MECHANISMS OF GROWTH HORMONE INHIBITION OF ADIPOSE TISSUE GROWTH

LIDAN ZHAO

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Honglin Jiang, Chair

Robert M. Akers

Benjamin A. Corl

David E. Gerrard

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ABSTRACT

Growth hormone (GH) is a poly-peptide hormone produced by the anterior pituitary. Growth hormone not only stimulates body and muscle growth but also inhibits adipose tissue growth. The overall objective of this study was to determine the mechanisms by which GH inhibits adipose tissue growth. Three studies were conducted to achieve this objective. The first study was conducted to determine if GH inhibits fat tissue growth by stimulating lipolysis. In this study, adipose tissue weight and adipocyte size were compared between GH-deficient growth hormone releasing hormone receptor (*Ghrhr*) homozygous mutant mice (i.e., *lit/lit* mice), *lit/+* mice, and *lit/lit* mice injected with GH. *lit/lit* mice had less body weight but more subcutaneous fat and larger adipocytes compared to *lit/+* mice at the same ages. GH treatment to *lit/lit* mice for four weeks partially reversed these differences. These data suggest that GH inhibits adipose tissue growth in mice at least in part by stimulating lipolysis. Additional data from this study suggest that GH indirectly stimulates lipolysis *in vivo* and this indirect mechanism is independent of β adrenergic receptors in the adipose tissue. The second study was conducted to investigate if GH inhibits fat tissue growth also by inhibiting adipogenesis. In this study, stromal vascular fraction (SVF) cells were isolated from subcutaneous fat of *lit/+* and *lit/lit* mice and were induced to differentiate into adipocytes *in vitro*. Oil Red O staining and gene expression analysis revealed that the SVF cells from *lit/lit* mice had

greater adipogenic potential than from *lit/+* mice. This suggests that GH inhibits adipose tissue growth also through inhibition of adipogenesis. Additional data from this study suggest that GH may inhibit adipogenesis by inhibiting the formation of adipogenic precursor cells in adipose tissue in mice. The third study was conducted to determine the role of the central component of GH receptor signaling, STAT5, in GH inhibition of differentiation of bovine preadipocytes. In this study, preadipocytes were isolated from subcutaneous fat of adult cattle and were induced to differentiate with or without GH. Based on Oil Red O staining, gene expression, glycerol-3-phosphate dehydrogenase (G3PDH) activity and acetate incorporation assays, GH inhibited differentiation of bovine preadipocytes into adipocytes. GH induced phosphorylation of STAT5 in differentiating bovine preadipocytes. Overexpression of constitutively active STAT5 through adenovirus mimicked the effect of GH on differentiation of bovine preadipocytes. These data support a role of STAT5 in mediating the inhibitory effect of GH on differentiation of bovine preadipocytes into adipocytes. Overall, GH inhibits adipose tissue by both stimulating lipolysis and inhibiting adipogenesis; GH stimulates lipolysis through an indirect mechanism that is independent of the β adrenergic receptors; GH inhibits adipogenesis through a direct mechanism that may involve the transcription factor STAT5.

Keywords: Adipose tissue, Growth hormone, Adipogenesis, Lipolysis

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INTRODUCTION

White adipose tissue plays a critical role in regulating energy balance. The major function of white adipose tissue is to store energy in the form of triglycerides during excessive energy intake and restore it during negative energy balance. Obesity is the primary disease of adipose tissue. Over the past decades, obesity has become a worldwide health problem (Letonturier, 2007). Excessive body weight increases the risks of cardiovascular disease, type 2 diabetes, hypertension and metabolic abnormalities (Ahmadian et al., 2007). Understanding the molecular mechanisms underlying adipose tissue growth is of prime importance for control of obesity. Adipose tissue in meat production is an energetically expensive tissue to grow. Thus, understanding the molecular mechanisms underlying adipose tissue growth could have significant implications for improving production efficiency in meat animals. Growth hormone (GH) is a polypeptide hormone secreted by the anterior pituitary. It exerts a broad range of physiological roles in development, growth and metabolism. It has been well documented that GH inhibits fat growth in many animal models (Berryman et al., 2010; Maison et al., 2004; Schlegel et al., 2006). The possible ways by which GH inhibits fat tissue growth involve regulating adipogenesis, lipolysis and lipogenesis. However, the cellular and molecular mechanisms by which GH regulates these processes are still unclear. Therefore, the overall goal of the studies reported in this dissertation was to determine how GH regulates adipogenesis and lipolysis at the cellular and molecular levels.

Chapter I

Literature Review

INTRODUCTION

Adipose tissue is a connective tissue scattered throughout the body. Two types of adipose tissue were considered to exist in mammals: white adipose tissue and brown adipose tissue. Most recently, a third type of adipose tissue, beige fat, was found (Ishibashi and Seale, 2010). White adipose used to be considered as an energy storage organ, but it is now considered as also an endocrine and immune tissue (Schaffler and Scholmerich, 2010). The endocrine and immune functions of adipose tissue are achieved by secretion of hormones and cytokines including leptin, adiponectin, visfatin, tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (Kiess et al., 2008; Schaffler and Scholmerich, 2010).

Adipose tissue growth is the result of hypertrophy (increase in cell size) and hyperplasia (increase in cell number) (Hirsch and Batchelor, 1976). In adults, increase in adipose tissue mass usually begins with hypertrophy (Spalding et al., 2008). However, the volume of the individual adipocyte cannot increase infinitely; as a result, new fat cells are developed to meet the increased requirement of energy storage (Spalding et al., 2008).

The molecular mechanisms of formation of new adipocytes or increase in adipocyte size have been studied intensively in adipocyte cell lines or primary adipocytes. A complex and integrated network of signaling pathways modulates the process of adipogenesis, lipolysis and lipogenesis. In this review, the cellular, molecular, and

hormonal mechanisms that mediate adipose growth will be discussed with a focus on the roles of growth hormone (GH) in these processes.

