# EXTRA-THYROIDAL ACTION OF TSH ON ADIPOCYTE INSULIN SIGNALING

By

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## ABSTRACT

In subclinical hypothyroidism (SH), high levels of circulating thyroid stimulating hormone (TSH) maintain normal thyroid hormone levels, despite mild thyroid failure. SH is associated with cardiovascular disease and insulin resistance, although the underlying pathophysiology is not fully understood. We hypothesized that TSH may inhibit insulin action in adipocytes. To investigate this relationship, we studied primary human differentiated adipocytes. Abdominal subcutaneous adipose tissue samples were obtained (approved by OHSN-REB) from 16 weight-stable patients undergoing elective abdominal surgery. We stimulated adipocytes differentiated from stromal preadipocytes with 5 mU/ml TSH and/or 100 nM insulin, and assessed acute insulin signaling, lipogenesis and glucose uptake. Immunoblot analysis revealed that TSH suppressed insulin-stimulated Akt phosphorylation by 45% (n=5; p = 0.01). When adipocytes were pre-incubated with conventional protein kinase C (cPKC) inhibitor Gö6976, TSH inhibition was blocked. Our data indicate that TSH inhibits insulin-stimulated lipogenesis (up to 37%), but depends on BMI. Insulin-stimulated glucose uptake was enhanced by 36% and also correlated with BMI. This data suggests that TSH can modulate adipocyte insulin signaling.

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# TABLE OF CONTENTS

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ABHD5 | = | α/β hydrolase fold domain 5 |
| ACC | = | Acetyl-CoA carboxylase |
| AMPK | = | 5'adenosine monophosphase-activated protein kinase |
| ATGL | = | Adipose triglyceride lipase |
| BSA | = | Bovine serum albumin |
| cAMP | = | Cyclic adenosine monophosphate |
| cGMP | = | Cyclic guanosine monophosphate |
| CHD | = | Coronary Heart Disease |
| ChREBP | = | Carbohydrate-response-element-binding protein |
| cPKC | = | Conventional protein kinase C |
| CREB | = | cAMP response element binding protein |
| C/EBPα | = | CCAAT-enhancer-binding protein alpha |
| DAG | = | Diacylglycerol |
| DGAT | = | Diacylglycerol transferase |
| DNL | = | De novo lipogenesis |
| Erk 1/2 | = | Extracellular signaling regulated kinases 1/2 |
| FA | = | Fatty acid |
| FAS | = | Fatty acid synthase |
| FBS | = | Fetal bovine serum |
| GPCR | = | G-protein coupled receptor |
| GSV | = | GLUT4 storage vesicles |
| HOMA-IR | = | Homeostasis model assessment for insulin resistance |
| HSL | = | Hormone sensitive lipase |
| $IP_3$ | = | Inositol triphosphate |

| | | |
|---|---|---|
| IR | = | Insulin receptor |
| IRS | = | Insulin receptor substrate |
| L-T4 | = | Levo-thyroxine |
| MFS | = | Major facilitative superfamily |
| mTORC | = | Mammalian target of rapamycin complex |
| NEFA | = | Non-esterified fatty acids |
| NF-κB | = | Nuclear factor kappa B |
| OH | = | Hypothyroidism |
| PDK-1 | = | 3-phosphoinositide dependent protein kinase-1 |
| PH | = | Pleckstrin homology |
| PIP2 | = | Phosphatidylinositol (4,5) bisphosphate |
| PIP3 | = | Phosphatidylinositol (3,4,5) triphosphate |
| PI3K | = | Phosphatidylinositol-3-kinase |
| PKA | = | Protein kinase A |
| PKC | = | Protein kinase C |
| PKG | = | Protein kinase G |
| PLC-β | = | Phospholipase C-β |
| PPARγ | = | Peroxisome proliferator-activated receptor gamma |
| PRAS40 | = | Proline-rich Akt substrate 40 |
| PTEN | = | Phosphatase and tensin homologue |
| rhTSH | = | Recombinant human TSH |
| SDS-PAGE | = | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SH | = | Subclinical Hypothyroidism |
| SH2 | = | Src homology 2 |

| | | |
|---|---|---|
| SREBP | = | Sterol element binding protein |
| TCA | = | Tricarboxylic acid cycle |
| TG | = | Triacylglycerol |
| TNF-α | = | Tumor necrosis factor alpha |
| TSC1/2 | = | Tuberous sclerosis 1/2 |
| TSH | = | Thyroid stimulating hormone |
| TSHR | = | Thyroid stimulating hormone receptor |
| TZD | = | Thiazolidinediones |
| T3 | = | Tri-iodothyronine |
| T4 | = | Thyroxine |
| UCP-1 | = | Uncoupling protein-1 |
| WAT | = | White adipose tissue |

## LIST OF FIGURES AND ILLUSTRATIONS

# INTRODUCTION

## Subclinical Hypothyroidism

Subclinical hypothyroidism (SH) is a condition defined biochemically by high circulating levels of thyroid stimulating hormone (TSH) in the presence of normal thyroid hormone levels (Weiss et al., 2011). In this condition, the thyroid gland begins to fail due to harmful contributors such as thyroid auto-immunity (Hashimoto's thyroiditis) or iodine deficiency (Weiss et al., 2011). As a result, the output of the thyroid hormones thyroxine (T4) and tri-iodothyronine (T3) decreases. This reduction in T4/T3 levels disrupts the hypothalamic-pituitary-thyroid (HPT) axis (**Figure 1**). T3 and T4 act on the hypothalamus and pituitary to inhibit TSH output. When T3/T4 levels drop, this inhibition is alleviated, and the pituitary increases TSH production. When thyroid failure is mild, the higher TSH levels can compensate to restore thyroid hormone levels to normal. When TSH cannot prevent the thyroid hormone levels from dropping below normal due to severe thyroid failure, overt hypothyroidism (OH) occurs (Razvi et al., 2005). This progression to OH occurs in ~2 to 5% of SH patients per year (Surks MI, Ortiz E, Daniels GH,et al, 2004).

T3 is the biochemically active form and comprises 20% of total thyroid hormone produced (Weiss et al., 2011). The thyroid hormones are bound to serum-binding proteins (thyroxine-binding globulin, transthyretin, and albumin) for transport to the periphery. Once at the target tissues, T4 can be converted to the bioactive T3 by 5'-monodeiodinase (Weiss et al., 2011). Low levels of T3/T4 can cause a number of symptoms including muscle weakness, intolerance to cold, depression,

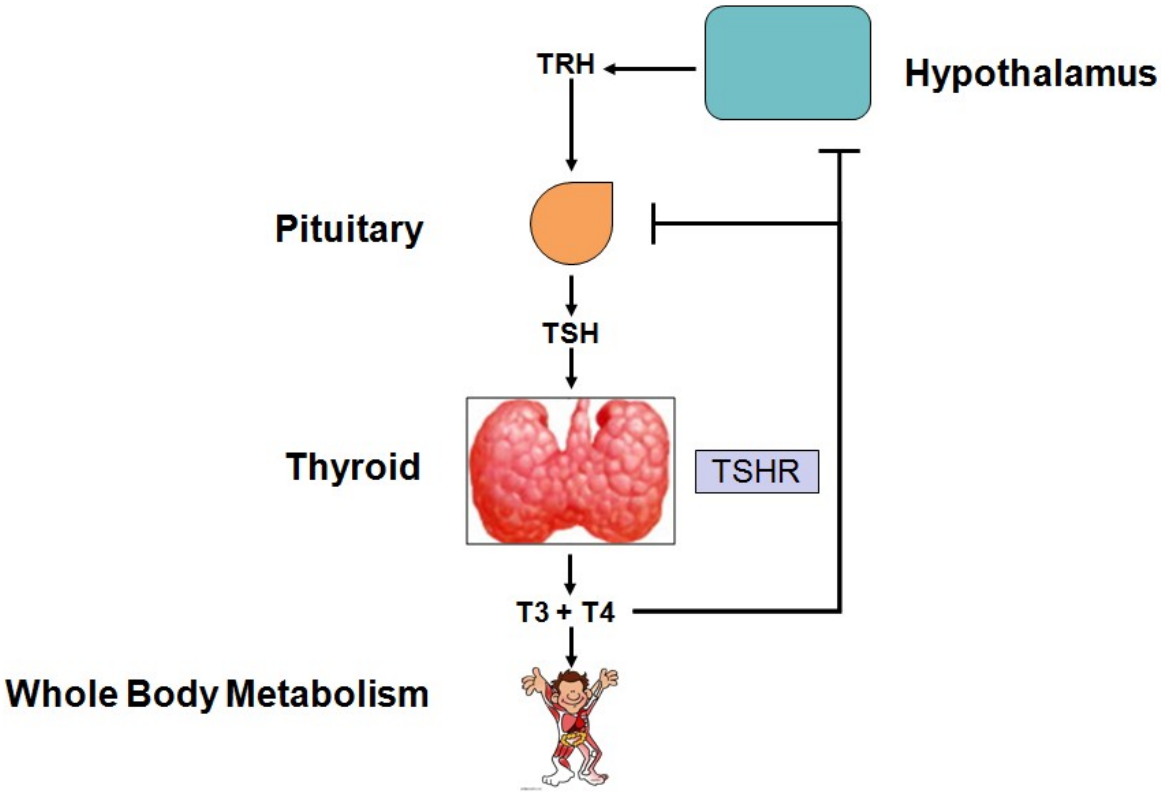

TRH
Hypothalamus
Pituitary
TSH
Thyroid
TSHR
T3 + T4
Whole Body Metabolism

**Figure 1: The hypothalamic-pituitary-thyroid (HPT) axis**. The HPT axis signals through the hypothalamus to the pituitary using TRH to release TSH. TSH causes the thyroid to release thyroid hormones T3 and T4 that act on its effector tissues throughout the body. T3 and T4 also act in a classical negative feedback loop, inhibiting the hypothalamus and the pituitary in order to control the amount of TSH secretion. Abbreviations: TRH = TSH releasing hormone; TSH = thyroid stimulating hormone; TSHR = thyroid stimulating hormone receptor; T3 = triiodothyronine; T4 = thyroxine.

and elevated blood cholesterol levels (Razvi et al., 2005), (M Anne Pollock et al., 2001), (Torun et al., 2009). OH can be treated with levothyroxine (L-T4) to compensate for poor thyroid gland T3/T4 production (Weiss et al., 2011). Patients with SH may display variable and milder symptoms (Pearce et al., 2013).

Longitudinal epidemiological studies drew attention to SH and the cardiovascular system. The 1977 Whickham survey was one of the first to document the prevalence of thyroid disorders in the community (Tunbridge et al., 1977). In general, SH is reported in about 5-15% of the general population and is more prevalent in women and in the elderly (Razvi et al., 2010), (Roberts and Ladenson, 2004). A 20 year follow-up to the Whickham survey showed that patients with SH had an increased probability of developing OH (Vanderpump et al., 1995). Many other studies followed over the years, and a meta-analysis based on data from 55,287 adult participants demonstrated that SH was associated with an increased risk of coronary heart disease (CHD) events and CHD mortality (Rodondi et al., 2010).

Several studies have also reported that SH is associated with insulin resistance. Using the homeostasis model assessment for insulin resistance (HOMA-IR) and the quantitative insulin sensitivity check index (QUICKI), one research group found that SH was independently predictive of IR in a study of 126 patients with rheumatoid arthritis (Dessein et al., 2004). Furthermore, TSH levels have also been independently associated with insulin resistance in women with polycystic ovary syndrome (Mueller et al., 2009). In the same year, Maratou et al. published a study that assessed the sensitivity of glucose metabolism to insulin in

patients using an oral glucose tolerance test (Maratou et al., 2009). Subjects with overt or SH had higher insulin levels when compared to euthyroid subjects for the same glucose elevation following the challenge (Maratou et al., 2009). These studies suggest that SH may be linked to insulin resistance.

The mechanisms behind these correlations to SH are still under investigation. Since SH is a relatively common condition linked to negative outcomes, molecular insights are needed in order to understand the link between SH and these diseases. There is evidence to suggest that high levels of circulating TSH can affect tissues other than the thyroid. Many of these tissues express the TSH receptor and activation of the receptor has been shown to occur in immune, bone and adipose cells (Csaba and Phíllinger, 2009), (Nannipieri et al., 2009), (Heemstra et al., 2008). For example, TSH activates lipolysis in adipocytes (Gagnon et al., 2010).

For this thesis, the main focus will be on the effect of high TSH on adipocytes. Adipose tissue is an endocrine organ that releases factors called adipokines to communicate with other tissue types in whole body metabolism. High levels of TSH may disrupt adipocyte function and could contribute to the negative cardiometabolic consequences of SH.

**Adipocyte Function**

Adipocytes store metabolic fuel in the form of triacylglycerol (TG). These cells also secrete large number of adipokines which affect a wide variety of physiologic and pathologic processes in the body (Suganami et al., 2012), (Lafontan, 2008). Adipocytes have been separated into types: white and brown.

White adipocytes are classical fat energy storage units that release FAs for oxidation in other tissues when energy is needed (Trayhurn and Beattie, 2001). Furthermore, white adipocytes play a role in thermal insulation, inflammation, and glucose metabolism (Trayhurn and Beattie, 2001). For example, white adipose tissue (WAT) of marine mammals (called blubber) can help conserve heat in a cold environment (Trayhurn and Beattie, 2001). Furthermore, white adipocytes can secrete pro-inflammatory factors such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) or anti-inflammatory factors such as adiponectin (Trayhurn and Beattie, 2001). The relative balance of these adipokines can help determine whether the WAT environment leans more towards a pro-inflammatory or an anti-inflammatory state.

Brown adipose tissue is a key site of thermogenesis instead of energy storage (Harms and Seale, 2013). Brown adipocytes are rich in mitochondria and their ability to produce heat is primarily controlled by the thermogenic uncoupling protein-1 (UCP1) (Meyer et al., 2010). The recognition of functional brown fat in adult humans (which had previously only been shown in infants) has accelerated interest in its ability to expend energy instead of storing it (Harms and Seale, 2013), (Lidell et al., 2013).

Adipose tissue can also be classified anatomically. Adipose tissue that is present beneath the skin is known as subcutaneous whereas adipose tissue that surrounds organs such as the stomach or intestines is labelled visceral. Subcutaneous WAT stores more than 80% of total body fat in the abdominal, gluteal and femoral regions (Lee et al., 2013). Visceral WAT is located in the intra- and retro-peritoneal depots and make up to 10-20% of total body fat in men and 5-10%

in women (Lee et al., 2013). In adult humans, brown adipose tissue can be found in cervical-supraclavicular, perirenal, and paravertebral regions (Lee et al., 2013).

In general, most of the energy reserves in white adipocytes are stored as TG in unilocular lipid droplets which comprise 95% of the cell volume (Lafontan, 2008), (Lee et al., 2013). TGs are esters of glycerol in which three FAs are individually esterified to each of the three hydroxyl groups present on the glycerol backbone (Baud and Lepiniec, 2008). TG forms within adipocytes through two major routes: *de novo* lipogenesis (DNL) or through the uptake and re-esterification of fatty acids (FA) from the plasma (Lafontan, 2008).

**Lipogenesis**

DNL is defined by the production of FAs from carbohydrates such as glucose. Once glucose enters the adipocyte through a glucose transporter, it can undergo glycolysis and be converted into two pyruvate molecules. Subsequently, pyruvate can enter the tricarboxylic acid cycle (TCA) in which citrate (a TCA cycle metabolite) can be converted into cytosolic acetyl-CoA by ATP-citrate lyase (Baud and Lepiniec, 2008). These acetyl-CoA molecules act as precursors for fatty acid synthesis. Acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC), which is the rate-limiting step of FA synthesis (Steinberg, 2009). ACC is primarily under the negative regulation of 5' adenosine monophosphate-activated protein kinase (AMPK), which phosphorylates ACC and inhibits production of malonyl-CoA (Steinberg, 2009). When inhibition of ACC is relieved (under conditions such as insulin stimulation), DNL is activated. Fatty acid synthase (FAS) can then use

acetyl-CoA and malonyl-CoA as substrates to form palmitate (Steinberg, 2009). Palmitate can also undergo modifications such as elongation and/or desaturation in order to create other FA molecules (Steinberg, 2009).

