytes. It is possible that a higher concentration of BSA present would not only promote TSH-stimulated lipolysis, but promote FA stability in the medium. Such speculation would need to be tested using various concentrations of BSA in TSH-stimulated adipocyte lipolysis.

e. Potential Re-esterification

The exact fate of FAs after TSH-stimulated lipolysis in the conditions used remains unknown. As mentioned, it is possible FA re-esterification in 3T3-L1 adipocytes is occurring, and that it could be so fast as to negate any increased release of FA produced by TSH-stimulation over a 4 hour period. *In vivo*, 57% of FA released from rats in a fasted state were re-esterified (Kalderon, Mayorek, Berry, Zevit, & Bar Tana, 2000). Our culture

medium utilizes a physiologically relevant low glucose concentration of 5 mM instead of the conventional 25mM glucose used *in vitro*. *In vitro* glucose values have been shown to affect the inflammatory capabilities of adipocytes and TNF-α stimulated lipolysis (Green, Rumberger, Stuart, & Ruhoff, 2004; Yeop Han et al., 2010). However, isoproterenol-stimulated lipolysis and FA release are unaffected *in vitro* by glucose, therefore changes in glucose concentrations are likely not the explanation (Green et al., 2004; Raclot & Oudart, 2000). Rosenstock *et al*. demonstrated that over the long term, insulin reduced isoproterenol-stimulated FA release by nearly 90% through increased re-esterification (Rosenstock, Greenberg, & Rudich, 2001). During 3T3-L1 adipocyte differentiation in our studies, 1 μM of insulin is used in the first 4 days to help facilitate adipogenesis. As the cells are stimulated with TSH 5 days post-exposure to these increased levels of insulin, it is unlikely that insulin is the cause for the lack of accumulation of FA in the medium.

In order to revisit our hypothesis in the murine model, understanding the fate of FAs released through TSH-stimulated lipolysis of 3T3-L1 adipocytes is necessary. Although it is not established if re-esterification is the actual cause of the lack of FA accumulation in the medium in our 3T3-L1 adipocyte studies, the ability of murine adipocytes to greatly increase re-esterification exists and remains a potential explanation.

f. Cytokines on Adipocytes

In 3T3-L1 differentiated adipocytes, TSH increased glycerol release in prf-DMEM containing both 1%CS and 1%BSA in murine cells. TNF-α pre-treatment also increased glycerol release. This suggests TNF-α modifies the lipolytic machinery to enhance TG breakdown through reduction in perilipin A protein levels. When 3T3-L1 differentiated

adipocytes were pre-treated with TNF-α before TSH stimulation, the increase caused by TSH appears to be somewhat attenuated as the overall increase does not equal the sum of the TNF-α and TSH effects individually. Bezaire *et al.* showed that TNF-α pre-treatment resulted in a marked reduction in forskolin stimulated TG-hydrolase activity and impaired perilipin phosphorylation in multipotent stem cells (Bézaire et al., 2009). The overall level of glycerol release was higher for the forskolin with TNF- α pre-treatment than the forskolin stimulation alone and this agrees with our results. As TSH activates adenyl cyclase activity, it can be inferred that TNF-α might reduce TSH effectiveness much in the same manner.

IL-6 pre-treatment did not affect glycerol release in 3T3-L1 differentiated adipocytes on its own, and did not interfere with TSH-stimulated glycerol release. The cells were pre-treated for 24 hours with IL-6 before having the medium changed and conditioned over 4 hours without IL-6. IL-6 has been shown to increase lipolysis through lipolytic protein regulation and increased glycerol release over the 4 hours should have been observed if protein expression was altered. In the literature, values used for IL-6 stimulation of lipolysis were much higher than those used in my study (Petersen et al., 2005; Päth et al., 2001). It would be reasonable to conclude that the levels of IL-6 used in this study were insufficient to alter the protein expression and cause an increase in glycerol. Other studies have demonstrated that high plasma IL-6 concentrations positively correlated with isoproterenol-stimulated lipolysis (Morisset, Huot, Légaré, & Tchernof, 2008). Twenty four hour IL-6 and isoproterenol co-stimulation of *in vitro* human differentiated adipocytes has also been shown to significantly increase glycerol release (Päth et al., 2001). Interestingly, this observation seems to come in contrast to observations from Bézaire *et al*. on the effects of TNF-α. However, Päth *et al.* did not analyze the data in the same manner, thereby leading to these