ADIPOSE TISSUE

Adipose tissue composition

Adipose tissue is a special connective tissue that serves as the major form of energy storage. It is scattered throughout the body including under the skin, around internal organs, in the bone marrow and in the mammary gland. Adipose tissue contains not only adipocytes but also stromal vascular fractions, which include macrophages, fibroblasts, pericytes, blood cells, endothelial cells, and adipose precursor cells (Tang et al., 2008). Adipocytes account for 35-70% of total cells in adipose tissue (Ailhaud et al., 1992; Tang et al., 2008).

It was considered that there are two types of fat tissue in mammal: white adipose tissue and brown adipose tissue. The adipocytes from white adipose tissue contain a single large lipid droplet encompassing 90% of the cell volume. As a result, the nucleus and other organelles are pushed to the periphery of the cell. Due to large lipid content, adipose tissue contains less water than the other types of tissues (Hull, 1966). The mature brown fat cells contain multiple lipid droplets and more mitochondria (Hull, 1966) (Figure 1.1). Both white adipose tissue and brown adipose tissue have critical functions at different stages of life. The major function of white adipose tissue is to store energy, whereas, the brown adipose tissue is for dissipating energy through the action of uncoupling protein 1 (UCP-1). Infants have large depots of brown fat located primarily around core organs. The tissue acts to provide heat after exposure in the environment. It

was believed that brown adipose tissue was diminished in adults and had no physiologic relevance (Cannon and Nedergaard, 2004; Cypess et al., 2009). However, recent studies have shown that adults do possess metabolically active brown fat (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009). In rodents most brown fat is located in the interscapular region, and is maintained throughout life (Symonds and Stephenson, 1999). Recently, a type of brown adipocyte-like cells called beige adipocytes were found in white adipose tissue (Ishibashi and Seale, 2010). Beige cells express very low basal level of uncoupling protein-1 (UCP-1), which is similar to white adipocytes. The UCP-1 is highly expressed upon cyclic AMP stimulation in beige cells, which is responsible for thermogenesis (Wu et al., 2012). The origins of beige adipocytes are still unclear, but it has been suggested that beige cells can be generated from progenitor cells by prostaglandin (Vegiopoulos et al., 2010), and they are also converted from white adipocytes (Ishibashi and Seale, 2010). In this study, we focused on the white adipose tissue.

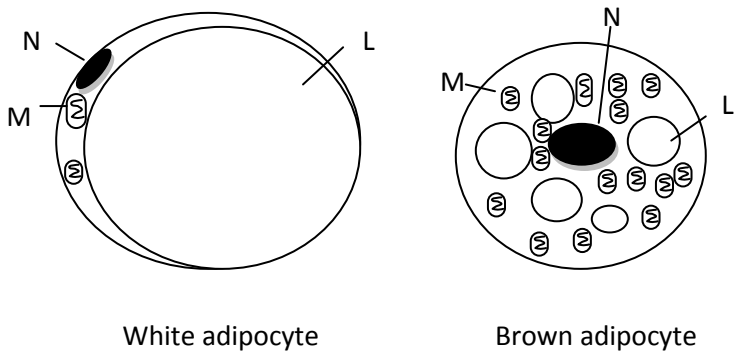

Figure 1.1. Adipocytes from white adipose tissue and brown adipose tissue. The white adipocyte has a large lipid droplet with nucleus and other organelles in the

peripheral region. The brown adipocyte has multiple lipid droplets and numerous mitochondria. L: lipid droplet; M: mitochondria; N: nucleus

Physiological role of adipose tissue

The primary role of white adipose tissue is to store energy as triacylglycerol when excessive energy is fed and consumed. On the other hand, triacylglycerol are broken down from the stored lipid into free fatty acids when energy is needed. Adipose tissue triglyceride is an efficient form of energy storage. One gram of fat can store 9 kcal of energy, which is more than twice the energy stored in carbohydrate or protein (4 kcal/g). An adult with 15 kg of fat can store 110,000 kcal of energy, which could provide 2,000 kcal daily for about two months (Jensen, 1997). The details regarding lipid storage and release will be discussed later. In addition to providing stored energy, adipose tissue also insulates the body and protect vital organs. Subcutaneous fat is directly below the skin and thus prevents heat loss in a cold environment. The visceral fat around the internal organs provides some protection from external forces.

Many studies indicate that fat tissue also plays a crucial role in the control of metabolism through paracrine and endocrine factors. The signaling molecules synthesized by adipocytes are called adipokines, which include hormones, growth factors and cytokines. Leptin is a hormone that is primarily secreted by mature adipocytes and functions in regulating body fat mass. Leptin binds to its receptor in the hypothalamus to inhibit appetite, and lack of leptin leads to uncontrolled food intake and resulting in obesity (Van Harmelen et al., 1998). Adiponectin is another hormone expressed exclusively by mature adipocytes. Decreased adiponectin is found in obesity, which

increases risk for diabetes and cardiovascular disease (Kiess et al., 2008). Visfatin is a more recently identified protein secreted by adipose tissue. It is assumed to bind to the insulin receptor directly and to mimic insulin functions (Fukuhara et al., 2005).

Due to the importance of adipose tissue in immunity, many researchers now define adipose tissue as a part of the innate immune system. First, subcutaneous fat acts as a barrier to prevent the pathogens from entering through the skin. Concentrations of free fatty acids around the fat cells are most likely very high, particularly during lipolysis. This high local fatty acid concentration is toxic to pathogenic bacteria, whereas, adipocytes and preadipocytes are resistant to this cytotoxic effect (Guo et al., 2007). Second, adipose tissue is capable of releasing complement components, cytokines and macrophage chemotactic factors that regulate immune cells. Lipopolysaccharide (LPS) is ofen used in studies to induce inflammation. Stimulation of adipocytes by LPS leads to secretion of proinflammatory factors including tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β) (Schaffler and Scholmerich, 2010). During inflammatory stress, adipose tissue is the major tissue expressing IL-6 (Starr et al., 2009).