Alternatively, FAs can be acquired from the diet, which are then carried through the blood to adipose tissue by chylomicrons as TG. Since chylomicrons are too large to penetrate the endothelial lining of adipose tissue capillaries, they are processed in the luminal space by lipoprotein lipase (secreted from the adipocytes) (Lafontan, 2008). As a result, non-esterified fatty acids (NEFA) are released from the chylomicron TG, and can move through the endothelial lining to the adipocyte. These NEFA molecules can then be processed back into TG.

Three FA molecules can be esterified onto a glycerol backbone provided by cytosolic glycerol-3-phosphate dehydrogenase acting on dihydroxyacetone-3-phosphate (a glycolysis metabolite). The rate-limiting step for this process is diacylglycerol transferase (DGAT), which catalyzes the addition of the third FA moiety to diacylglycerol (DAG) (Lafontan, 2008). This ultimately forms TG which can then be stored in the adipocyte.

In WAT, carbohydrate-response-element-binding protein (ChREBP) is the dominant transcriptional regulator of DNL and controls FAS and ACC (Rosen and Spiegelman, 2014). ChREBP activity is induced by insulin-activated glucose transporter GLUT4 activity in the adipocyte (Herman et al., 2012). Recently, expression of isoform ChREBP-β in human subcutaneous WAT was shown to be predictive of insulin sensitivity (Herman et al., 2012). Roberts et al. showed that

DNL is downregulated as adipocytes expand and that a strong negative relationship exists between adipocyte size and insulin sensitivity, independent of BMI (Roberts et al., 2009). Furthermore, DNL in WAT has been shown to be predictive of metabolic health, whereas Glut 4 and DNL enzymes of obese patients are significantly reduced (Eissing et al., 2013). Eissing et al.'s study also showed that weight loss by bariatric surgery increased DNL associated enzymes and Glut4 expression (Eissing et al., 2013). These studies suggest that DNL in WAT positively correlates with insulin sensitivity and the healthy metabolic state, thus making adipocyte DNL a potential therapeutic target.

Another important regulator of lipid synthesis is mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a nutrient sensing protein and, when active, will phosphorylate eukaryotic translation initiation factor 4E and S6 kinase to promote protein synthesis (Bakan and Laplante, 2012). mTORC1 can activate sterol element binding protein-1c (SREBP-1c), which controls several lipogenic genes in conjunction with ChREBP (Ferre and Foufelle, 2010), (Bakan and Laplante, 2012). mTORC1 can be stimulated through activation of the insulin signaling pathway. Akt, a serine/threonine kinase that is a part of the insulin signaling pathway, can phosphorylate and inhibit proline-rich Akt substrate 40 kDa (PRAS40) and tuberous sclerosis 1/2 (TSC1/2). PRAS40 and TSC1/2 are negative regulators of mTORC-1, thus inhibiting these two factors can activate mTORC1 and SREBP-1c (Bakan and Laplante 2012).

## Insulin Signaling

Insulin stimulates uptake of glucose into adipose tissue for energy storage (Rowland et al., 2011). After a meal, insulin is secreted from the β-cells located in the islets of Langerhans in the pancreas. Insulin stimulates the uptake of glucose from the blood into adipose and skeletal muscle tissue (Rowland et al., 2011). Insulin stimulates GLUT4 glucose transporter translocation to the plasma membrane of these insulin sensitive cell types to allow glucose entry (Rowland et al., 2011). Insulin also increases lipid synthesis and decreases lipolysis in adipocytes.

Insulin is a polypeptide hormone that contains two chains: the A chain (21 residues) and the B chain (30 residues) (Hua, 2010). The two chains are linked by three disulfide bridges; all three play an important role in the protein's stability and function (Hua, 2010). The binding of insulin to the insulin receptor (IR) initiates an intracellular signaling cascade that controls aspects of cellular metabolism, growth and survival. IR exists in two isoforms that differ by the presence (B) or absence (A) of 12 amino acids on the carbonyl terminus of the α-subunit (Siddle, 2011). IR-B is the more prominent isoform in adipose tissue (Siddle, 2011). IR is a large transmembrane glycoprotein containing four subunits (two α and two β) and belongs to the tyrosine kinase superfamily (Ward et al., 2008), (Hua, 2010). The exact mechanism by which insulin binds to its receptor is still unknown, but it is believed that one molecule of insulin binds to 2 α subunits (Brandt et al., 2001), (Hua, 2010).

Following activation of the insulin receptor by insulin, the receptor tyrosine kinase trans-phosphorylates its β subunits (Rowland et al., 2011). The activated

insulin receptor also interacts with and phosphorylates cytosolic insulin-receptor substrates 1 and 2 (IRS-1 and IRS-2) to create binding sites for Src homology 2 (SH2) domains of other proteins (Rowland et al., 2011), (Siddle, 2011). Phosphatidylinositol-3-kinase (PI3K) is then recruited through its SH2 domains to the tyrosine-phosphorylated IRS proteins and catalyzes the conversion of phosphatidylinositol (3,4,5) triphosphate (PIP3) from phosphatidylinositol (4,5) bisphosphate (PIP2) on the cytosolic leaflet of the plasma membrane (Rowland et al., 2011). PIP3 then acts as a docking site for serine/threonine kinase Akt by binding to its pleckstrin homology (PH) domain (Rowland et al., 2011). Binding to the PH domain causes a conformational change in Akt and allows for Akt activation via phosphorylation of $Thr^{308}$ by 3-phosphoinositide dependent protein kinase 1 (PDK-1) (Rowland et al., 2011). To achieve full activation, a second phosphorylation occurs at Ser473 by mammalian target of rapamycin complex 2 (mTORC2); other potential Ser473 kinases are still under debate (Bozulic and Hemmings, 2009). Akt phosphorylates a wide range of substrates including glycogen synthase kinase-3, TBC1D4, mTORC1, and FoxO transcription factors to mediate cell responses (Bozulic and Hemmings, 2009). For example, phosphorylation of TBC1D4 by Akt increases translocation of Glut4 to the plasma membrane in muscle and adipose tissue (Rowland et al., 2011).

**Glucose Uptake**

Whereas GLUT4 expression is restricted to muscle and adipose tissue, glucose uptake also occurs in other human tissue types due to the other 13 related members of the GLUT family (Thorens and Mueckler, 2010). The GLUT protein

family is part of the Major Facilitative Superfamily (MFS) of passive membrane transporters that catalyze the facilitated diffusion of hexoses (such as glucose) across the plasma membrane (Thorens and Mueckler, 2010). Generally, glucose uptake and subsequent glucose phosphorylation to glucose-6-phosphate is considered the rate limiting step in glucose metabolism and the process plays a key role in maintaining whole body glucose homeostasis (Leto and Saltiel, 2012). In particular, GLUT1-4 have been studied extensively due to their importance in diseases such as diabetes, glucose-galactose malabsorption, Fanconi-Bickel syndrome, and De Vivo disease (Pascual et al., 2004),(Pascual et al., 2004; Sun et al., 2012).

Crystal structures of the GLUT protein family are not yet available, but a recent publication reported three related crystal structures of XylE, an *Escherichia coli* homologue of GLUT1-4 (Sun et al., 2012). GLUT proteins are approximately 500 amino acids long and a majority of the amino acids responsible for D-glucose recognition were invariant compared to the D-glucose bound XylE (Sun et al., 2012). The authors proposed that these similarities (~50% between XylE and GLUT1-4) are due to functional and mechanistic conservations. This study may allow further structure-based modeling of GLUT1-4 (Sun et al., 2012).

In this thesis, I will focus on the transporters expressed in the adipocyte (GLUT1 and GLUT4). GLUT1 is ubiquitously expressed in most cells (highly represented in erythrocytes and brain), and often together with other GLUTs (Wertheimer et al., 1991), (Samih et al., 2000). In rat FRTL-5 thyroid cells, GLUT1 has been shown to take up glucose in response to TSH or insulin (Samih et al.,

2000). The increase in glucose uptake is mediated by an increase in GLUT1 translocation to the plasma membrane (Samih et al., 2000). In adipocytes, GLUT1 modulates basal glucose uptake and its action is non-insulin dependent (Kim et al., 2007). However, the effect of TSH on GLUT1 transport in adipocytes has not been studied. GLUT1 regulation appears to be cell-specific and does not simply mediate basal glucose uptake levels in all cell types.

GLUT4, on the other hand, is primarily expressed in muscle and adipocyte cells and its activation via insulin results in a rapid increase in glucose uptake. In humans, approximately 80-90% of insulin-stimulated glucose uptake occurs in skeletal muscle and 5-10% in adipose tissue (Thiebaud et al., 1982), (Leto and Saltiel, 2012). Although only a minor amount of glucose is taken up by adipocytes with insulin stimulation, this process is important due to its impact on whole body metabolism. For example, in response to changes in glucose uptake, adipocytes can produce adipokines that regulate muscle, liver and brain metabolism (Abel et al., 2001), (Leto and Saltiel, 2012). Selective deletion of adipocyte GLUT4 expression and insulin-stimulated glucose transport in mice cause insulin resistance in muscle and liver (Abel et al., 2001). Therefore, alteration of insulin-stimulated glucose uptake in adipocytes can directly influence whole body metabolism.

In adipocytes, only 5% of GLUT4 transporters are expressed on the plasma membrane under basal conditions; the rest are contained in intracellular GLUT4 storage vesicles (GSVs) (Leto and Saltiel, 2012). Evidence suggests that these GSVs remain physically tethered to the microtubule cytoskeleton in order to prevent the vesicles from reaching the plasma membrane (Fletcher et al., 2000). Once

activated by insulin, Akt phosphorylates and inactivates TBC1D4, a protein containing a GTPase-activating domain for small G proteins called Rabs (Sano et al., 2003), (Miinea et al., 2005). Rabs, in their GTP-bound form, act as components of vesicle trafficking pathways and promote membrane fusion through the regulation of small transmembrane proteins called SNAREs (Miinea et al., 2005), (Barr, 2013). In the inactive GDP-bound form, Rabs associated with GSVs are unable to promote GLUT4 translocation (Sakamoto and Holman, 2008). TBC1D4's GTPase-activating domain activity has been shown to be selective towards various Rabs (-2A, -8A, -8B, -10, -14) (Miinea et al., 2005). Inactivation of TBC1D4 by Akt phosphorylation relieves suppression of Rabs, which has been shown to be required for GLUT4 translocation to the plasma membrane (Sano et al., 2003). GSV fusion to the plasma membrane is mediated by the assembly of SNARE complexes, which have also been proposed to be controlled by insulin (Leto and Saltiel, 2012), (Bryant and Gould, 2011). Kinesin motor proteins (KIF3 in adipocytes) interact with Rabs to transport GSVs back and forth along cytoskeletal tracks to the plasma membrane (Leto and Saltiel, 2012). Insulin increases the amount of GLUT4 present at the cell membrane by accelerating the exocytosis of these GSVs to the plasma membrane.

**Lipolysis and GPCR-related Signaling**

In addition to synthesizing and storing FAs as TG, adipocytes can release NEFAs to provide energy for the body when required. This process is called lipolysis and involves the breakdown of TG into glycerol and three FAs. Lipolysis is under the control of lipases and lipid droplet-associated proteins, which promote the hydrolysis of TG (Lafontan, 2008). For example, perilipin is a lipid droplet-

associated protein and suppresses lipolysis by blocking lipase access to the lipid droplet (Lafontan, 2008). Perilipin can be phosphorylated by hormone sensitive lipase (HSL) after stimulation through lipolytic pathways, resulting in the fragmentation and dispersal of the lipid droplet (Lafontan, 2008).

HSL is activated through cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) or cyclic guanosine monophosphate (cGMP)-dependent protein kinase G (PKG) (Ducharme and Bickel, 2008), (Lafontan, 2008). In contrast to HSL, adipocyte triglyceride lipase (ATGL) is activated by a cofactor called α/β hydrolase fold domain 5 (ABHD5) (Lafontan, 2008).

TG's are broken down into DAGs and then into monoacylglycerols. ATGL primarily controls the breakdown into DAG (the first step) whereas HSL controls the breakdown into monoacylglycerol (the second step) (Ducharme and Bickel, 2008). Monoacylglycerol lipase controls the final step of TG lipolysis and converts monoacylglycerols into glycerol and its fatty acid component (Ducharme and Bickel, 2008).

During times of stress, catecholamines act on β-adrenergic receptors (β1, β2, and β3) in order to release FAs into the circulation (Lafontan, 2008). These receptors are part of the G-protein coupled receptors (GPCR) family and are the beginnings of lipolytic signaling (Jalink and Moolenaar, 2010). An example of a GPCR is the TSH receptor (TSHR).

GPCRs are seven transmembrane domain structures that signal via heterotrimeric G effector proteins (Jalink and Moolenaar, 2010). After ligand

binding, GPCRs activate their respective G proteins at the plasma membrane (Jalink and Moolenaar, 2010). These G proteins are the molecular on/off switches of GPCRs, consisting of α, β and γ subunits and are responsible for activating the receptor's downstream target effectors (Jalink and Moolenaar, 2010). When the receptor is stimulated by a ligand, GDP is exchanged for GTP on the G protein α-subunit ($G_{\alpha s}$), which leads to its dissociation from the β and γ subunits (Slessareva et al., 2006). $G_{\alpha s}$ can then activate enzymes that produce second messengers, e.g. cAMP (Slessareva et al., 2006). In contrast, G proteins such as $G_{\alpha i}$ can inhibit levels of cAMP production (Jalink and Moolenaar, 2010).

Generally, phosphorylation of the receptor's cytoplasmic tail, followed by arrestin binding, leads to signal termination and receptor internalization by dynamin-dependent mechanisms (Jalink and Moolenaar, 2010). Dynamin is a membrane scission GTPase that is believed to be responsible for separating the forming endosome from the plasma membrane through a twisting force (Doherty and McMahon, 2009). Separation of GPCRs from the plasma membrane canonically results in signal attenuation.

However, dynamin-dependent endocytosis of some GPCRs is believed to be required for the full activation of their signaling pathways (Jalink and Moolenaar, 2010). For these GPCRs, internalization of the receptor does not halt cAMP production, but continues to stimulate adenylyl cyclase activity through $G_{\alpha s}$ (or inhibit through $G_{\alpha i}$) (Jalink and Moolenaar, 2010). This allows for more persistent cellular responses (Jalink and Moolenaar, 2010).

## TSH Signaling

TSHR is required for thyroid hormone production, as well as for regulating the gland's development growth and overall function (Latif et al., 2009). The TSHR holoreceptor (764 residues) undergoes multiple post-translational events including being cleaved into two subunits (A and B) and N-glycosylation (Michalek et al., 2009). 50 amino acids are removed between residues 316 and 366 when the receptor is cleaved, and this cleavage results in two subunits bound together with disulfide bounds (Michalek et al., 2009). The A domain is present outside the plasma membrane (ectodomain), and the B domain represents the transmembrane and the relatively short intracellular domains (Michalek et al., 2009). The major binding site of TSH to its receptor is located in the ectodomain, which is comprised of nine leucine-rich repeats (LRRs) and an N-terminal tail (Latif et al., 2009). These LRRs are composed of β strands and α helix turns and resemble a horseshoe (Latif et al., 2009). The ectodomain is connected to the transmembrane domain by a hinge region (residues 277-418) (Mizutori et al., 2008). Mutagenesis and deletion-based analysis show that this hinge region is important for ligand binding affinity and signal transduction (Mizutori et al., 2008).

In thyroid follicle cells, TSHR increases cAMP levels through activation of adenylyl cyclase, which is sustained after internalization of the receptor (Jalink and Moolenaar, 2010). G proteins such as $G_{\alpha s}$, $G_{\alpha q}$, $G_{i/o}$ and $G_{12/13}$ directly interact with the receptor transmembrane region in order to propagate TSH signaling (Michalek et al., 2009), (Buch et al., 2008). In the case of $G_{\alpha s}$, activation of adenylyl cyclase and

cAMP generation stimulates PKA and cAMP response element binding protein (CREB) (Michalek et al., 2009).