different conclusions. Conclusions from Päth *et al.* are based on the raw data and compared the isoproterenol stimulation to the IL-6 and isoproterenol co-stimulation. The results from Päth *et al.* indicate the glycerol release from IL-6 and isoproterenol co-stimulation appears to be an additive effect of the individual parts. Bézaire *et al*. computed forskolin-stimulated glycerol fold increase over their respective basal levels (with or without TNF-α) before comparing the values. The basal lipolysis after TNF-α pre-treatment is higher than basal lipolysis of untreated cells. The difference in basal levels caused the forskolin-stimulated fold increase to be lower in the TNF-α condition and this allowed them to draw the conclusion that TNF-α attenuates forskolin stimulation. Although TNF- α and IL-6 cannot be compared directly, their mechanistic effect on lipolysis is similar. One can speculate then that a sufficient concentration of IL-6, capable of promoting lipolysis, would affect β-adrenergic stimulation of lipolysis in a manner similar to TNF-α. To consider the studies I attempted, it would suggest that sufficient IL-6 levels should have increased TSH stimulation in an additive manner or through slight attenuation as observed by TNF-α. This conjecture would need to be confirmed experimentally however.

If proper conditions are presented to allow for TSH-ACM to initiate a pro-inflammatory response in macrophages, a co-culture system may offer further support as an adipose tissue type model. This model would provide a paracrine interaction similarly present *in vivo*. The co-culture system has demonstrated that an increasing presence of macrophages, a phenomenon noted in the progression of obesity, demonstrates increased inflammation by way of increased IL-6 secretion (Bouwman, Diepersloot, & Visseren, 2009). While no intercellular effectors were suggested by Bouwman *et al.*, others have suggested FAs to be the key regulator in this process (Suganami et al., 2005). As previously

mentioned, adipocytes require the extracellular release of the FA before re-uptake and re-esterification can occur. A co-culture system might allow the FA an opportunity to interact with macrophages over the 4 hour stimulation prior to re-esterification. Therefore, a co-culture system could demonstrate how TSH facilitates a pro-inflammatory response in macrophages through real time interaction.

g. Clinial Significance

My main challenge in this thesis was the difficulty in attaining significance in many of the studies, notably those using human subcutaneous differentiated adipocytes. These are primary cell cultures and are subject to inter-donor variation, thereby increasing the experimental variation. Increasing the number of experiments in studies using the human primary cells would help account for the variability by reducing the standard error of the mean. Also, the expression of TSH receptors on the differentiated adipocytes could be assessed as a source of variation. However, TSH receptor protein expression is difficult to detect through immunoblotting. Therefore using indirect measures could help determine TSHR responsiveness. CREB phosphorylation, an established cAMP target in human subcutaneous differentiated adipocytes (Antunes, Gagnon, Langille, & Sorisky, 2008), could help ensure donor samples are all responding accordingly.

h. ELISA in place of PCR

Another limitation to the studies conducted was the use of PCR for detection of pro-inflammatory cytokine changes. PCR is a very sensitive process that is subject to error from the lengthy processing procedure of the samples. Steps were taken to ensure that the RNA

was not degraded or compromised prior to reverse transcription and amplification. However, it was difficult to obtain consistent quantification of the cDNA products despite these steps. Furthermore, while PCR is capable of detecting transcriptional changes, it does not guarantee direct translation into increased cytokine secretion. PCR primers are inexpensive and allow for the quantification of a variety of products, but an ELISA would provide a more definitive answer in a shorter time period. ELISA detection can be a costly procedure that requires cytokine and species specific kits, thereby limiting the range of proteins that can be investigated. Consequently, it may be advisable to focus on one or two cytokines significant to SCH inflammation, such as TNF-α and IL-6, and use ELISA kits to determine the macrophage inflammatory response to TSH-stimulated differentiated adipocytes.

CONCLUSION

The purpose of this study was to observe the effect of TSH-stimulated adipocyte condition media on macrophage cytokine mRNA expression. Initial steps were taken to characterize the lipolysis and the conditions required for its actions. These conditions were used to produce media for use in investigating the inflammatory response of macrophages. Failure to see the predicted increase in pro-inflammatory cytokine mRNA expression in macrophages after exposure to TSH-stimulated adipocyte conditioned medium led us to investigate the models employed and the methods used. This exploration was intended to offer support or possibly an explanation for the observed findings. Indeed preceding studies and the scientific literature available supported our observations.