Adipose tissue and animal production

In meat animal production, fat is an expensive addition. The lipids in fat tissue store about twice energy as in proteins and carbohydrates. This means that more food intake is required to deposit the same amount of lipids than proteins or carbohydrates. Additionally, because consumers do not like fat, most of the subcutaneous fat is trimmed from the carcasses during processing. Thus, the deposition of fat is costly. In the past

years, animal scientists have worked on genetic selection of lean animals to reduce fat. However, loss of fat causes decreased palatability and flavor of the meat. To a large extent, the flavor of meat comes from marbling or intramuscular fat (IMF) (Schwab et al., 2006). Therefore, researchers are also working to develop dietary additives to improve the marbling score. Conjugated linoleic acids (CLA), as an example, is a group of isomers of linoleic acid, which has been shown to reduce backfat and increase marbling in pigs (Barnes et al., 2011; Wiegand et al., 2001).

Although dietary additives can help to improve intramuscular fat, there is still no efficient way to increase intramuscular fat without also changing the peripheral fat accumulation. The IMF content is positively correlated to backfat thickness (Warriss et al., 1990). During positive energy balance, excess energy is deposited in the peripheral tissues, including muscle, after adipose tissue hypertrophy (Ravussin and Smith, 2002). So the simple way to increase marbling is to increase whole body fat. Thus, it is necessary to understand the process of adipocyte formation and eventually to improve production efficiency.

Adipose tissue growth and control

Adipocyte hyperplasia and hypertrophy are responsible for fat tissue growth (Hirsch and Batchelor, 1976). Hyperplasia refers to the recruitment of multipotent stem cells to the adipocyte lineage and production of new adipocytes during adipogenesis. Hypertrophy refers to the increase of triacylglycerol storage in the existing mature adipocytes, which is the result of lipogenesis and lipolysis.

Adipogenesis

Origin of adipocytes

Adipocytes derive from mesenchymal stem cells (MSC, figure 1.2), which also can be differentiated into myocytes, osteoblasts and chondrocytes depending on the medium conditions (Dicker et al., 2005). Similar cells are also identified later in adipose tissue (Dicker et al., 2005). Adipose tissue derived- and bone marrow derived- MSCs express the similar profiles of cell surface markers: CD13, CD29, CD44, CD90, CD105, SH-3, and STRO-1 (De Ugarte et al., 2003). The adipocytes developed from these two origins display similar lipolysis and secretory functions (Dicker et al., 2005).

Adipogenesis can be divided into two phases (Rosen and MacDougald, 2006), one is called commitment or determination which involves the conversion of mesenchymal stem cells to adipocyte precursor cells, preadipocytes (Figure 1.2). During this process, mesenchymal stem cells become restricted to an adipose lineage. The other phase is the terminal differentiation, during which the preadipocytes turn into mature functional adipocytes (Figure 1.2) Adipocyte differentiation involves many gene expression events, and understanding the underlying mechanisms is an important in understanding fat tissue biology.

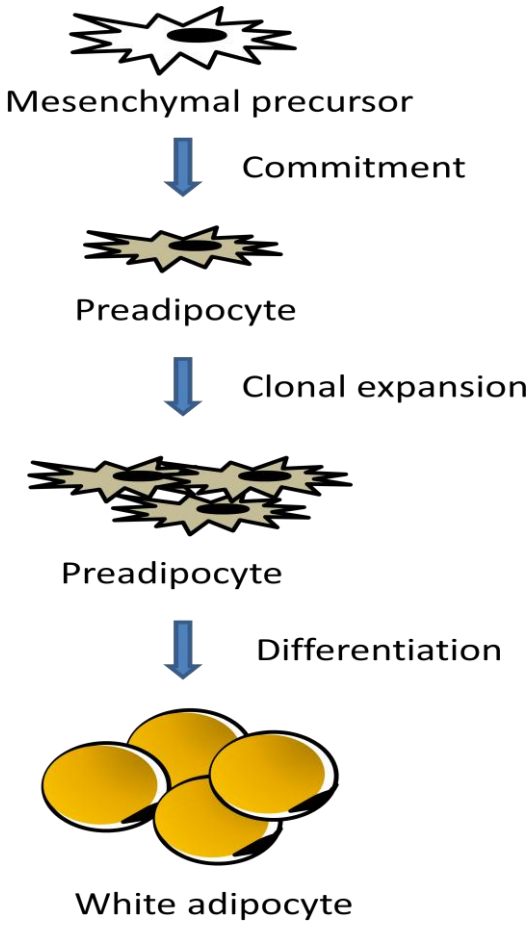

Figure 1.2. Phases of adipogenesis. Preadipocytes arise from mesenchymal stem cells. After committing to the fat cell lineage, preadipocytes proliferate and differentiate into functional adipocytes.

Growth arrest and clonal expansion

Growth arrest of preadipocytes is required for adipocyte differentiation. In cultured cell lines growth arrest is usually achieved after 100% confluency; however, cell-cell contact is not absolutely required for growth arrest. Primary rat preadipocytes plated at low density in serum free medium or 3T3-F442A cells in suspension still undergo differentiation (Gregoire et al., 1998; Pairault and Green, 1979). Two transcription factors, CCAAT enhancer binding protein (C/EBP) α and peroxisome

proliferator activated receptor (PPAR) γ, appear to be involved in the growth arrest. Umek et al. (1991) have shown that expression of C/EBPα in mouse preadipocytes caused a direct cessation of mitotic growth. Antisense C/EBPα prevented growth arrest in 3T3L1 cells (Lin and Lane, 1992). P21/SDI-1, a cell cycle regulator functioning in growth arrest associated with differentiation, is involved in C/EBPα regulating growth arrest (Timchenko et al., 1996). Additionally, activation of PPARγ was sufficient to induce growth arrest in adipogenic cells (Altiok et al., 1997).