Whereas most molecular targets of TSHR are targeted by the $G_{\alpha s}$-cAMP pathway, binding of $G_{\alpha q}$ to TSHR leads to the formation of inositol trisphosphate ($IP_3$) and DAG, which cause an increase in intracellular $Ca^{2+}$ and an activation of protein kinase C (PKC) (Michalek et al., 2009), (Back et al., 2013). $IP_3$ and DAG formation from PIP2 is controlled by $G_{\alpha q}$ activation of phospholipase C-β (PLC-β) (Latif et al., 2009). Downstream effectors of PKC include nuclear factor kappa-B (NF-κB) and extracellular signaling-regulated kinases (Erk1/2) (Latif et al., 2009).

PKC isoforms can be separated into three major groups. The conventional PKCs (α, βI, βII, and γ) are classified by their dependence on DAG and intracellular $Ca^{2+}$ levels (Huang et al., 2012). Novel PKCs (δ, ε, v and θ) are dependent only on DAG levels, whereas atypical PKCs (ζ and ι/λ) are not sensitive to either of those two factors (Huang et al., 2012).

Recently, TSH has been implicated in regulating adipocyte responses. In 2002, Davies et al. reviewed the sites of extra-thyroidal TSHR expression (Davies et al., 2002) including five studies from 1992 to 1999 that showed TSHR expression in mammalian adipocytes. Although controversial at first, many laboratories have confirmed the existence of TSHR on human adipocytes (Lu et al., 2012), (Nannipieri et al., 2009). TSH can cause significant changes in adipocyte physiology. In particular, our laboratory has shown that TSH increases lipolysis as measured by NEFA and glycerol release from human differentiated adipocytes and 3T3-L1

adipocytes (preadipocyte mouse cell line) (Gagnon et al., 2010), (Thrush et al., 2012).

New studies continue to demonstrate the importance of TSH in adipocyte physiology. One study gave a dose of recombinant human TSH (rhTSH) to patients with no thyroid in order to observe the effects of TSH on non-thyroidal targets (Santini et al., 2010). The study showed that rhTSH stimulation increased serum leptin levels, a pro-inflammatory adipokine secreted by white adipocytes (Santini et al., 2010). rhTSH administration has also been shown to promote secretion of other adipocyte proinflammatory factors such as interleukin-6 and monocyte chemoattractant protein-1 release (Antunes et al., 2005), (Gagnon et al., 2014). These studies point towards a role for TSH inducing a pro-inflammatory adipocyte phenotype. TSH has also been shown to promote adipogenesis in mouse embryonic stem cells (Lu and Lin, 2008). These studies clearly show that TSH is a relevant molecule in adipocyte physiology. More research is needed in order to further define the role of TSH in adipocyte function.

**Model Systems**

In order to study the connections among adipose tissue, TSH and insulin signaling, an accurate *in vitro* model of human adipocytes is required. I used human preadipocytes isolated from the stromal vascular fraction for the experiments performed in this thesis. Difficulties using this model include a limited passage number (<5), slower growth, and donor to donor variation. Preadipocytes can be differentiated into mature white adipocytes (a process called adipogenesis) *in vitro*

using a cocktail of adipogenic inducers including fetal bovine serum (FBS), dexamethasone, isobutylmethylxanthine, and insulin (Farmer, 2006) These inducers act through a complex signaling pathway in order to activate two important adipogenic transcription factors: peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα) (Farmer, 2006).

PPARγ is a nuclear receptor that is the master regulator of adipogenesis due to its ability to control hundreds of genes responsible for the formation and maintenance of the mature adipocyte phenotype (Farmer, 2005). Activation of PPARγ by thiazolidinediones (TZDs) in both mouse and humans has been shown to induce adipogenesis (Adams et al., 1997). Differentiation of abdominal subcutaneous, but not intra-abdominal omental, adipocytes is enhanced with TZD treatment (Adams et al., 1997).

C/EBPα has also been shown to activate adipogenesis in mouse fibroblastic cells when expressed ectopically (Freytag et al., 1994). C/EBPα helps regulate terminal differentiation of preadipocytes, but is not absolutely required for adipogenesis, unlike PPARγ (Rosen et al., 2002). Rosen et al. showed that an immortalized cell line lacking PPARγ could not be stimulated to activate adipogenesis upon C/EBPα induction, while ectopic PPARγ could (Rosen et al., 2002). Overall, these two transcription factors act as end targets for initiating and maintaining the adipogenic process.

Whereas studies using human preadipocytes are more relevant to the human population, one of the most well used models to study adipocyte physiology is the

murine 3T3-L1 preadipocyte cell line, which is committed to the adipocyte lineage (Farmer, 2006). They do not suffer from donor to donor variations and are less costly than freshly isolated cells (Poulos et al., 2010). 3T3-L1 preadipocytes can also be passaged multiple times without losing their ability to differentiate (Poulos et al., 2010). Although the 3T3-L1 preadipocyte cell line is useful, human differentiated adipocytes provide a more relevant model for examining human-related disease conditions.

## Rationale

Given the association of insulin resistance, cardiovascular disease, and SH (high blood levels of TSH), and the fact that adipocytes express TSHR; TSH may be acting on adipocytes. Adipocytes are a major insulin target. Previous work has shown that β-adrenergic receptor signaling can inhibit insulin signaling in adipocytes (Issad et al., 1995), (Klein et al., 1999). Therefore, I propose that TSH signaling molecules interfere with insulin signaling in adipocytes. The adipocyte plays a central role in whole body metabolism and energy homeostasis. By studying the effects of TSH on insulin action in adipocytes, I hope to elucidate mechanisms underlying insulin resistance in subclinical hypothyroidism.

## Hypothesis

TSH inhibits insulin action in human adipocytes.

## Objectives

1. Determine if TSH inhibits the insulin signaling pathway.

2. Identify the molecular mechanisms underlying the ability of TSH to inhibit insulin action.

3. Determine if TSH inhibits insulin-stimulated lipogenesis and glucose uptake.

# METHODS

## Human Subcutaneous Preadipocyte Isolation and Culture

Human abdominal subcutaneous samples were obtained from 16 consenting patients (1 male; 15 female) undergoing elective abdominal surgery (approved by the Ottawa Health Science Network Research Ethics Board). The mean age of the patients was 52 ± 10 years and the mean body mass index was 32 ± 10 $kg/m^2$ (±SD). The stromal preadipocytes were isolated as previously described (Gagnon et al., 2003). Connective tissue and capillaries were removed by dissection and tissue was digested with collagenase CLS type 1 (200 U/g of tissue; Worthington) on a rotary shaker at 37°C for 1 hour in the absence of serum. Following digestion, the tissue was processed via size filtration using a sterile 200 µm nylon filter to remove debris. The filtered tissue was then centrifuged at 200xg for 20 minutes to remove any floating mature adipocytes. 10% fetal bovine serum (FBS, Hyclone) was then added to the infranatant for cell viability. To further ensure removal of mature adipocytes, progressive size filtration was done using 100 µm, 50 µm and 25 µm nylon filters. After filtration, the tissue was centrifuged at 200xg for 20 minutes and red blood cell lysis buffer (155 mM $NH_4Cl$, 5.7 mM $K_2HPO_4$, 0.1 mM EDTA, pH 7.3) was added to the pellet for 5 minutes.

Dulbecco Modified Eagle Medium/Low Glucose (5mM) (DMEM; Hyclone) supplemented with 10% FBS and antibiotics {0.1 mg/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco) and 50 U/ml Nystatin (Calbiochem)} (henceforth referred to as growth medium) was added to the isolated stromal cells and the cells were centrifuged at 200g for 5 minutes to remove any cellular debris. Cells were then

counted using a Neubauer hemacytometer. Preadipocytes were expanded in growth medium for a maximum of 3 passages before differentiation or cryopreserved in liquid nitrogen until required.

**Human Subcutaneous Adipocyte Differentiation**

Preadipocytes were seeded at a density of 3 x $10^4$ cell/$cm^2$ for a maximum of 3 passages in growth medium. Differentiation was induced using growth medium supplemented with 0.85 µmol/L insulin (Sigma), 100 µmol/L indomethacin (Sigma), 0.5 µmol/L dexamethasone (Steraloids), and 0.25 mmol/L isobutylmethylxanthine (Sigma) for 14 days. Visual inspection using a Nikon Eclipse TS-100 microscope showed that approximately 60-70% of the cells differentiated into adipocytes. On day 14, the differentiation medium was removed; the cells were washed once with growth medium and placed in growth medium for 2 days prior to treatment.

**Lipogenesis**

Differentiated adipocytes in a 12 well plate were washed once with DMEM prior to treatment. Cells were stimulated with vehicle control, 5 mU/ml bovine TSH (Sigma), 100 nM insulin (Sigma) or a combination of TSH and insulin for 4 hours in the presence of 0.2 µCi/ml $^{14}$C-glucose (American Radiolabeled Chemicals) in phenol-free DMEM (Gibco) supplemented with 4% fatty acid free bovine serum albumin (BSA) (Roche), 0.1 mg/ml streptomycin and 100 U/ml penicillin (henceforth referred to as experimental medium). The use of $^{14}$C-glucose allowed the observation of incorporation of the labeled carbons into both the glycerol backbone as well as the individual fatty acids, in an insulin-stimulated fashion. Preliminary

time course studies demonstrated that a four hour insulin stimulation time point was adequate to produce a significant lipogenic response. Medium was removed and centrifuged at 500xg for 5 min at 4°C, without braking. 25 µl of the supernatant was added to 4 ml of Ecolume$^{TM}$ in scintillation vials to quantify the amount of $^{14}C$ to ensure equal loading per well. The remaining supernatant was aliquoted and stored at -80°C for later lipolysis measurement.

Cells were washed 3 times with phosphate buffer saline (PBS) and treated with 1ml of isopropanol/n-heptane (2:3 v/v) for 30 minutes to extract the cellular lipid fraction. The extraction was repeated for 15 minutes with 0.5 ml of isopropanol/n-heptane and both fractions were transferred to glass tubes covered by parafilm and vortexed. 500 µl of the fraction was removed and placed in scintillation vials, which were dried using $N_2$ stream and 4 ml of Ecolume$^{TM}$ was added to measure radioactivity. The remaining extract was dried with a speed vacuum or by $N_2$ stream and stored at -20°C for measurement of TG levels. After letting the residual solvent evaporate, the remaining protein was solubilised in 150 µl of Laemmli buffer (Laemmli, 1970). The dish contents were scraped with cell scrapers, and DNA was sheared using 25 G1/2 needles with 1 ml syringes. The lysate was boiled for 5 minutes to denature the protein. Protein was stored at -20°C and measured with the modified Lowry reaction, using BSA as a standard.

**TG Quantification**

Cellular TG was measured by resuspending the remaining extract not measured for radioactivity (the cellular lipid fraction) in 300 µl isopropanol and using

triolein (MP biomedicals, Inc.) as a standard (Gagnon et al., 2010). 10 µl of extract was diluted in 50 µl of isopropanol on a 96 well plate. 30 µl of saponification reagent (1.78 M KOH, 25% v/v isopropanol) was added and the solutions were incubated for 10 minutes at room temperature. Subsequently, 60 µl of sodium metaperiodate and 60 µl of acetyl acetone were added and incubated for 15 minutes at 65°C. Following a cooling period of 10 minutes, samples were assessed using a spectrophotometer at 405 nm.

**Lipolysis**

Lipolysis was measured based on the glycerol released into the medium. Glycerol was quantified using the Glycerol Reagent A protocol (Zen-Bio). A standard curve was created (3.125-200 uM) using a 1 mM glycerol standard stock solution (Sigma). An equal volume of test sample (diluted accordingly) and reconstituted glycerol reagent A (Sigma) were mixed together on a 96 well plate. The plate was then incubated at room temperature for 15 minutes and the optical density of each well was measured at 540 nm.

**Insulin signaling**

Differentiated adipocytes in 35 mm dishes were washed once with DMEM prior to treatment. For time course studies, cells were acutely stimulated with vehicle control, 5 mU/ml bovine TSH (Sigma), 100 nM insulin (Sigma) or a combination of TSH and insulin for 5, 15 and 30 minutes. Protein was collected using Laemmli buffer (Laemmli, 1970) containing phosphatase inhibitors (50 mM NaPPi, 500 mM NaF, 500 mM pH 8 EGTA, 100 mM $Na_3VO_4$) and immunoblot

analysis was performed. Protein was measured using the modified Lowry reaction, with BSA as the standard.

In some cases, cells were pre-incubated with vehicle control or 5 mU/ml bovine TSH (Sigma) for 1 hour**.** Following pre-incubation, cells were acutely stimulated with vehicle control or 100 nM insulin (Sigma) for 5 minutes. In other cases, a 1 hour pre-treatment with control ($H_2O$) or conventional PKC α and β1 inhibitor Gö6976 (Calbiochem) was performed prior to pre-treatment with TSH and insulin stimulation. In both cases, protein was collected using Laemmli buffer containing phosphatase inhibitors and immunoblot analysis was performed.

**Immunoblot Analysis**

Equal amounts of protein (varied from 20-50 ug depending on patient sample) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membrane. Non-specific sites were blocked (using 3% BSA for phosphotyrosine antibody and 5% milk for other antibodies) and the membrane was probed with the following antibodies: αPy20 (1:2000; Cell Signaling Technology), PhosphoAkt Ser473 (1:1000; Cell Signaling Technology), Akt (1:1000; Cell Signaling Technology), phosphoserine PKC substrate (1:1000; Cell Signaling), phosphoPKC δ Thr505 (1:500, Cell Signaling), ERK 1/2 (0.25 ug/ml; millipore) and TSHR (1:500, Abcam ). This was followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using enhanced chemiluminescence. Relative densitometry was measured with AlphaEaseFC

software (Alpha Innotech, San Leandro, CA), and data were expressed as integrated optical density units.

**Glucose Uptake**

Glucose uptake was measured according to Lee et al, with modifications as described (Lee et al., 2012). Differentiated adipocytes on a 12 well plate were washed twice with 37°C PBS and then placed in Krebs Ringer HEPES (KRH) buffer supplemented with 5.6 mM glucose for 2 hours at 37°C. Subsequently, vehicle control or 5 mU/ml bovine TSH (Sigma) was added and the cells were incubated for 1 hour at 37°C. Media was removed and cells were placed in KRH buffer (no glucose) and stimulated with vehicle control, 5 mU/ml bovine TSH (Sigma), 10 nM insulin (Sigma) or a combination of TSH and insulin for 30 minutes at 37°C. Radioactive label solution (2 µCi/ml $^{3}$H-2-deoxy-glucose (PerkinElmer) and 0.2 mM unlabeled 2-deoxy-glucose) was added then for 10 or 20 minutes at 37°C. Unlike glucose, 2-deoxy-glucose cannot be phosphorylated and will not proceed through glycolysis. This allows for measurement of glucose uptake.

Cells were then placed on ice, and media were removed for counting. The cells were washed three times with ice-cold PBS and placed in lysis buffer containing 20 mM Tris pH 7.4, 150 mM NaCl and 1% Triton X-100. Cells were lysed for 30 minutes on a rotary shaker and then scraped and transferred to microfuge tubes. $^{3}$H-incorporation was determined by counting two aliquots (150-200 µl) of lysate per sample. 4 ml of Ecolume$^{TM}$ was added to count for radioactivity. Remaining lysates were stored at -20°C overnight. Protein was determined using

the remainder of the lysate and measured using the modified Lowry reaction, with BSA as the standard.