This hypothesis remains to be clarified. A careful examination of TSH-stimulated FA release, a more thorough understanding of FAs in macrophage pro-inflammatory activation and a re-evaluation of TSH-ACM on macrophages would need to be investigated prior to drawing any firm conclusions on the hypothesis investigated. Consequently, with the knowledge gained from these experiments, I believe that a re-evaluation of the hypothesis is warranted.

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CURRICULUM VITAE

Summary of Qualifications

- B.Sc. in Physiology, pending completion of M.Sc. in Biochemistry
- Over two years of management and leadership related experience
- Strong interpersonal skills and ability to work with all age groups and backgrounds
- Bilingual in English and French

Education

Master's in Science, Biochemistry 2009-2011
University of Ottawa, Ottawa Canada

- Conducted biochemical research on the effect of TSH-stimulated adipocytes on macrophage inflammation; supervised by Dr Alexander Sorisky
- Frequent preparation of presentations on results and relevant journal articles
- Teacher assistant in biochemistry teaching lab, instructing and marking students on the performance of DNA isolation, PCR and electrophoresis

Baccalaureate in Science, Honours Physiology *Magna cum laude* 2002-2006
University of Ottawa, Ottawa, Canada

- Conducted research project on the desensitization of dopamine receptor D1 by G protein-coupled receptor kinase 5 (GRK5); supervised by Dr. Mario Tiberi

Honours and Awards

University of Ottawa

- Admission scholarship 2009-2011
- Dean's Honour List 2002-2004
- Admission Scholarship 2002-2005

Abstracts

- Durand, J., Gagnon, A. and Sorisky, A. The Role of TSH-Stimulated Adipocyte Lipolysis in Macrophage Activation. 10th Annual OHRI Research Day (November 18th, 2010). *(poster)*
- Durand, J., Gagnon, A. and Sorisky, A. Role of PKCδ in TSH-stimulated cytokine release. 9th Annual OHRI Research Day (November 27th, 2009). *(poster)*

Work Experience

Medical Management Consultant 2011-present
Ottawa, Canada

- Developing a business and medical work model for a novel clinical practice
- Preparing documentation for administrative and clinical duties for support staff
- Investigating Electronic Health Record programs for client desired applications

Office Manager 2008-2009
Assistant Manager 2007-2008
Medical Assistant 2006-2007
Appletree Medical Centre, Ottawa, Canada

- Managed a support staff of ten to fifteen; coordinated and assisted twelve to twenty physicians and specialists
- Responsible for training, scheduling, payroll, work flow, quality control, conflict resolution, medical inventory and public relations
- Capable at multi-tasking in a volatile and high paced work environment
- Took initiative in implementing new policies and procedures to increase clinic efficiency
- Instrumental in providing feedback and assisting to change in our Electronic Medical Records program EMR Advantage
- Assisted physicians in a clinical setting with patient registration and preparation
- Took patient histories and performed introductory medical skills as required
- Trained in allergy testing, subcutaneous, intramuscular and intradermal injections
- Trained foot care specialist in treating corns, calluses and plantar warts

University Tutor 2006-2007
Ottawa, Canada

- Help and instruct students in biology, chemistry and biochemistry courses

Child Care Worker 2002-2006
Hunt Club-Riverside Park Community Centre, Ottawa, Canada

- Influential in several creative changes to program thereby increasing staff involvement and camaraderie
- Organize various physical, mental and artistic activities for children ages 4 to 13
- Required interpersonal problem solving skills to address conflicts between parents, staff and children

Volunteer Experience

Parkinson's Society Superwalk Event Registrar 2006-present
Ottawa Parkinson's Society, Ottawa, Canada

- Review and compile all registration forms completed by registration volunteers
- Verify donation amounts and clarify discrepancies
- Assist in other financial tasks as required

Peer Helper 2004-2006

Peer Help Centre, University of Ottawa, Ottawa, Canada

- Dealt with student population in providing academic, social and personal support
- Trained in active listening, stress management, suicide awareness and prevention, sexual harassment and mental health
- Certified in ASIST (Applied Suicide Intervention Skills)

Hemodialysis Unit Assistant 2003-2004

The Ottawa Hospital, Ottawa, Canada

- Provided personal assistance to patients during dialysis
- Communication skills necessary to provide emotional and physical care

APPENDIX A: Research Collaboration Acknowledgement

Figure 6: Dr. AnneMarie Gagnon performed and collected data for three of the four experiments required for this figure.