Preadipocyte cell lines go through at least one round of DNA replication and cell doubling after growth arrest, and inhibition of DNA synthesis prevents the formation of fat cells (Amri et al., 1986). However, primary preadipocytes from human adipose tissue do not need cell division to enter the differentiation process (Entenmann and Hauner, 1996). These cells may have already undergone cell division *in vivo*, and may correspond to a later stage of adipocytes development.

Differentiation of preadipocytes into adipocytes

Dramatic morphology change occurs during adipocyte differentiation. The fibroblastic preadipocytes change to spherical shape. Adipocyte differentiation involves a set of sequential gene-expression events. Genes related to adipogenesis are highly expressed and preadipocyte genes are silenced during differentiation. Over the past two decades, two transcription factor families, C/EBP and PPAR, have been widely studied. C/EBPβ and C/EBPδ are two specific transcription factors involved in the early stage of adipocyte differentiation. A transient increase in the expression of C/EBPβ and C/EBPδ stimulates the expression of PPARγ (Gregoire et al., 1998), which plays a central role in differentiation. PPARγ then activates C/EBPα, which in turn gives a positive feedback to

PPARγ. The increase of C/EBPα mRNA occurs slightly before the expression of adipocytes-specific genes (Gregoire et al., 1998). Both C/EBPα and PPARγ are involved in maintaining the differentiation.

Transcriptional regulation of adipogenesis

PPARγ

The PPARs were originally identified as nuclear hormone receptors that induced the proliferation of peroxisomes in cells (Dreyer et al., 1992). The PPAR family is composed of PPARα, PPARβ/δ, and PPARγ. PPARs act as transcription factors regulating the expression of genes. Among PPAR family members, PPARγ is highly enriched in adipose tissue and considered as the master regulator of adipogenesis. The PPARγ gene is transcribed from two alternative promoters; as a result, two isoforms are generated: PPARγ1 and PPARγ2 (Zhu et al., 1995). Both isoforms are expressed abundantly in adipose tissue. PPARγ1 is also detected at low levels in liver, spleen, skeletal muscle and heart, and PPARγ2 is almost adipocyte specific (Vidal-Puig et al., 1996).

Like other nuclear hormone receptor family members, PPARγ must heterodimerize with another nuclear hormone receptor called retinoid X receptor (RXR). After dimerization and ligand activation, they bind to the target genes containing specific response elements called PPREs (Fajas et al., 1998). PPARγ binding primarily occurs in distal intergenic regions and introns, and a few sites in proximal promoters (Fajas et al., 1998).

PPARγ functions as a ligand-gated transcription factor. The biological ligands for PPARγ are still unclear. Several metabolites have been found as potential ligands including polyunsaturated fatty acids and eicosanoids, but the affinities are low (Tontonoz and Spiegelman, 2008). 15-deoxy-Δ12, 14 prostaglandin J 2 (15-dPGJ2) is also proposed to be a remarkable activator of PPARγ, but the levels of 15-dPGJ2 *in vivo* are insufficient to be an endogenous ligand (Tontonoz and Spiegelman, 2008). There are synthetic ligands for PPARγ as well. Thiazolidinediones (TZD) are antidiabetic drugs which have been found to increase the PPARγ/RXRα heterodimer binding activity on the adipocyte protein 2 (aP2) promoter (Harris and Kletzien, 1994; Tontonoz et al., 1994a). Currently two members of TZD, rosiglitazone and pioglitazone, are widely used for the treatment of type 2 diabetes.

PPARγ is highly expressed in both white and brown adipose tissue. PPARγ alone can initiate the entire adipogenic program (Tontonoz et al., 1994b). Both *in vivo* and *in vitro* studies have shown that adipose cells cannot form without function of PPARγ. PPARγ null mice died during embryogenesis due to placental dysfunction (Kubota et al., 1999; Rosen et al., 2002). PPARγ-deficient mice generated by the tetraploid-rescue approach failed to form all forms of fat and showed profound hepatic steatosis (Barak et al., 1999). Jones et al. (2005) used the Cre/loxP strategy to generate the adipose-specific PPARγ knockout mice, which displayed abnormal formation and function of both brown and white adipose tissues. The fibroblasts from PPARγ null mice are not capable of undergoing adipogenesis *in vitro*; infecting them with PPARγ expressing retrovirus can rescue the adipogenic potential of those cells (Rosen et al., 2002). In addition, during differentiation of human preadipocytes into mature cells, PPARγ gene expression

increased significantly in the presence of its agonist rosiglitazone; however, preadipocytes failed to differentiate when cells were incubated with PPARγ antagonist (Leyvraz et al., 2010).

Identifying the genes regulated by PPARγ is helpful to understand the importance of PPARγ in adipogenesis. Several studies have attempted to find the PPARγ-regulated genes by microarray (Perera et al., 2006; Sears et al., 2007). Using chromatin immunoprecipitation (ChIP), a genome-wide analysis of PPARγ binding site was conducted in 3T3-L1 adipocytes and 5299 PPARγ binding genomic regions were found (Lefterova et al., 2008). The large number of binding sites in the genome suggests the importance of PPARγ in adipocytes.