**Statistical Analysis**

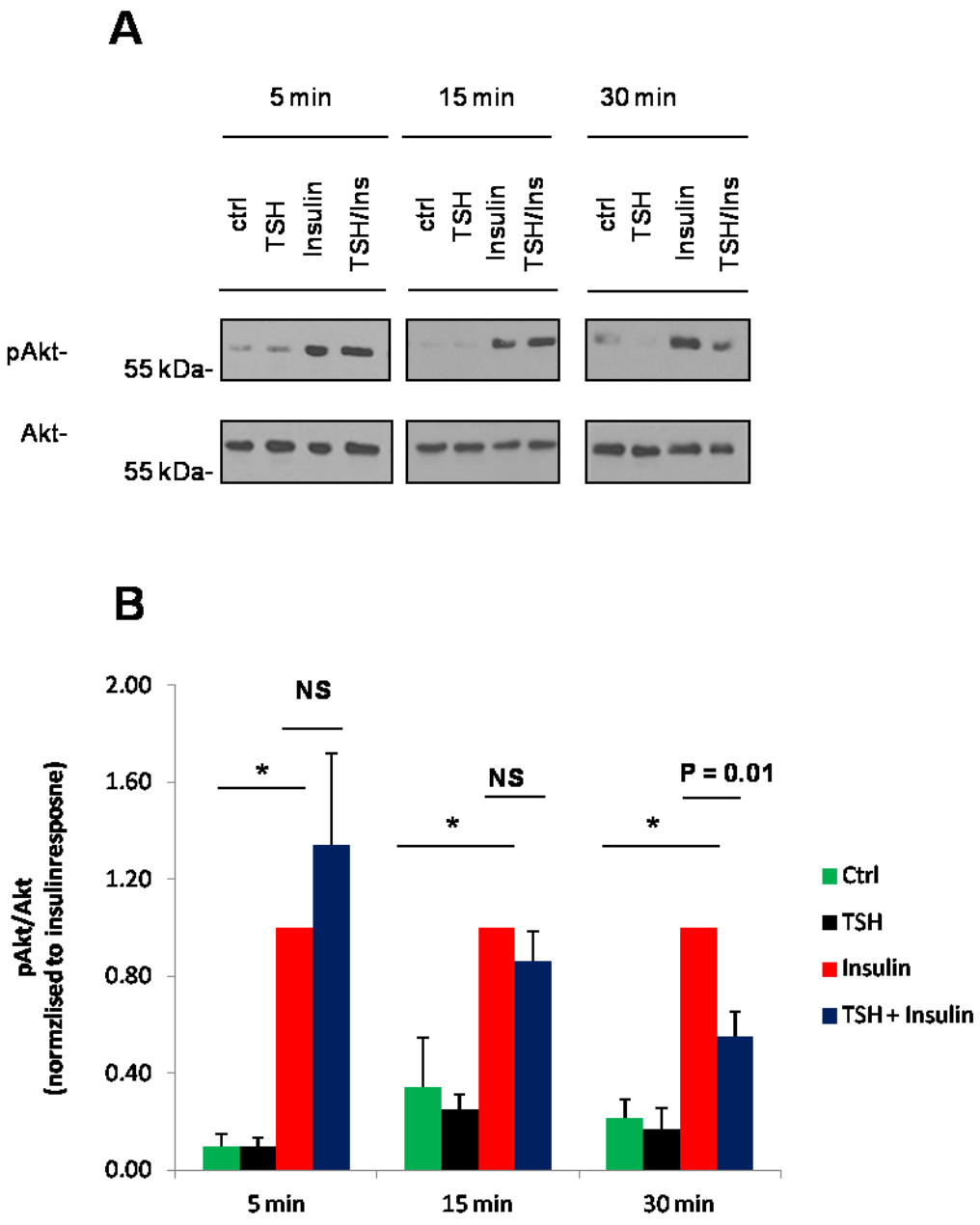
Data was analyzed using two way ANOVA with Tukey's post-hoc tests, using Microsoft Excel 2007 Analysis Plug-in. Pearson R correlation was used to assess correlations. P-values less than 0.05 were taken as significant.

## RESULTS

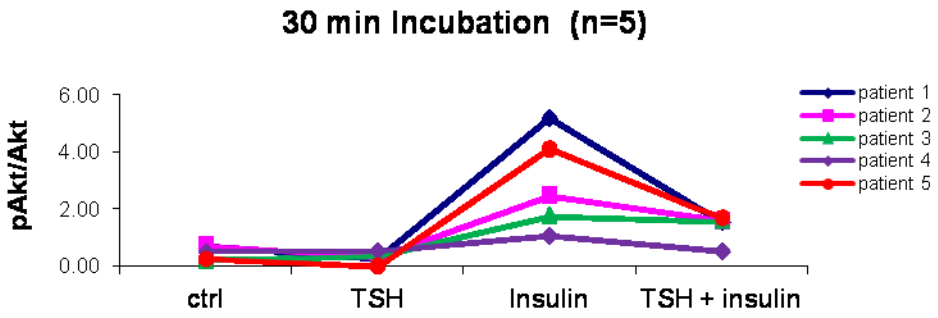
### Effect of TSH on adipocyte insulin signaling

In order to observe the effect of TSH on insulin signaling in adipocytes, differentiated human adipocytes were treated with 100 nM insulin in the absence or presence of 5mU/ml TSH, or vehicle control (0.01 N HCl or water respectively) for 5, 15 and 30 minutes. Subsequently, cellular protein was solubilised and immunoblot analysis was performed to probe for Ser473 Akt phosphorylation (**Figure 2**). As expected, stimulation with insulin alone significantly increased Akt phosphorylation at all time points (**Figure 2A and 2B**). At 30 minutes, TSH caused a significant 45% decrease in insulin-stimulated Akt phosphorylation. This demonstrates that TSH can inhibit insulin-stimulated Akt phosphorylation, a key part of the insulin signaling pathway. Results were normalized to the insulin response alone, due to variation between the different patient samples (**Figure 2C-E**). No BMI or age-dependent effects on the inhibition of insulin-stimulated Akt phosphorylation or on the insulin response itself were observed (**Figure 2F-I**).

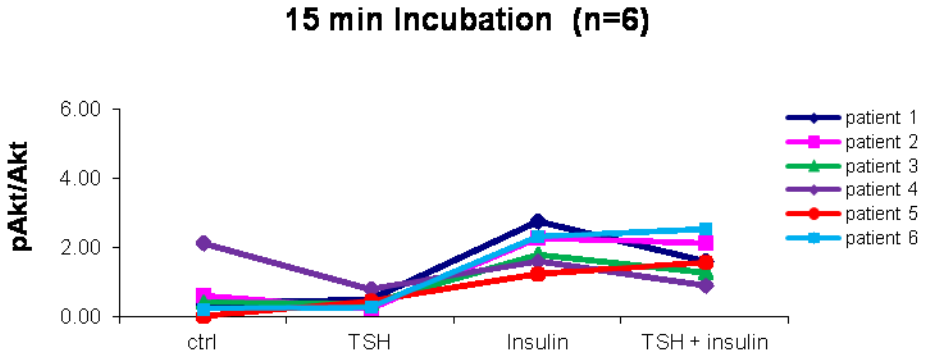
The above studies were done with TSH added simultaneously with insulin. Adipocytes were pre-incubated with TSH in order to see if this would augment its inhibition of the insulin response. Differentiated adipocytes were pre-treated with 5 mU/ml TSH or vehicle (water) for 1 hour and then acutely stimulated with 100 nM insulin or vehicle (0.01 N HCl) for 5 minutes. Cellular protein was solubilized, and Ser473 Akt phosphorylation was assessed via immunoblot analysis. TSH inhibited insulin-stimulated Akt phosphorylation by 50% after 5 minutes of insulin stimulation (**Figure 3A**).


A
5 min
15 min
30 min
ctrl
TSH
Insulin
TSH/Ins
pAkt-
55 kDa-
Akt-
55 kDa-
B
pAkt/Akt
(normzlised to insulinresposne)
2.00
1.60
1.20
0.80
0.40
0.00
NS
*
NS
P = 0.01
5 min
15 min
30 min
Ctrl
TSH
Insulin
TSH + Insulin

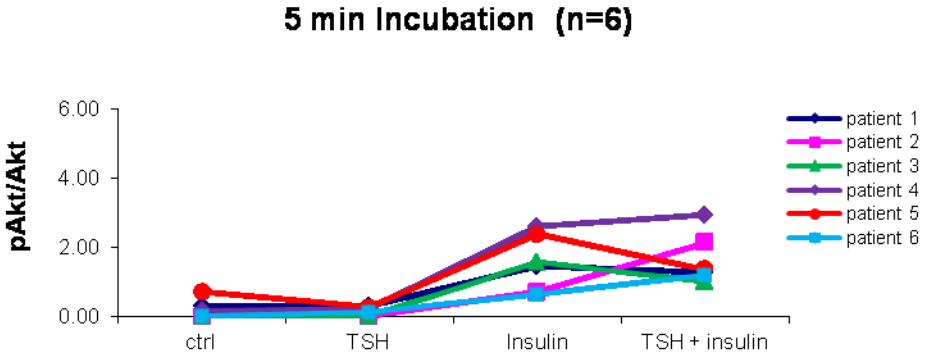
C


30 min Incubation (n=5)
pAkt/Akt
6.00
4.00
2.00
0.00
ctrl
TSH
Insulin
TSH + insulin
patient 1
patient 2
patient 3
patient 4
patient 5

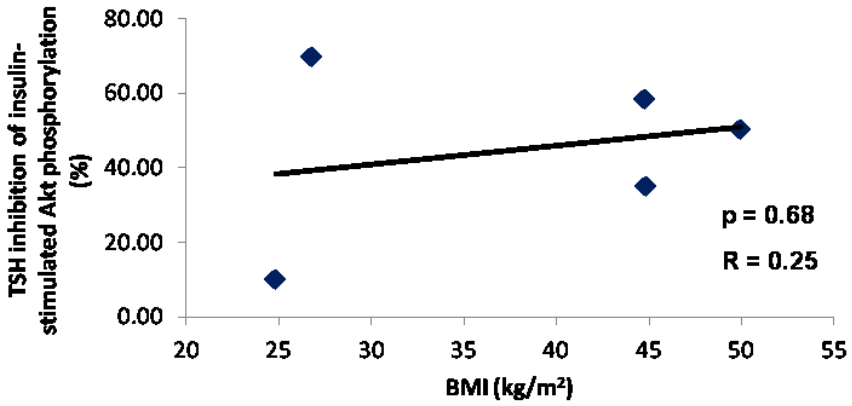
D


15 min Incubation (n=6)
pAkt/Akt
6.00
4.00
2.00
0.00
ctrl
TSH
Insulin
TSH + insulin
patient 1
patient 2
patient 3
patient 4
patient 5
patient 6

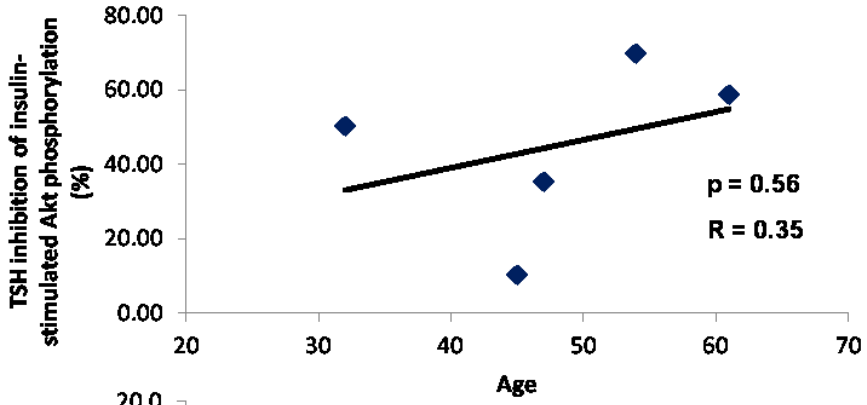
E


5 min Incubation (n=6)
pAkt/Akt
6.00
4.00
2.00
0.00
ctrl
TSH
Insulin
TSH + insulin
patient 1
patient 2
patient 3
patient 4
patient 5
patient 6

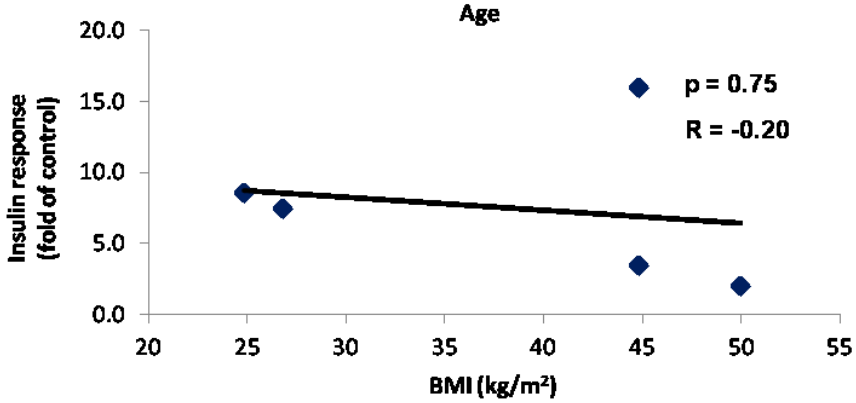
F


TSH inhibition of insulin-stimulated Akt phosphorylation (%)
80.00
60.00
40.00
20.00
0.00
20
25
30
35
40
45
50
55
BMI (kg/m²)
p = 0.68
R = 0.25

G


TSH inhibition of insulin-stimulated Akt phosphorylation (%)
80.00
60.00
40.00
20.00
0.00
20
30
40
50
60
70
Age
p = 0.56
R = 0.35

H


Insulin response (fold of control)
20.0
15.0
10.0
5.0
0.0
20
25
30
35
40
45
50
55
BMI (kg/m²)
p = 0.75
R = -0.20

I

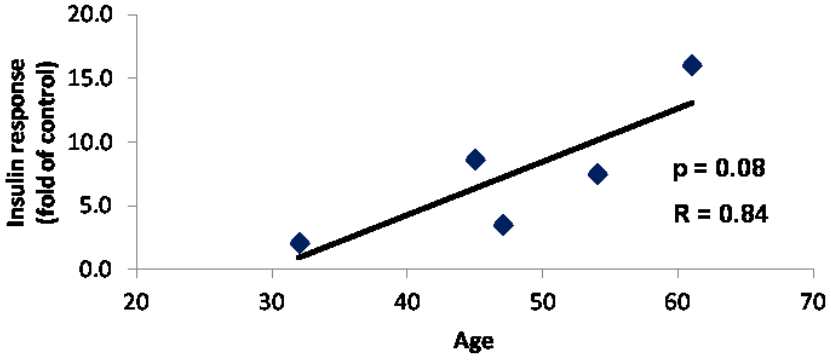

Insulin response (fold of control)
20.0
15.0
10.0
5.0
0.0
20
30
40
50
60
70
Age
p = 0.08
R = 0.84

**Figure 2: TSH inhibits insulin-stimulated Akt phosphorylation in human differentiated adipocytes**. Human differentiated adipocytes were stimulated for 5, 15 or 30 minutes with 100 nM insulin in the presence or absence of 5 mU/ml TSH. Protein was solubilized, and Akt phosphorylation and mass was assessed by immunoblot analysis. Representative blots are shown (A). Akt phosphorylation was quantified by densitometry, and normalized to Akt mass (C-E). Data from 5-6 experiments are expressed as means +/-SE, normalized to the insulin response; this normalized value varied by 11-26% across the individual experiments (B). Each experiment uses a different patient. % inhibition of insulin-stimulated Akt phosphorylation by TSH is shown as a function of BMI (F) and age (G). Insulin response is shown as a function of BMI (H) and age (I) and was expressed as a fold of control. Statistical analysis was by 2 way ANOVA with Tukey's post-hoc tests. * $p < 0.05$. NS = not significant.