C/EBPs

C/EBPs belong to a family of highly conserved basic-leucine zipper proteins. Three C/EBP family members are involved in adipogenesis. C/EBPβ and C/EBPδ are expressed during early stage of adipogenesis (Yeh et al., 1995), and stimulate the expression of PPARγ. C/EBPα plays an important role at the later stages of differentiation and in maintaining the high levels of PPARγ expression. C/EBPα is highly expressed in liver and adipose tissue, and is also detectable in other organs, such as lung, kidney, small intestine, brain and hematopoietic cells (Birkenmeier et al., 1989). Both *in vivo* and *in vitro* models proved the importance of C/EBPs. To investigate the role of C/EBP family members during adipocyte differentiation, C/EBPβ and C/EBPδ deficient mice were generated. About 85% of C/EBPβ and C/EBPδ deficient mice died at the early neonatal stage; for those survived, their epidydimal fat pad was significantly smaller than

that of wild type mice (Tanaka et al., 1997). C/EBPα knockout mice died shortly after birth due to liver hypoglycemia. Transgenic mice expressing C/EBPα only in the liver showed an absence of subcutaneous, perirenal, and epididymal white fat, but mammary gland fat and BAT were present in those mice (Linhart et al., 2001). Failure to develop WAT was also observed in the mice expressing the dominant-negative leucine zipper containing protein, in which all the C/EBP family members and all other leucine zipper proteins cannot bind to their target genes (Reitman et al., 1998). All these *in vivo* data show that C/EBPs are required during adipogenesis.

Additionally, embryonic fibroblasts from mice lacking both C/EBPβ and C/EBPδ fail to differentiate into adipocytes (Tanaka et al., 1997). Other than expression at early stage of differentiation, C/EBPβ was also found to occupy C/EBP sites in mature adipocytes and required for adipocyte gene expression (Lefterova et al., 2008). 3T3-L1 and 3T3-F442A are two widely used cell lines in adipogenesis research. Blocking C/EBPα expression through the introduction of antisense RNA into 3T3-L1 cells suppressed the expression of adipocyte genes and also prevented the triglyceride accumulation during differentiation (Lin and Lane, 1992). Stable transfection of 3T3-L1 cells with C/EBPα gene induced the expression of several endogenous adipocyte-specific genes and lipid accumulation (Lin and Lane, 1994). These studies indicate that C/EBPα is necessary for 3T3-L1 preadipocytes to differentiate into adipocytes. However, C/EBPα has no ability to promote adipogenesis without PPARγ (Rosen et al., 2002).

In general, C/EBPβ and C/EBPδ induce low levels of PPARγ and C/EBPα, and then PPARγ and C/EBPα stimulate each other's expression to maintain the differentiation. Therefore, PPARγ and C/EBPα play a central role in differentiation, and cross-regulation

between PPARγ and C/EBPα is a key component of the transcriptional control of adipogenesis. Lack of PPARγ expression reduced the expression of C/EBPα, and C/EBPα deficient cells expressed reduced levels of PPARγ (Wu et al., 1999). The majority of adipocyte genes are not only regulated by PPARγ but also by C/EBPs. A genome-wide ChIP-chip experiment demonstrated the cooperation between PPARγ and C/EBPα. They found that most genes up-regulated in adipogenesis have both PPARγ and C/EBPα binding sites within 50 kb of the transcription starting site, only a few are PPARγ-specific (Lefterova et al., 2008). The precise mechanism by which PPARγ and C/EBPα cooperate remains to be elucidated.

Krüppel-like transcription factors (KLFs)

Krüppel-like transcription factors (KLFs) are a group of zinc-finger transcription factors implicated in many biological processes, including proliferation, differentiation and development. KLFs bind to CACCC elements and GC-rich regions of DNA to regulate the transcription. Seventeen members of the KLF family have been discovered in mammalian cells. Recent studies suggest a potential role for some KLFs in adipogenesis.

KLF2 is a negative regulator of adipocyte differentiation, which is highly expressed in adipose tissue. Results from both primary cells and cell lines showed that KLF2 was expressed in preadipocytes but not mature adipocytes (Banerjee et al., 2003). Overexpression of KLF2 in 3T3-L1 cells inhibited differentiation, and this inhibitory effect was caused by down-regulating the PPARγ expression and direct repression of PPARγ2 promoter activity (Banerjee et al., 2003). KLF5 is induced at the early stage of adipocyte differentiation by C/EBPβ and δ; the increase of KLF5 mRNA expression

could be detected 1 h after induction of differentiation and reached a maximum within 3 h (Oishi et al., 2005). $KLF5^{+/-}$ embryonic fibroblasts exhibited less capacity of differentiation than fibroblasts from wild type mice (Oishi et al., 2005). KLF15 is another member that serves as an inducer of adipocyte differentiation. Overexpression of KLF15 induces lipid accumulation, PPARγ expression and glucose transporter 4 (GLUT4) expression. RNAi-mediated depletion of KLF15 in 3T3-L1 cells resulted in inhibition of adipogenesis (Gray et al., 2002; Mori et al., 2005). KLF15 can act synergistically with C/EBPα to increase the activity of the PPARγ2 promoter in 3T3-L1 adipocytes (Mori et al., 2005).

Signal transducers and activators of transcription (STAT)

Signal transducers and activators of transcription (STAT) proteins are transcription factors activated by numerous cytokines and growth factors, such as interleukins, growth hormone (GH) and prolactin (PRL). Seven STAT family members have been identified so far, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT5a was first known as Mammary Gland Factor (MGF) because of its role in promoting prolactin-induced transcription of milk protein genes in mammary epithelium (Schmitt-Ney et al., 1992). It was renamed STAT5 due to its similarity in sequence to others of the STAT family (Groner and Gouilleux, 1995). In the following years, two types of STAT5 were identified, called STAT5a and STAT5b (Azam et al., 1997; Liu et al., 1995). The transcription factors STAT5a and STAT5b are encoded by two separate genes but are more than 90% identical in amino acid sequence. The STAT5 proteins are involved in a variety of signaling pathways in hepatocytes, mammary epithelial cells, and the immune cells. All STAT family members are highly

conserved in the molecular structure and contain several domains. The N terminal domain is followed by a α-helical coiled-coil domain, in which 4 α-helixes are coiled together. The coiled-coil domain is followed by a β-barrel domain and a linker domain, which connects to the C terminus. The N terminus mediates cooperative DNA binding (Xu et al., 1996), and the coiled-coil domain is critical for nuclear import (Iyer and Reich, 2008). The C terminus contains a Src homology 2 (SH2) domain and a transactivation domain. All STATs have a phosphotyrosine residue located within 30 amino acids of the C-terminal to the SH2 domain, which is critical for phosphorylation by Janus kinases (JAKs). The transactivation domain is the most divergent part in the STAT family. The unphosphorylated STAT5 forms antiparallel dimers in cytoplasm through hydrophobic interaction of the 4 α-helixes as well as the β-barrel domains (Neculai et al., 2005). Activation of STAT5 induces rearrangement and generation of phosphorylated dimers formed through interaction of the SH2 domains (Hennighausen and Robinson, 2008).

The modulation and function of STAT5 during adipogenesis and in mature adipocytes have been studied by several groups in the past decades. The first study on STATs expression in 3T3L1 cells showed that STAT1, 3, and 5 were markedly elevated during differentiation, but only STAT5 expression was highly correlated with adipocyte phenotype (Stephens et al., 1996). Increased expression of STAT3 and STAT5 were also found in human differentiating preadipocytes (Harp et al., 2001). The expression of STAT5 protein in 3T3L1 cells was highly correlated with the expression of C/EBPα and PPARγ (Stewart et al., 1999). However ectopic expression of STAT5a has been shown to inhibit adipogenesis in 3T3L1 cells (Nanbu-Wakao et al., 2002). Fetal bovine serum (FBS) is an important component of differentiation in 3T3L1 cells; using an activator of

STAT5 protein can replace the requirement of FBS in adipogenesis (Stewart et al., 2004). Therefore, STAT5a and STAT5b are sufficient to induce the expression of early adipogenic markers in nonprecursor cells (Floyd and Stephens, 2003). 3T3-F442A cells are a preadipocyte cell line that can be induced to differentiate in the serum-free medium which includes GH, insulin and epidermal growth factor (EGF) as the essential components of differentiation. In these cells, GH-dependent differentiation requires JAK2/STAT5 activation, and blocking STAT5 activation severely attenuated the ability of GH to promote differentiation (Yarwood et al., 1999). Also, constitutively active STAT5a was able to replace GH to stimulate adipogenesis in 3T3-F442A preadipocytes (Shang and Waters, 2003). All these studies suggest that STAT5 is involved in adipocyte development.

Hormonal regulation of adipogenesis

The differentiation of preadipocytes can be successfully induced *in vitro*. Insulin is one of the inducers essential for differentiation. However, preadipocytes express low levels of insulin receptors, and insulin functions through insulin-like growth factor-1 (IGF-1) receptor signaling in the early stages of adipogenesis. Differentiation results in increased expression of insulin receptors (Rubin et al., 1978). Insulin receptor knockout mice had low fat mass indicating the stimulatory effect of insulin (Bluher et al., 2002). Insulin modulated differentiation involves the expression of various proteins related to lipid accumulation, such as GLUT4 and fatty-acid-synthesizing enzymes.

Insulin-like growth factor is required by adipogenesis. It has been reported that IGF-1 stimulated adipogenesis in rat, rabbit, and porcine preadipocytes (Deslex et al., 1987; Nougues et al., 1993; Ramsay et al., 1989). In mouse preadipocyte Ob1771 cells,

autocrine or paracrine IGF-1 could induce differentiation (Kamai et al., 1996). Similar results were also found in 3T3L1 preadipocyte cell lines (Schmidt et al., 1990).

The downstream signaling of insulin/IGF-1 is important for adipogenesis. Insulin receptor substrates (IRS) are a group of proteins phosphorylated by insulin receptor kinase. Deficiency of both IRS-1 and IRS-3 in mice caused severe lipotrophy (Laustsen et al., 2002). Among insulin signaling, PKB/AKT has been found to be pivotal in 3T3-L1 adipocyte differentiation (Xu and Liao, 2004). AKT-activated mammalian target of rapamycin complex 1 (mTORC1) caused increase of mRNA and protein expression of PPARγ (Zhang et al., 2009).

Glucocorticoid is a steroid hormone secreted by the adrenal cortex in response to stress. It has diverse effects on immunity, metabolism, and fetal development. Glucocorticoid was found to be both adipogenic and lipolytic in adipose tissue (Campbell et al., 2011). Deletion of dexamethasone, a synthetic glucocorticoid, from differentiation medium resulted in a lack of adipose conversion in rabbit and human preadipocytes (Nougues et al., 1993; Zilberfarb et al., 2001). During early stage of differentiation, dexamethasone stimulates CEBPα and PPARγ expression in human adipocytes indicating the importance of dexamethasone in differentiation.

Prenatal development

Adipogenesis occurs both in prenatal and postnatal stages. In mice and rats, WAT is not visible during embryonic development and at birth, whereas in humans, rabbits, guinea pigs and pigs both BAT and WAT could be macroscopically detected at birth (Ailhaud et al., 1992). In humans, the key period in adipogenesis is the second trimester.

Primitive organs of WAT in humans can be found in various sites including buccal, neck, shoulder, gluteal, and perirenal area (Ailhaud et al., 1992). Initially, mesenchymal cells deposit in those area with the network of capillaries, then ovoid bodies composed of fat cells containing small lipid droplets are formed (Hausman et al., 1980). Finally, the size of the fat cell clusters is increased. Besides lipid-containing cells, there are also lipid-free cells which can accumulate fat after birth (Kiess et al., 2008). During adipose tissue development, angiogenesis occurs at the same time and coordinates with fat cell formation. Many angiogenic and adipogenic factors including zinc α2-glycoprotein (ZAG), leptin, adiponectin, and IGF-1 are considered to regulate the development of both capillaries and adipocyte cluster (Kiess et al., 2008).