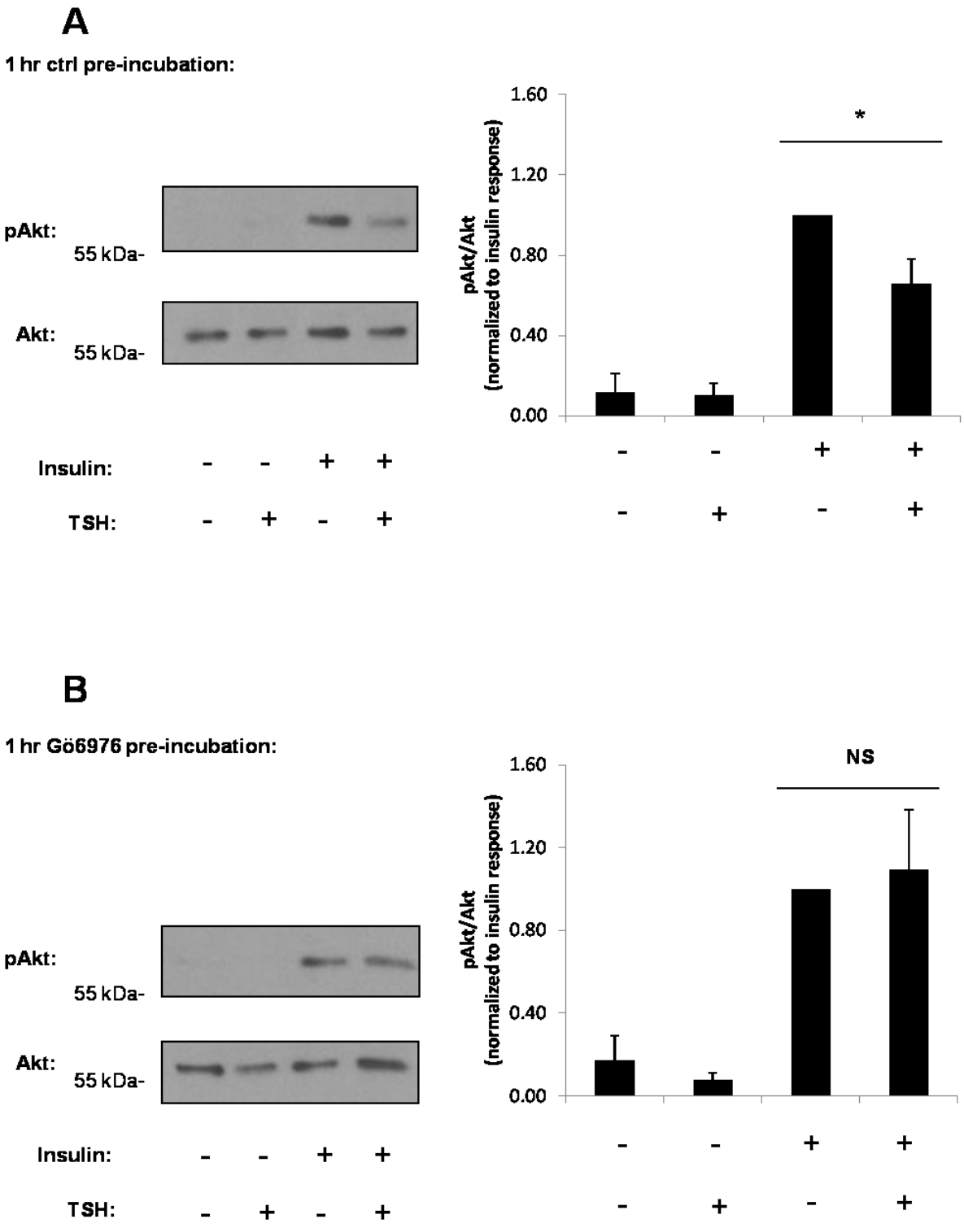

A
1 hr ctrl pre-incubation:
pAkt:
55 kDa-
Akt:
55 kDa-
Insulin: - - + +
TSH: - + - +
pAkt/Akt (normalized to insulin response)
1.60
1.20
0.80
0.40
0.00
*
- - + +
- + - +
B
1 hr Gö6976 pre-incubation:
pAkt:
55 kDa-
Akt:
55 kDa-
Insulin: - - + +
TSH: - + - +
pAkt/Akt (normalized to insulin response)
1.60
1.20
0.80
0.40
0.00
NS
- - + +
- + - +

**Figure 3: Conventional PKC inhibitor Gö6976 blocks TSH inhibition of insulin stimulated Akt phosphorylation in human differentiated adipocytes**. Human differentiated adipocytes were pre-incubated with 1 μM Gö6976 for 1 hour. Subsequently, 5 mU/ml TSH was added for 1 hour and then acutely stimulated with 100 nM insulin for 5 minutes. Protein was solubilized, and Akt phosphorylation and mass was assessed by immunoblot analysis. Representative blots from one experiment are shown. Akt phosphorylation was quantified by densitometry, and normalized to Akt mass. Results from 6 experiments are expressed as means +/- SE normalized to the insulin response for ctrl (A) and Gö6976 (B); this normalized value varied by 12-29% across the individual experiments. Each experiment uses a different patient. Statistical analysis was by 2 way ANOVA with Tukey's post-hoc tests. * $p < 0.05$. NS = not significant.

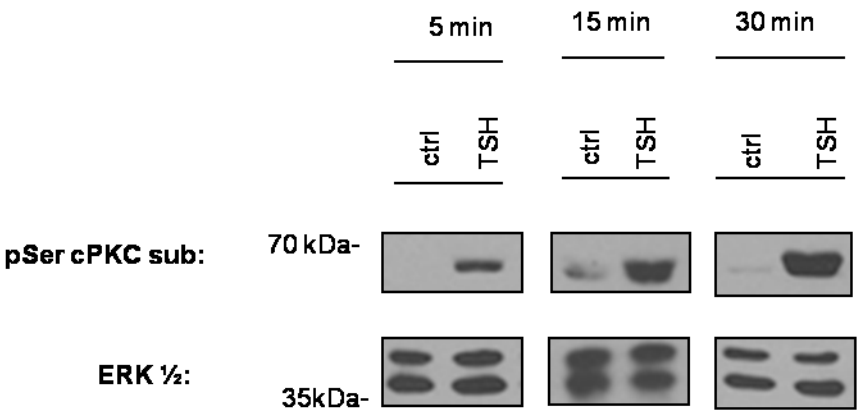
This was much earlier than what was previously observed when TSH was added together with insulin. These results suggest that TSH inhibits insulin signaling in human adipocytes.

**Effect of TSH-activated cPKC on adipocyte insulin signaling**

Previous work in rat adipocytes and human adipose tissue showed that conventional PKCs (cPKC) can inhibit insulin signaling by upstream serine phosphorylation of the C-terminus of IRS-1 ($Ser^{336}$) (Issad et al., 1995), (Klein et al., 1999), (Liberman et al., 2008). The authors also showed that this would inhibit Akt phosphorylation as it is downstream of IRS-1 (Klein et al., 1999). Our laboratory has previously reported that TSH can activate cPKC in human differentiated adipocytes (Thrush et al., 2012). To confirm that TSH activates cPKC activity under the conditions used in my studies, differentiated human adipocytes used in Figure 2 were stimulated with TSH for 5, 15 and 30 minutes. PKC phosphorylation was assessed by an antibody that detects cellular proteins that have been phosphorylated at serine residues surrounded by arginine and lysine at the -2 and +2 positions and a hydrophobic residue at the +1 position (Cell Signaling Cat. #2261). TSH significantly increased serine phosphorylation of a 63 kDa cPKC substrate by 26 fold at 5 minutes and 7 fold at 30 minutes. Although the 15 minute TSH stimulation did trend towards an increase in phosphorylation of this cPKC substrate, it was not significant (**Figure 4**).

After confirming that TSH can activate cPKC, cPKC was inhibited in order to determine if TSH inhibition of insulin-stimulated Akt phosphorylation would be

A


5 min
15 min
30 min
ctrl
TSH
ctrl
TSH
ctrl
TSH
pSer cPKC sub:
70 kDa-
ERK ½:
35kDa-

B

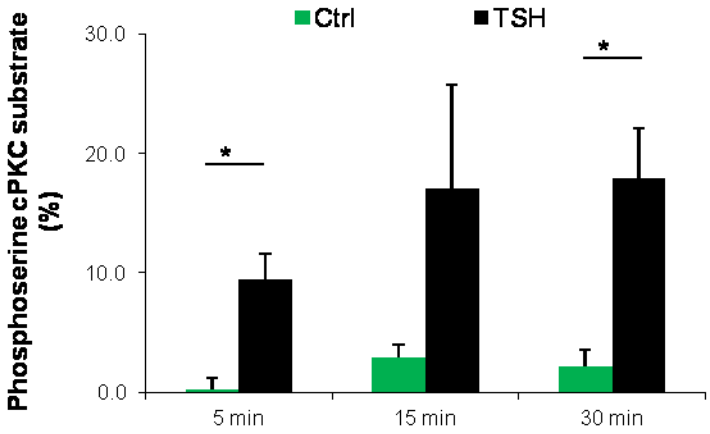

Ctrl
TSH
Phosphoserine cPKC substrate (%)
30.0
20.0
10.0
0.0
*
*
5 min
15 min
30 min

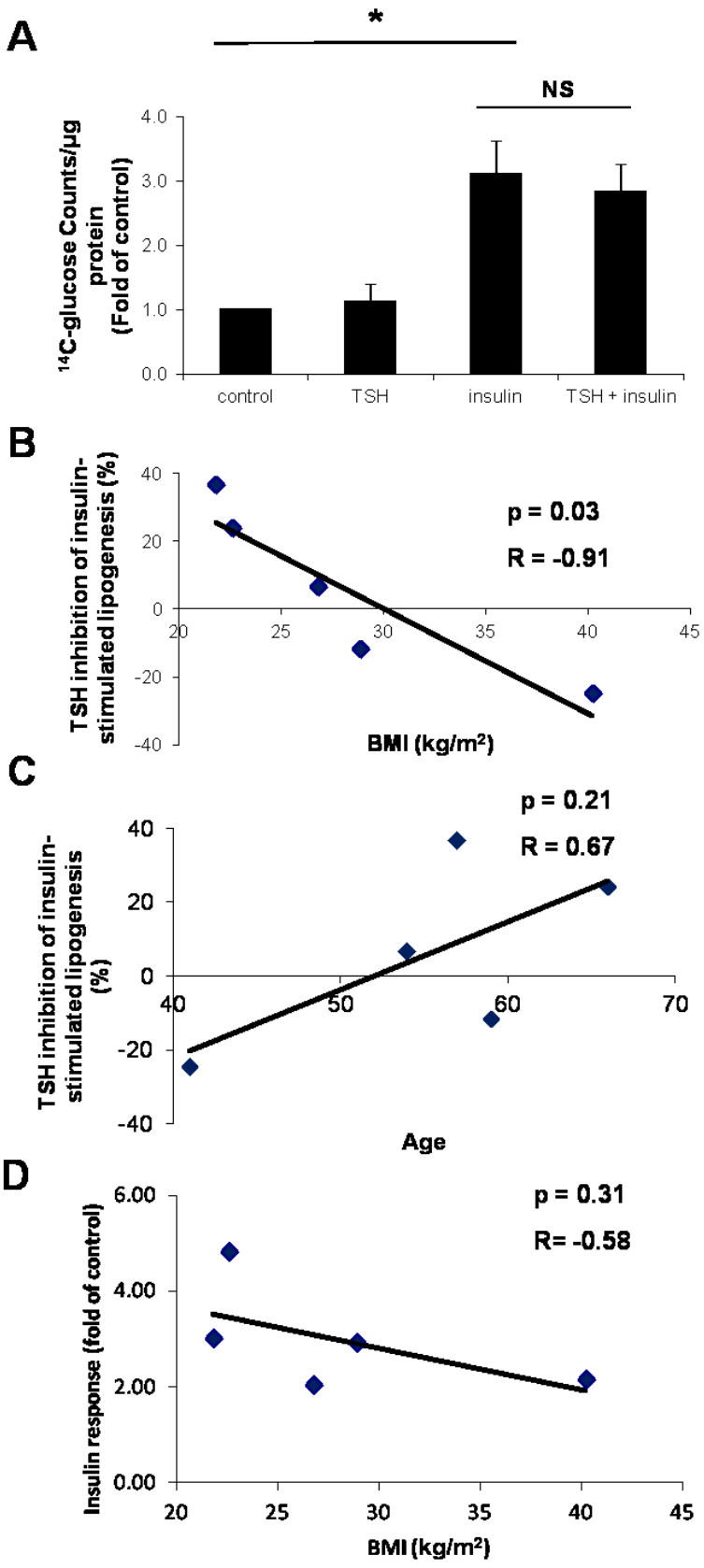
**Figure 4: TSH stimulates serine phosphorylation of a cPKC substrate in human differentiated adipocytes.** Human differentiated adipocytes were stimulated for 5, 15 or 30 minutes with 5 mU/ml TSH. Protein was solubilized, and serine cPKC phosphorylation and ERK 1/2 mass were assessed by immunoblot analysis. Representative blots are shown (A). cPKC substrate serine phosphorylation was quantified by densitometry and expressed as optical density units. Results from 5-6 experiments are expressed as mean +/- SE (B). Each experiment uses a different patient. Statistical analysis was by 2 way ANOVA with Tukey's post-hoc tests. * $p < 0.05$. NS = not significant.

affected. Human differentiated adipocytes were pre-treated with cPKCα and βI inhibitor Gö6976 (Calbiochem CAS 136194-77-9) for 1 hour before pre-incubating with TSH for 1 hour. As before, the cells were then stimulated with100 nM insulin for 5 minutes and Akt phosphorylation was assessed by immunoblot analysis. Gö6976 pre-incubation was able to completely prevent the 34.2% TSH inhibition of insulin-stimulated Akt phosphorylation (**Figure 3B**).

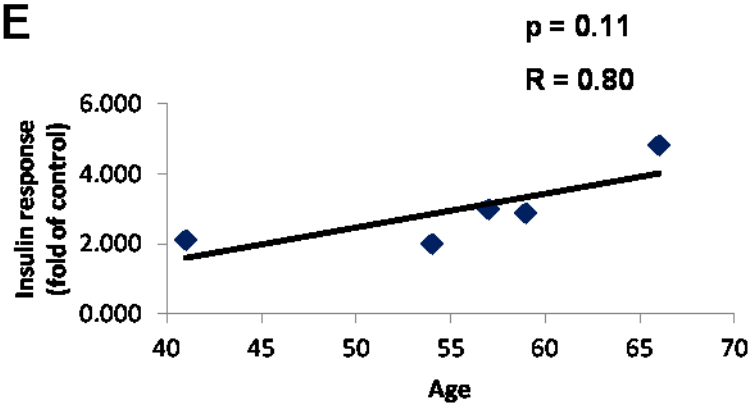
**Effect of TSH on insulin-regulated adipocyte lipogenesis**

To investigate the effects of TSH on insulin-regulated cellular processes, differentiated human adipocytes were stimulated with 5 mU/ml TSH or vehicle in the presence or absence of 100 nM insulin containing 0.2 μCi $^{14}$C-glucose for 4 hours. Intracellular lipids were extracted and counted for $^{14}$C radioactivity using liquid scintillation spectrometry in order to measure insulin-stimulated lipogenesis. When all 5 patients were analyzed together, there was no effect of TSH on insulin-stimulated lipogenesis (**Figure 5A**). A significant negative correlation was found ($r = -0.91$; $p = 0.03$) between BMI and the inhibition of insulin-stimulated lipogenesis by TSH (**Figure 5B**). No significant correlation was observed with age (**Figure 5C**). To ensure that this inhibition was not due to an effect of BMI on the insulin response, these two variables were compared and no significant correlation was found (**Figure 5D**). In addition, no significant correlation was observed between age and the insulin response (**Figure 5E**).

To confirm equal amounts of adipocyte differentiation between each condition, TG levels were measured and no significant changes were found (**Figure**


A
*
NS
$^{14}$C-glucose Counts/µg protein (Fold of control)
4.0
3.0
2.0
1.0
0.0
control
TSH
insulin
TSH + insulin
B
TSH inhibition of insulin-stimulated lipogenesis (%)
p = 0.03
R = -0.91
40
20
0
-20
-40
20
25
30
35
40
45
BMI (kg/m$^2$)
C
TSH inhibition of insulin-stimulated lipogenesis (%)
p = 0.21
R = 0.67
40
20
0
-20
-40
40
50
60
70
Age
D
Insulin response (fold of control)
p = 0.31
R= -0.58
6.00
4.00
2.00
0.00
20
25
30
35
40
45
BMI (kg/m$^2$)

E


p = 0.11
R = 0.80
Insulin response (fold of control)
6.000
4.000
2.000
0.000
40
45
50
55
60
65
70
Age

F

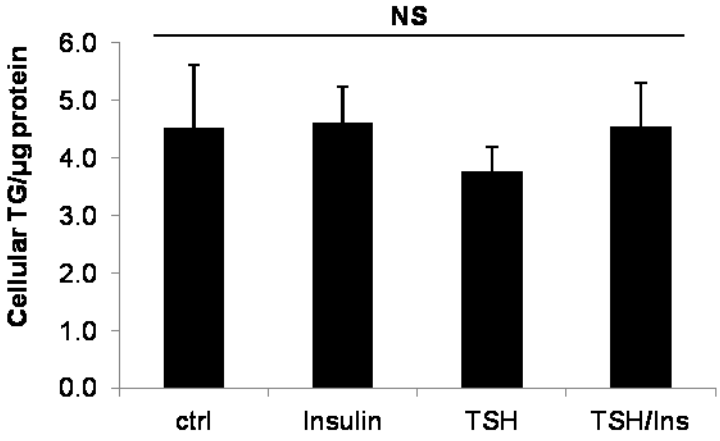

NS
Cellular TG/µg protein
6.0
5.0
4.0
3.0
2.0
1.0
0.0
ctrl
Insulin
TSH
TSH/Ins

**Figure 5: TSH inhibition of adipocyte insulin-stimulated lipogenesis negatively correlates with body mass index (BMI).** Human differentiated adipocytes were stimulated for 4 hours with 100 nM insulin and/or 5 mU/ml TSH in medium containing 0.2 µCi $^{14}$C-glucose. The intracellular lipid fraction was isolated and $^{14}$C incorporation was counted using liquid scintillation spectrometry. $^{14}$C counts were normalized to the total amount of protein and expressed as fold of control (A); this normalized value varied by 52% across the individual experiments. % inhibition of insulin-stimulated lipogenesis by TSH as a function of BMI (B) and age (C). Insulin response as a function of BMI (D) and age (E) was expressed as a fold of control. Intracellular lipid fraction levels of triacylglycerol were also measured (D). Results are the mean +/- SE of 5 experiments, each performed in duplicate. Each experiment uses a different patient. Statistical analysis was by 2 way ANOVA with Tukey's post-hoc tests for (A) and (F) or Pearson R correlation for (B-E). * $P < 0.01$. NS = not significant.