It has been shown that nutrition affects fetal adipose tissue development. In sheep, glucose infusion to fetus increased body fat depot (Stevens et al., 1990). Increased maternal nutrition during late pregnancy also increased fat mass of offspring (Muhlhausler et al., 2007). This increased fat mass may be related to an increase in the expression of PPARγ in fetal fat. Similar result was also found in rats. Pups born to mothers fed high fat and high sugar diet during gestation accumulated more intramuscular fat with adipocyte hypertrophy (Bayol et al., 2005). So changes in nutrient availability could alter rates of adipogenesis.

Lipolysis

Lipid droplets

Lipids are stored in the fat cells as triglyceride (TAG) which contains 3 mol of fatty acid and 1 mol of glycerol. The unilocular cytosolic lipid droplets are surrounded by

a phospholipid monolayer and coated with many proteins. The most abundant coating proteins are PAT family proteins which are named after perilipin, adipophilin, and the tail-interacting protein of 47 kDa (TIP47). TIP47 is found to coat small lipid droplets, and adipopholin and perilipin are found on larger droplets (Wolins et al., 2005). Perilipin is one of the best-studied proteins coating on lipid droplets and is essential for lipolysis. Non-phosphorylated perilipin suppressed lipolysis by blocking access of lipases to the TAG. During negative energy balance, perilipins are highly phosphorylated by PKA upon adrenergic stimulation, and therefore, attract hormone sensitive lipase to the lipid droplets (Sztalryd et al., 2003). Isolated adipocytes from perilipin-null mice showed increased basal lipolysis because of absence of protective coating provided by perilipin, meanwhile, they also exhibited attenuated lipolytic activity upon isoproterenol stimulation (Tansey et al., 2001). Adipocytes express two types of perilipin, A and B. Perilipin A is required by hormone sensitive lipase (HSL) translocation during PKA-activated lipolysis (Sztalryd et al., 2003). In addition to PAT proteins, some other cellular proteins were also found in lipid droplet fractions. Caveolin, for example, has been shown to localize to lipid droplets and regulate lipolysis (Cohen et al., 2004).

The process and key enzymes of lipolysis

Lipolysis is the step-wise process catalyzed by specific enzymes to cleave one ester bond at a time and released free fatty acid at each step with diglycerol, monoglycerol and glycerol (Figure 1.3). During energy shortage, free fatty acid released from adipocytes can be transported to other organs for β-oxidation, and therefore, to generate ATP. Free fatty acid and glycerol can be utilized by the liver for ketogenesis and gluconeogenesis, respectively. One of the key enzymes involved in lipolysis is HSL.

Catecholamine and insulin are two major hormones regulating HSL. HSL hydrolyzes a variety of substrates including triglyceride and diglyceride; the hydrolase activity of HSL towards diglyceride is much higher than the other (Duncan et al., 2007). HSL was considered to be the rate-limiting enzyme in triglyceride hydrolysis. The function of HSL was studied in HSL-null mice (Haemmerle et al., 2002; Wang et al., 2001). They found that HSL-null mice have similar body weight but reduced abdominal fat compared with wild type mice, and adrenergic-stimulated release of free fatty acids was decreased both *in vivo* and *in vitro*. Additionally, diglycerides, instead of triglycerides, were accumulated in adipocytes of HSL deficient mice. However, HSL-null mice had normal basal lipolysis suggesting additional lipolytic pathways in adipocytes.

In 2004, another triglyceride lipase named adipocyte triglyceride lipase (ATGL) or desnutrin was identified by several groups (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004). The ATGL is a triglyceride-specific lipase expressed predominantly in adipose tissue (Zimmermann et al., 2004). It is responsible for the first step of lipolysis. siRNA-mediated knockdown of ATGL in 3T3-L1 adipocytes decreased both basal and stimulated lipolysis (Kershaw et al., 2006). ATGL-knockout mice exhibited impaired lipolysis and massive accumulation of triglycerides in adipose tissue and muscle (Schoiswohl et al., 2010). On the other hand, transgenic mice overexpressing ATGL showed increased lipolysis and fatty acid oxidation in adipocytes, as well as reduced adipocyte triglyceride content and attenuation of diet-induced obesity (Ahmadian et al., 2009). All of these results support that ATGL plays an important role in triglyceride lipolysis. Additionally, ATGL is found to play a predominant role in basal lipolysis, but not catecholamine-induced lipolysis (Ryden et al., 2007).

The last step of lipolysis is catalyzed by monoglyceride lipase (MGL), which is the major function of MGL. Few studies were related to MGL, because it is not the rate-limiting enzyme. Removal of MGL by immunoprecepitation significantly decreased glycerol release and increased monoglyceride accumulation (Fredrikson et al., 1986). Another role of MGL is to hydrolyze 2-monoglyceride generated by lipoprotein lipase (LPL) (Tornqvist et al., 1978).

Hormonal control of lipolysis

The development of mature adipose tissue is triggered by ubiquitous angiogenic and adipogenic factors. Dynamic changes of adipose tissue are regulated by various hormones (Christiaens and Lijnen, 2009). Several major hormones, catecholamine, insulin, glycocorticoid and growth hormone, have been described in this review.

Catecholamines are the major hormones stimulating lipolysis (Arner, 1999; Jocken and Blaak, 2008). During negative energy balance, catecholamines are increased by the sympathetic nervous system. The processes in which a catecholamine regulates lipolysis begins with binding to its receptors. There are two types of adrenergic receptors: α receptor (including α1 and α2) and β receptor (including β1, β2, and β3). Catecholamine binds to both α and β receptors. Adrenergic receptors are G protein-coupled receptors controlling intracellular cyclic AMP (cAMP). The β-receptor is coupled to stimulatory G protein (Gs) and increases the cAMP level, which then activates protein kinase A (PKA), which phosphorylates HSL and perilipin. As a result, lipolysis is stimulated by catecholamine. Whereas, the α_2 receptor is antilipolytic due to coupling to inhibitory G protein (Gi) and decreases cAMP production. In human fat cells, the

lipolysis is mainly regulated by β_1- and β_2-receptors, but in rodent adipocytes it is mainly regulated by β_3-adrenergic receptors (Jocken and Blaak, 2008).