**5F**). Furthermore, lipolysis was measured in order to confirm that TSH was active. Glycerol levels in the media showed a 3-fold increase in response to TSH stimulation (**Figure 6**). Since a correlation between BMI and TSHR expression has been reported (Nannipieri et al., 2009), (Fu et al., 2012), TSHR expression was measured via immunoblotting. In the 5 patients examined, no significant correlation was seen between the TSHR and BMI (**Figure 7**).

**Effect of TSH on insulin-stimulated adipocyte glucose uptake**

The effect of TSH on insulin-stimulated glucose uptake was investigated. A radioactively labelled $^{3}$H-2-deoxyglucose molecule was used as a tracer to measure glucose uptake, as described (Lee et al., 2012). Briefly, differentiated human adipocytes were serum-starved for 2 hours, followed by a 1 hour pre-treatment with 5 mU/ml TSH. The cells were stimulated for 30 minutes with 10 nM insulin and then incubated with $^{3}$H-2-deoxyglucose for 10 or 20 minutes. The cells were lysed and counted for $^{3}$H using liquid scintillation spectrometry. There was a significant 2-fold increase in 2-deoxyglucose uptake at both 10 and 20 minute incubation times (**Figure 8A and 8B**). In the presence of TSH, insulin-stimulated 2-deoxyglucose uptake was significantly augmented by 36% at 20 minutes, but not at 10 minutes. The effect of age or BMI on the response was also examined and a significant negative correlation was found ($r = -0.94$; $p = 0.02$) between BMI and the augmentation of insulin stimulated 2-deoxyglucose by TSH at 20 minutes, but not at 10 minutes (**Figure 8C**). No significant correlation was seen between age and the augmentation response (**Figure 8D**). Similarly to the lipogenesis data, no significant

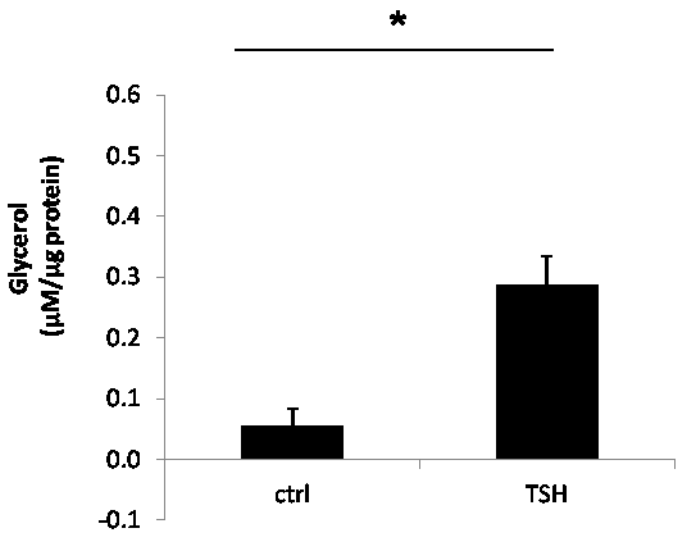
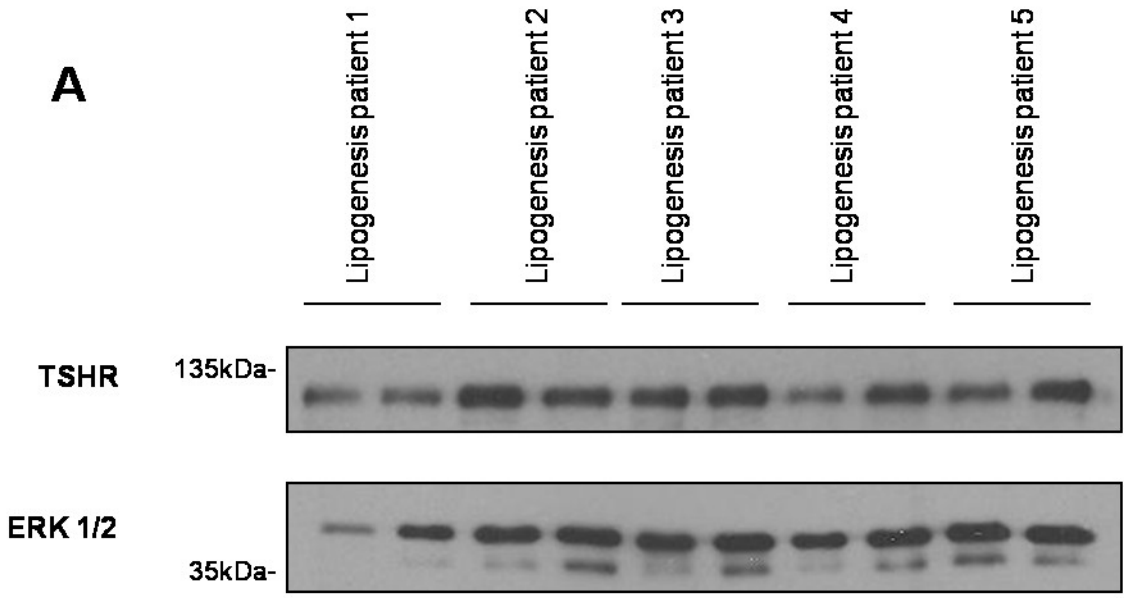

*
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
Glycerol
(µM/µg protein)
ctrl
TSH

**Figure 6: TSH stimulates adipocyte lipolysis.** Human differentiated adipocytes were stimulated for 4 hours with 5 mU/ml TSH. Media was taken and glycerol levels were measured in the medium as a marker of lipolysis. Results are the mean +/- SE of 4 experiments, each performed in duplicate. Each experiment uses a different patient. Statistical analysis was by 2 way ANOVA with Tukey's post-hoc tests. * $P < 0.01$. NS = not significant.


A
Lipogenesis patient 1
Lipogenesis patient 2
Lipogenesis patient 3
Lipogenesis patient 4
Lipogenesis patient 5
TSHR
135kDa-
ERK 1/2
35kDa-

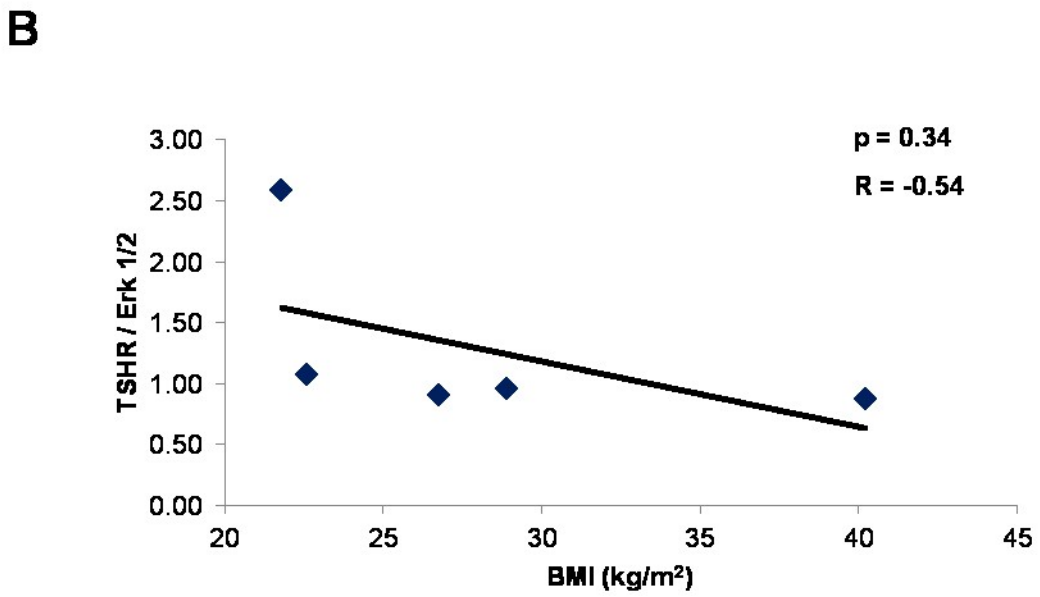
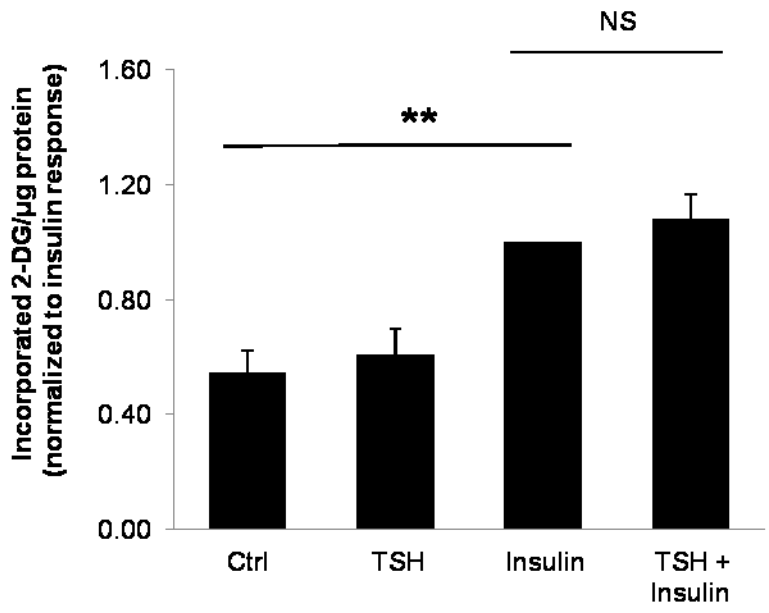
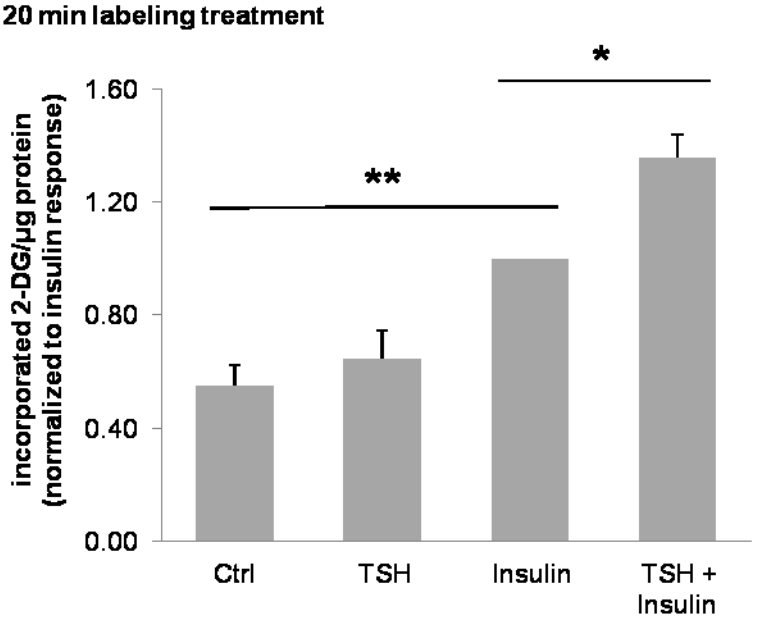

B
p = 0.34
R = -0.54
TSHR / Erk 1/2
3.00
2.50
2.00
1.50
1.00
0.50
0.00
20
25
30
35
40
45
BMI (kg/m²)

**Figure 7: TSH receptor protein levels in human differentiated adipocytes are not significantly correlated with BMI.** Protein was solubilized from human differentiated adipocytes used in the control samples from the lipogenesis experiment. TSH receptor and ERK 1/2 mass was assessed by immunoblot analysis (A). TSH receptor protein expression was normalized to ERK 1/2 and shown as a function of BMI. Pearson R correlation is shown (B).

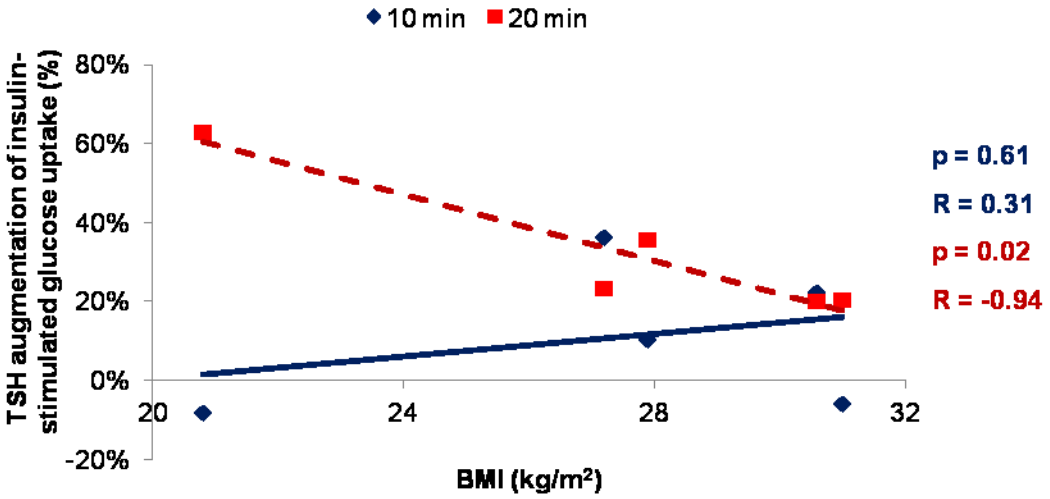
**A**


10 min labeling treatment
NS
**
Incorporated 2-DG/µg protein (normalized to insulin response)
1.60
1.20
0.80
0.40
0.00
Ctrl
TSH
Insulin
TSH + Insulin

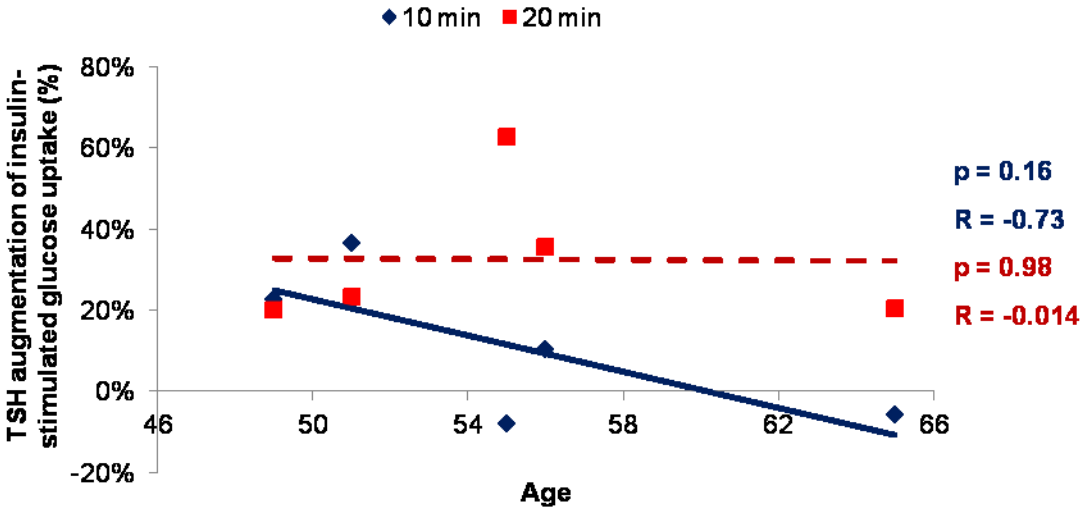
**B**


20 min labeling treatment
*
**
incorporated 2-DG/µg protein (normalized to insulin response)
1.60
1.20
0.80
0.40
0.00
Ctrl
TSH
Insulin
TSH + Insulin

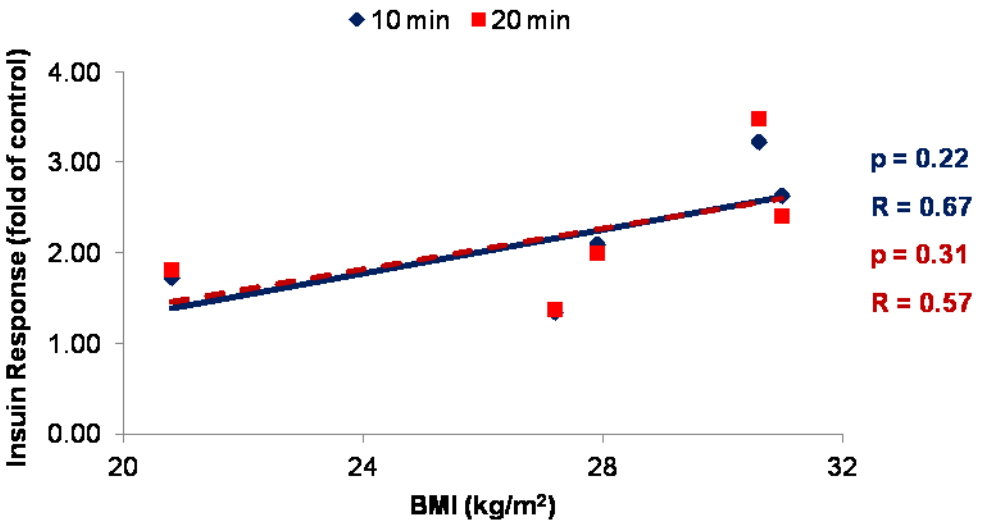
C


10 min
20 min
TSH augmentation of insulin-stimulated glucose uptake (%)
80%
60%
40%
20%
0%
-20%
20
24
28
32
BMI (kg/m²)
p = 0.61
R = 0.31
p = 0.02
R = -0.94

D


10 min
20 min
TSH augmentation of insulin-stimulated glucose uptake (%)
80%
60%
40%
20%
0%
-20%
46
50
54
58
62
66
Age
p = 0.16
R = -0.73
p = 0.98
R = -0.014

E


10 min
20 min
Insuin Response (fold of control)
4.00
3.00
2.00
1.00
0.00
20
24
28
32
BMI (kg/m²)
p = 0.22
R = 0.67
p = 0.31
R = 0.57

F

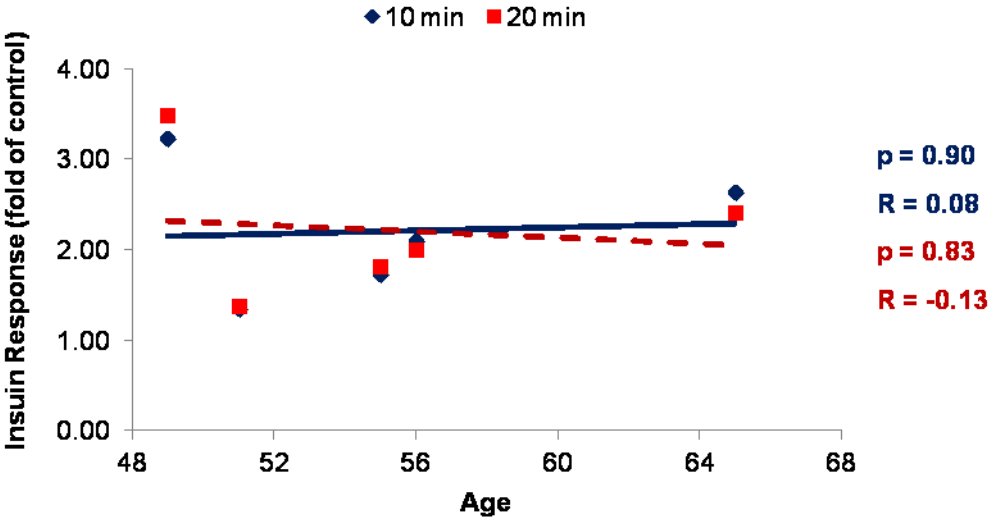

10 min
20 min
Insuin Response (fold of control)
4.00
3.00
2.00
1.00
0.00
48
52
56
60
64
68
Age
p = 0.90
R = 0.08
p = 0.83
R = -0.13

**Figure 8: TSH stimulates adipocyte insulin-stimulated glucose uptake and this stimulation negatively correlates with BMI.** Human differentiated adipocytes were serum-starved for 2 hours in KRH buffer in the presence of 5.6 mM glucose and then pre-treated for 1 hour with 5 mU/ml TSH. Media was removed and the cells were stimulated with 10 nM insulin in KRH buffer for 30 minutes. Subsequently, 2 µCi/ml $^{3}$H-2-deoxy-glucose tracer was added in the presence of 0.2 mM unlabeled 2-deoxy-glucose (2-DG) for 10 or 20 minutes. The cells were lyzed and $^{3}$H incorporation was measured in order to estimate total 2-DG uptake. $^{3}$H counts were expressed as incorporated 2-DG per cellular protein and normalized to the insulin response for 10 minutes (A) and 20 minutes (B) tracer incubations. This normalized insulin response value varied by 9-17% across the individual experiments. % stimulation of insulin-stimulated glucose uptake by TSH was plotted against BMI (C) and age (D). Insulin response was plotted against BMI (E) and age (F) and was expressed as a fold of control. Results are the mean +/- SE of 5 experiments, each measured in duplicate. Each experiment uses a different patient. Statistical analysis was by 2 way ANOVA with Tukey's post-hoc tests for (A) and (B) or Pearson R correlation for (C-F). * $P < 0.05$. ** $P < 0.01$. NS = not significant.

correlation was seen between the insulin response and BMI or age (**Figure 8E and 8F**).

## DISCUSSION

Adipocyte insulin resistance has become an increasingly challenging problem. Insulin resistance in adipocytes can cause the secretion of pro-inflammatory factors that can induce insulin resistance in other organs such as liver and skeletal muscle (Abel et al., 2001). Since high levels of TSH have been shown to correlate with insulin resistance and cardiovascular disease (Mueller et al., 2009), (Rodondi et al., 2010), prevention and treatment of SH has become an important area of discussion. Currently, the molecular mechanism linking insulin resistance and SH is unknown. The goal of this thesis is to elucidate potential adipocyte-related mechanisms that explain this relationship.

### Molecular Signaling

To investigate the link between SH and insulin resistance, we used a preadipocyte-derived adipocyte *in vitro* model. The resulting adipocytes were incubated with high levels of TSH in order to mimic SH conditions, and stimulated with insulin to analyze the insulin signaling pathway. We assessed phosphorylation of Akt, as a measure of insulin signaling, due to its central role in insulin signaling. Specifically, phosphorylation of Akt residue Ser473 was assessed as it is a major marker of Akt activation (Rowland et al., 2011).

We predicted that TSH would inhibit insulin signaling since high TSH levels have been previously correlated with insulin resistance (Mueller et al., 2009). We performed a time course and observed that TSH inhibits insulin-stimulated Akt phosphorylation after a 30 minute treatment. We normalized TSH inhibition to each individual patient's insulin response to Akt phosphorylation in order to reduce inherent patient variability.

We investigated the effect of TSH pre-condition on insulin signaling by treating with TSH for 1 hour prior to insulin stimulation. This approach was designed based on the idea that inhibitors can 'prime' a cell before stimulation. Pre-treating adipocytes with TSH would also mimic a chronic high TSH state similar to SH. Therefore, pre-treating with TSH represents a more relevant model for examining the effect of TSH on insulin signaling/Akt phosphorylation. TSH pre-incubation was able to inhibit insulin-stimulated Akt phosphorylation at 5 minutes, an earlier time point compared to when TSH was added together with insulin (30 minutes).

In order to investigate whether TSH-activated cPKC is the molecular culprit behind the inhibition of insulin-stimulated Akt phosphorylation, we used a molecular inhibitor strategy. To confirm that TSH activates cPKC in our cells, we showed that acute TSH treatment is able to enhance cPKC activity during a 30 minute time course as measured by serine phosphorylation of a cPKC substrate. The most prominent band was chosen as an indicator of cPKC activity.

We pre-treated the cells with Gö6976, a PKCα and PKCβ1 inhibitor, and examined its effect on insulin-stimulated Akt phosphorylation. We showed that this

inhibitor was able to completely restore insulin signaling, suggesting that PKCα and PKCβI are required for inhibition by TSH of insulin-stimulated Akt phosphorylation.

One way to improve our experimental design would be to analyze other parts of the insulin signaling pathway. TSH could be acting upstream of Akt phosphorylation on other components of the pathway including IRS-1 or PI3K. Previous work in adipose tissue has shown that activation of cPKC can inhibit insulin signaling via serine phosphorylation at $Ser^{336}$ of IRS-1 (Liberman et al., 2008). Serine phosphorylation of IRS-1 can inhibit its tyrosine phosphorylation through steric hinderance, thus inhibiting the insulin signal. Furthermore, cPKC activation by GPCR agonists has been shown to inhibit Akt phosphorylation (Klein et al., 1999), (Kimura et al., 2013).

We were unable to determine if cPKC inhibits insulin-stimulated Akt phosphorylation through IRS-1 phosphorylation. No band was seen at the molecular weight of IRS-1 (180 kDa) with the phosphoserine PKC substrate antibody. This observation suggests that either TSH may not be inhibiting Akt phosphorylation through upstream inhibition of IRS-1 tyrosine phosphorylation, or the antibody used is not sensitive enough to detect serine phosphorylation of IRS-1 in our cell model.

We attempted to use a phosphotyrosine antibody to measure tyrosine phosphorylation of IRS-1 and IR in total cell lysates. Insulin-stimulation did not appear to result in increased tyrosine phosphorylation of IR and IRS-1. Since this is a well-known technique (Uchida et al., 2000), (Kanety et al., 1995), (Kanai et al., 1993), the inability to observe this response could be due to technical difficulties with

the assay. An alternative approach could use immunoprecipitation with an IRS-1 antibody to isolate and amplify the tyrosine phosphorylation signal.

A recent study also demonstrated that insulin signaling can be inhibited via cPKC activation of phosphatase and tensin homologue (PTEN) in 3T3-L1 adipocytes (Kimura et al., 2013). PTEN is a phosphoinositide 3-phosphatase that negatively regulates insulin signaling by dephosphorylating insulin-induced PIP3 to PIP2. This reduces Akt activation and GLUT4 translocation to the cell membrane (Rains and Jain, 2011). The Kimura et al. study also showed that cPKC-stimulation and activation of PTEN by a GPCR agonist results in the inhibition of insulin-stimulated Ser473 Akt phosphorylation (Kimura et al., 2013). In this study, PTEN activity was measured via PTEN phosphorylation (Ser380/Thr382/Thr383). The authors proposed that activation of PTEN causes inhibition of PIP3, thus reducing Akt phosphorylation (Kimura et al., 2013). In adipocytes, TSH could potentially reduce insulin signaling either through upstream PKC-mediated activation of PTEN or through IRS-1 serine phosphorylation.

There are some limitations in the studies that I undertook. I used super-physiological levels of TSH to stimulate the adipocytes. However, these doses are used for *in vitro* thyrocyte cultures, an accepted TSH target cell (Back et al., 2013), (Buch et al., 2008), (Brewer et al., 2007). It is not clear what explains the higher dose requirement *in vitro* versus *in vivo* for either thyrocytes or adipocytes.

I only measured Akt phosphorylation at Ser473 as a measure of insulin signaling. While Ser473 Akt phosphorylation is an important juncture in insulin

signaling, other insulin signaling molecules should be analyzed (PI3K activity and IRS-1 tyrosine phosphorylation for example). Akt phosphorylation at Thr308 by PDK1 is also an important part of insulin action and could also be measured. Additionally, Akt activity with an *in vitro* kinase assay could also be measured instead of using phosphorylation of Akt as a marker of Akt activation.

**Lipogenesis**

Following my studies on acute effects of TSH on insulin-signaling (as assessed by Akt phosphorylation), I wanted to determine the effect of TSH on longer-term adipocyte cellular processes. I examined lipogenesis as it is an important insulin-regulated adipocyte response linked to metabolic health (Eissing et al., 2013). Inhibition of insulin-stimulated Akt phosphorylation would be expected to reduce mTORC1 activity, due to relieving PRAS40 and TSC1/2 inhibition (both negative regulators of mTORC1) (Bakan and Laplante, 2012). Blocking mTORC1 activity would lead to inhibition of SREBP-1c, which controls fatty acid synthesis through ACC and FAS (Bakan and Laplante, 2012).

I used radioactively labelled $^{14}$C-glucose in order to measure lipogenesis. Radioactive isotopes are widely used to measure lipogenesis, and are less expensive than using a florescent assay kit (Lofgren et al., 2005), (Campbell et al., 2011), (Lee et al., 2012). The radioactive $^{14}$C-glucose compound used was universally labelled on all six carbon atoms. Therefore, the labelled carbons can be used to trace incorporation into the TG fraction through synthesis of the glycerol backbone or individual FAs.

We stimulated adipocytes with TSH and insulin together for four hours in the presence of tracer. Insulin significantly increased lipogenesis, and the presence of TSH had no effect on insulin-stimulated lipogenesis overall. Further analysis revealed a strong significant correlation between BMI and TSH inhibition of insulin-stimulated lipogenesis.

TSH had a stronger inhibitory effect at lower BMI values, whereas a higher BMI appeared to cause an enhancement in lipogenesis. The mechanism behind this observation is still unknown. No BMI effect on the insulin-stimulated lipogenesis response was observed, so the BMI effect appears to be related to the TSH response. Since BMI does not correlate with TSH inhibition of insulin-stimulated Akt phosphorylation, it may be that the BMI-dependent target is further downstream.

We also analyzed the effect of BMI on adipocyte expression of the TSH receptor. At the receptor level, the effect of BMI on TSHR expression is controversial. High BMI has been associated with lower expression levels of adipocyte TSHR (Nannipieri et al., 2009), while another study reported the opposite (BMI positively correlated with TSHR expression) (Lu et al., 2012). In the patients analyzed for lipogenesis in this thesis, BMI did not correlate with TSHR expression.

In the Nannipieri et al. study, a total of 119 patients were studied (107 obese with type 2 diabetes or impaired glucose tolerance, 12 lean non-diabetic), whereas the Lu et al. studied 120 patients but with no history of diabetes (Nannipieri et al., 2009), (Lu et al., 2012). The adipose tissue in the Nannipieri et al. study was taken from abdominal subcutaneous and visceral adipocyte depots (same observations

regarding TSHR occurred for visceral and subcutaneous depots) (Nannipieri et al., 2009). In the Lu et al. study, subcutaneous adipose tissue from the neck was used (Lu et al., 2012). Differences in adipocyte depot (neck vs. abdominal) and insulin resistance (diabetic vs. non-diabetic) may explain the seemingly conflicting TSHR expression data. Insulin resistance and adipocyte depot may play a significant role in adipocyte TSHR expression. My data are likely more comparable to the Nannipieri et al. study due to using the same adipocyte depot. However, the patients I used were metabolically healthy (non-diabetic).

In rat and 3T3-L1 adipocytes, inhibition of Akt activity with an allosteric inhibitor (Akti) has been shown to result in a reduction in the ability of insulin to stimulate lipogenesis (Berggreen et al., 2009). The authors proposed that insulin-stimulated lipogenesis was regulated through Akt phosphorylation of AMPK (Berggreen et al., 2009). Phosphorylation of AMPK would relieve its inhibition of ACC and promote lipogenesis (Berggreen et al., 2009). Furthermore, inhibition of Akt activity would reduce the activation of SREBP1-c, a key lipogenic transcription factor (Bakan and Laplante, 2012). Presumably, TSH inhibition of insulin-stimulated Akt phosphorylation should result in the inhibition of lipogenesis at all BMI (BMI did not correlate with insulin-stimulated Akt phosphorylation).

However, in my data, TSH inhibition of insulin-stimulated lipogenesis was BMI-dependent at a 4 hour time point. In the Berggreen et al. study, adipocytes were stimulated with insulin for only 30 minutes before measuring lipogenesis (Berggreen et al., 2009). The BMI effect on TSH inhibition of insulin-stimulated lipogenesis may be time-dependent, and may not be seen at 30 minutes. Since a 4

hour time point showed a BMI effect on TSH inhibition of insulin-stimulated lipogenesis, TSH inhibition of Akt phosphorylation may also become BMI-dependent at four hours. Further time points could be analyzed in order to investigate the effect of TSH on insulin-stimulated Akt phosphorylation and lipogenesis at shorter or longer incubation times.

Recent studies have suggested that TSH action on adipocytes is pro-inflammatory (Antunes et al., 2005), (Gagnon et al., 2014). Since adipocyte lipogenesis is associated with a healthy metabolic state (Eissing et al., 2013), the effect of TSH on insulin-stimulated lipogenesis may contribute to this inflammatory phenotype at lower BMIs.

In a single patient with BMI of 40.2 $kg/m^2$, it appeared that TSH enhanced insulin-stimulated lipogenesis. The explanation for this enhancement is unknown. More patients in this BMI range will have to be studied to determine whether this is reliably occurring or not.

**Glucose Uptake**

In addition to lipogenesis, we investigated the effect of TSH on insulin-stimulated glucose uptake. I pre-incubated the cells with TSH for one hour before insulin stimulation. Given the evidence that inhibition of Akt phosphorylation reduces insulin-stimulated adipocyte glucose uptake (Kleiman et al., 2009), I predicted that TSH would inhibit insulin-stimulated glucose uptake. However, TSH actually enhanced insulin-stimulated glucose uptake.

Much of the research investigating insulin signaling and adipocyte glucose uptake uses the 3T3-L1 model. In 3T3-L1 adipocytes, PKCβII has been implicated in insulin-stimulated GLUT4-related glucose uptake. Inhibition of PKCβII has been shown to attenuate insulin-stimulated adipocyte glucose uptake and block GLUT4 translocation to membrane (Kleiman et al., 2009). PKCβII was also shown to activate phosphorylation of Akt at Ser473 and that PKCβII co-localized with mTORC2 (Kleiman et al., 2009). Since phosphorylation of Akt at Ser473 is dependent on mTORC2, the authors propose that PKCβII either associates with mTORC2 for full activation or provides substrate specificity for mTORC2 (Kleiman et al., 2009).

Under this paradigm, TSH stimulation would enhance GLUT4-related insulin-stimulated glucose uptake through Akt activation by PKCβII. However, my data show that TSH inhibits acute insulin-stimulated Akt phosphorylation at 5 minutes with TSH pre-incubation. Since insulin-stimulated glucose uptake was enhanced by TSH at a 50 minute (30 minutes insulin + 20 minutes tracer), but not a 40 minute time point, the effect of TSH on insulin-stimulated Akt phosphorylation and glucose uptake may be time dependent. At a 50 minute insulin stimulation time point, Akt phosphorylation may be enhanced through TSH activation of PKCβII.

In contrast to GLUT4, one possibility is that TSH can enhance GLUT1-related glucose uptake. In thyrocytes, TSH increases GLUT1-related glucose uptake (Hosaka et al., 1992). Furthermore, phorbol myristate acetate (PMA) (a potent activator of cPKC and nPKC) has been shown to increase glucose uptake through an increase in GLUT1 at the plasma membrane in 3T3-L1 adipocytes (Bosch et al.,

2004). Bosch et al. observed that PMA-induced glucose transport was partially attenuated by inhibition of MAPK kinase. Gö6976 was able to relieve this attenuation, which implicates PKCα and/or PKCβI and subsequent MAPK phosphorylation to GLUT1-related glucose transport (Bosch et al., 2004) The authors also showed that PMA-induced glucose uptake resulted in an increase in GLUT1 mRNA and protein levels, whereas no change was observed in GLUT4 expression (Bosch et al., 2004)

TSH alone was not able to cause an increase in glucose uptake in my studies with human differentiated adipocytes. However, it did enhance insulin-stimulated glucose uptake. Insulin may be needed in order to activate additional pathways before any effect of TSH on GLUT1-related glucose uptake can be seen. The identity of these pathways is unknown. A summary of these observations is shown (**Figure 9**).

It is possible that GLUT1-related glucose uptake may occur to compensate for TSH inhibition of Akt phosphorylation. TSH enhancement of GLUT1-related glucose uptake may overcome TSH inhibition of insulin-stimulated Akt phosphorylation (and GLUT4-related glucose uptake). A net increase in insulin-stimulated glucose uptake by TSH would then be observed.

In order to test if GLUT1 is involved with TSH enhancement of insulin-stimulated glucose uptake, TSH-stimulated GLUT1-related uptake can be measured via the use of GLUT1 antibodies in conjunction with subcellular fractionation to isolate the plasma membrane. Protein and mRNA expression of the GLUT1 gene

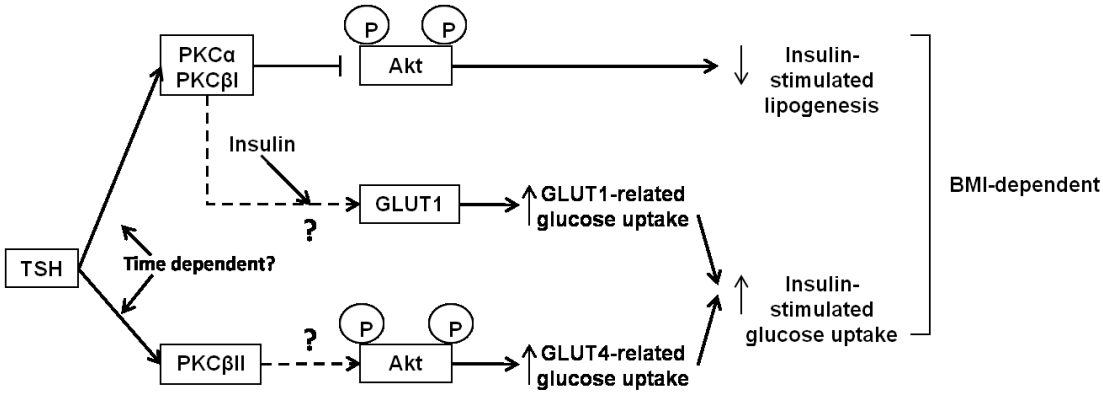

P
P
PKCα
PKCβI
Akt
Insulin-
stimulated
lipogenesis
Insulin
GLUT1
GLUT1-related
glucose uptake
BMI-dependent
?
TSH
Time dependent?
Insulin-
stimulated
glucose uptake
P
P
?
PKCβII
Akt
GLUT4-related
glucose uptake

**Figure 9: Potential effect of TSH on insulin-mediated adipocyte processes.** TSH binding to its receptor causes the stimulation of cPKC. Depending on stimulation time, TSH may either stimulate PKCα/PKCβI or PKCβII, which can lead to inhibition or enhancement of insulin-stimulated Akt phosphorylation respectively. Inhibition of insulin-stimulated Akt phosphorylation leads to a decrease in insulin-stimulated lipogenesis, whereas an enhancement of insulin-stimulated Akt phosphorylation leads to an increase in GLUT4-related glucose uptake. A separate pathway may involve an increase in GLUT1-related glucose uptake via PKCα/PKCβI. This process requires the presence of insulin, which may signal down a pathway separate from Akt phosphorylation. The effect of TSH on insulin-stimulated lipogenesis and glucose uptake appears to be BMI-dependent. Dotted lines represent conjecture based upon the literature, whereas normal lines represent well-established signaling pathways.

(SLC2A1) could also be measured in order to determine if TSH stimulation affects translation and/or transcription of GLUT1.

An inhibitor of GLUT1-related glucose uptake such as STF 31 (Chan et al., 2011) could be used to analyze the mechanism behind TSH enhancement of insulin-stimulated glucose uptake. This inhibitor would block an increase in glucose uptake via GLUT1; therefore, the effect of TSH on GLUT1-related glucose uptake could be ascertained.

A limitation in this study is that I measured overall insulin-stimulated glucose uptake, but did not track differences in GLUT4 and GLUT1 translocation. In theory, TSH could either promote insulin-stimulated GLUT4 translocation to the plasma membrane (through PKCβII) or increase GLUT1 translocation to the plasma membrane in order to enhance insulin-stimulated glucose uptake. In the future, subcellular fractionation could be used to isolate and analyze the low-density microsomes and plasma membrane fractions for GLUT4 and/or GLUT1 (Kondapaka et al., 2004).

In both insulin-stimulated lipogenesis and glucose uptake, the TSH effect on these two processes negatively correlated with BMI. TSH inhibits insulin-stimulated lipogenesis, but enhances insulin-stimulated glucose uptake. TSH enhancement of insulin-stimulated glucose uptake in adipocytes would be expected to increase lipogenesis. However, leaner patients show more TSH inhibition of lipogenesis compared to obese patients; despite the enhancement in glucose uptake by TSH. Without a process for storing the increase in cellular glucose levels, the extra

glucose might be shunted through a different pathway, such as oxidation in order to produce more energy. The mechanism by which adipocytes from leaner individuals are more responsive to TSH for its effects on these metabolic pathways is unknown. Further investigation is needed to precisely understand how BMI affects adipocyte TSH signaling.

**Patient Variability**

In this thesis, only human-derived adipocytes were used. Adipocytes derived from humans instead of cell-lines possess inherent patient variability. Unlike 3T3-L1 adipocytes, these patient-derived adipocytes correspond to individuals/patients who have different BMIs and ages. They are also influenced by their donor's lifestyle and diet.

My data shows that BMI does not correlate with adipocyte insulin response in the acute Akt phosphorylation, lipogenesis or glucose uptake data sets. Unless these data represent a false negative (β2-error), these data suggest that high BMI (obesity) is not correlated with an adipocyte insulin resistant-like state. Since the adipocytes used are differentiated from preadipocytes, the *in vitro* cell model may be more responsive to insulin (non-insulin responsive preadipocytes will not differentiate). A much larger sample size may be needed in order to observe any correlation between BMI and adipocyte insulin resistance.

Generally, aging is considered to be associated with the development of insulin resistance in WAT (Picard and Guarente, 2005). However, age did not

correlate with the acute Akt phosphorylation, lipogenesis or glucose uptake data sets.

## CONCLUSION

My data show that TSH has a significant impact on insulin signaling. In acute stimulation conditions, TSH inhibited insulin-stimulated Akt phosphorylation at Ser473. TSH stimulated cPKC activity, and a PKCα and PKCβI inhibitor prevented TSH inhibition of Ser473 Akt phosphorylation. TSH treatment also resulted in significant changes in adipocyte insulin-controlled cellular responses. TSH inhibited insulin-stimulated lipogenesis in a BMI-dependent manner. In contrast, TSH enhanced adipocyte insulin-stimulated glucose uptake. The negative effect of TSH on lipogenesis and its positive effect on insulin-stimulated glucose uptake were each inversely correlated with BMI

My data support the concept that TSH modulates adipocyte insulin signaling. TSH action on the adipocyte may play a role in the insulin resistance and higher risk of CVD in patients with SH. My data provide an initial framework for more research to help define the link between TSH and adipocyte signaling and function.

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# CURRICULUM VITAE

## Summary of Qualifications

- Master's of Science, Biochemistry
- Hon. B.Sc. with specialization in Biochemistry
- Four years of laboratory experience
- Over five years of management and leadership related experience
- Proficient in Microsoft Word, Excel, Powerpoint software
- Languages: English (fluent), French (working proficiency)

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## Education:

- Masters of Science, Biochemistry (expected) 09/2014
  *University of Ottawa. Ottawa, ON. Canada*

- Honours Bachelor of Science with Spec. in Biochemistry 05/2012
  *Magna Cum Laude* (cGPA: 3.81 on a 4.0 scale)
  *University of Ottawa. Ottawa, ON. Canada*

- Grade 9 Piano with Honours Certificate 10/2009
  *Royal Conservatory of Music, Ottawa, ON. Canada*

- Ottawa-Carleton Secondary School Diploma 06/2008
  Ontario Scholar (Graduating GPA: 91%)
  Arts Certificate in Music
  *Earl of March Secondary School. Ottawa, ON.*

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## Professional Experience:

- Research student 05/2011-09/2014
  *Supervisor: Dr. Alexander Sorisky*
  *Department of Chronic Disease*
  *Ottawa Hospital Research Institute. Ottawa, ON. Canada*
  Designed and conducted experiments towards a scientific project examining the extra-thyroidal effects of TSH on adipocyte insulin action. Working knowledge of immunohistochemistry, radioactive and fluorescent labeling, immunoblotting, protein and lipid determination and Alphaease

imaging software. Cell culture: primary and immortalized cell-lines. Performed literature reviews and data presentations.

- NSERC-funded summer research student 05/2010-08/2010
  *Supervisor: Dr. Daniel Figeys*
  *Department of Biochemistry, Microbiology and Immunology*
  *University of Ottawa. Ottawa, ON. Canada*
  Conducted experiments towards a scientific project examining the regulation of lipin-1 and lipid metabolism. Techniques included immunoblotting, silver nitrate staining, cell fractionation, agarose gel electrophoresis, DNA preparation, polymerase chain reactions (PCR) and restriction digestion. Cell culture: immortalized cell-lines.

- Teaching assistant/Lab demonstrator 01/2013-04/2014
  *University of Ottawa. Ottawa, ON. Canada*
  Supervised and taught students during 6 hr weekly lab session, answered students emails, marked weekly reports, presentations and the course's final project.

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**Awards**

- Admission Scholarship – Masters 09/2012-present
  *Department of Biochemistry, Microbiology and Immunology*
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- University of Ottawa Admission Scholarship 09/2008-04/2012
  *University of Ottawa. Ottawa, ON. Canada*

- Dean's Honour List - University of Ottawa 09/2008-08/2011
  *University of Ottawa. Ottawa, ON. Canada*

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**Abstracts**

- Felske D, Gagnon A, Sorisky A. Nov 2013. Extra-thyroidal effect of TSH on insulin-stimulated lipogenesis and Akt phosphorylation. 13th Annual OHRI Research Day. Ottawa ON, Canada. (Oral presentation)

- Felske D, Gagnon A, Sorisky A. June 2013. TSH Inhibits Insulin-Stimulated Human Adipocyte Lipogenesis in a BMI-Dependent Manner. 73rd Scientific Sessions-American Diabetes Association, Chicago IL, U.S.A. (Poster presentation)
- Felske D, Gagnon A, Sorisky A. November 2012. Extra-thyroidal action of TSH on adipocyte insulin signaling. 12th Annual OHRI Research Day, Ottawa ON, Canada. (Poster presentation)

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**Volunteer Work:**

- Home Church Compassion Coordinator 09/2014-present
  *The Meeting House Church. Ottawa, ON. Canada*

- Let's Talk Science Volunteer (ages 8-11) 09/2012-present
  *University of Ottawa. Ottawa, ON. Canada*

- Kidmax Teacher (ages 3-5 and ages 6-11) 05/2009-09/2014
  *The Meeting House Church. Ottawa, ON. Canada*

- Executive Chapter Officer of the Golden Key 09/2010-04/2011
  *University of Ottawa. Ottawa, ON. Canada*

- Bilingualism Center Group Leader 09/2008-12/2009
  *University of Ottawa. Ottawa, ON. Canada*

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**Extra-Curricular Activities:**

- Wedding Pianist 01/2013-06/2013
  *Kanata, ON. Canada*
- 6-on-6 soccer 05/2013-08/2013
  *Ottawa Sports and Social League. Ottawa, ON.*
- Kanata Street Hockey Club 05/2011-08/2012
  *Kanata, ON. Canada*
- Karate 09/2006-12/2008
  *Steve Anderson Karate School. Kanata, ON. Canada*