Insulin is an anti-lipolytic factor. The anti-lipolytic effect of insulin is also involved in the β-adrenergic receptors system (Jocken and Blaak, 2008). As described above, catecholamine activates the cAMP/PKA pathway to phosphorylate HSL and perilipin. Insulin has the opposite effect by inhibiting cAMP/PKA pathway. By binding to its receptor, insulin receptor substrate (IRS) is phosphorylated, which activates p85 of PI3K, then activates the Akt/PKB pathway (Kido et al., 2001). PKB induces the activation of phosphodiesterase, an enzyme changing cAMP to AMP. Consequently, the phosphorylation of HSL and perilipin is inhibited, therefore inhibits lipolysis. The importance of insulin-induced phosphodiesterase during lipolysis was demonstrated in cyclic nucleotide phosphodiesterase 3B-knockout mice (Choi et al., 2006). They found that phosphodiesterase 3B-knockout mice released more free fatty acids in the blood after stimulated by isoproterenol.

Glucocorticoid has been shown to increase adipose tissue lipolysis. Glucocorticoid treatment resulted in increases of circulating free fatty acids in humans and rodents (Djurhuus et al., 2002; Severino et al., 2002). The elevated lipolysis level by dexamethasone is associated with increased gene expression of HSL and ATGL (Campbell et al., 2011; Xu et al., 2009). As well as, dexamethasone induced phosphorylation and down-regulation of perilipin (Xu et al., 2009).

Nutritional control of lipolysis

Lipolysis is influenced by nutrient availability. Fasting or energy restriction acutely stimulates lipolysis. Food deprivation in rats for 18 hours increased basal lipolysis in isolated fat cell (Szkudelski et al., 2004). Fasting increased serum free fatty acid levels in humans (Salgin et al., 2009). The lipolysis stimulated by fasting may be associated with altering hormones. Catecholamine is the primary regulator responsible for fasting-induced lipolysis. Plasma epinephrine concentrations were elevated during fasting, which enhanced fasting-stimulated lipolysis (Vernon, 1992). By blocking the β-adrenergic receptors, the release in serum free fatty acids and glycerol was prevented (Vernon, 1992). Fasting increased fat tissue sensitivity to the catecholamine both *in vivo* and *in vitro* (Vernon, 1992). As a result, the activity of the lipolytic enzymes was increased.

It has been well known that serum insulin falls and serum glucagon rises during fasting, which facilitates lipolysis. Glucagon stimulates lipolysis, although the effect is weak compared to catecholamine. In isolated adipocytes from humans and rats, glucagon stimulates glycerol release in a dose dependent manner (Heckemeyer et al., 1983; Perea et al., 1995). The effect of glucagon on lipolysis may be mediated by direct activation of its receptors followed by an increase in adenylyl cyclase activity, resulting in cAMP increase (Duncan et al., 2007; Perea et al., 1995). On the other hand, refeeding induces an increase of insulin and a decrease of glucagon and epinephrine (Vernon, 1992). Therefore, re-feeding fasted animals attenuates fat tissue lipolysis.

Lipogenesis

The process and key enzymes of lipogenesis

Lipogenesis is the process whereby fatty acids are synthesized and esterified with glycerol to form the triglycerides (Figure 1.3). It was initially believed that liver was the organ responsible for lipogenesis from glucose. However, later studies have shown that adipose tissue itself was capable of synthesizing fatty acids (Chascione et al., 1987; Guo et al., 2000). Free fatty acids and glycerol are building blocks of triglyceride. During positive energy balance, triglyceride is synthesized in the endoplasmic reticulum (ER) by lipogenic enzymes. Free fatty acids are from either extracellular uptake or intracellular *de novo* synthesis. The backbone of the triglyceride, glycerol-3-phospahte, is derived from glycolysis or glyceroneogenesis.

Dietary triglyceride is packaged into chylomicrons by intestine and liver. Synthesized triglyceride is secreted as very low-density lipoprotein (VLDL). Chylomicrons and VLDL circulate in the body to provide fatty acids to other tissues including fat tissue. Lipoprotein lipase (LPL) is the enzyme that catalyzes the hydrolyzation of triglyceride in VLDL and chylomicrons, therefore provides free fatty acids for uptake by peripheral tissue (Shi and Burn, 2004). LPL is a member of lipase gene family that includes hepatic lipase, pancreatic lipase, and endothelial lipase (Wong and Schotz, 2002). In adipose tissue, LPL is synthesized in the adipocyte and transported to the luminal surface of vascular endothelial cells (Musliner et al., 1977).

In non-ruminant animals and humans, fatty acid synthesis in adipocytes starts with glucose, but in ruminant animals, acetate is the predominant carbon source for lipogenesis (Chascione et al., 1987; Hanson and Ballard, 1967; O'Hea and Leveille, 1969). Either glucose or acetate changes into acetyl-CoA, followed by malonyl-CoA

catalyzed by Acetyl-CoA carboxylase (Kersten, 2001). Fatty acid synthase (FAS) catalyzes the *de novo* synthesis of saturated fatty acid from acetyl-CoA and malonyl-CoA. Intracellular free fatty acids first need to convert to their acyl-CoA derivatives. This reaction is catalyzed by the acyl-CoA synthase. Then acyl-CoA is used as a substrate by two parallel triglyceride synthetic pathways in the endoplasmic reticulum (Shi and Burn, 2004).

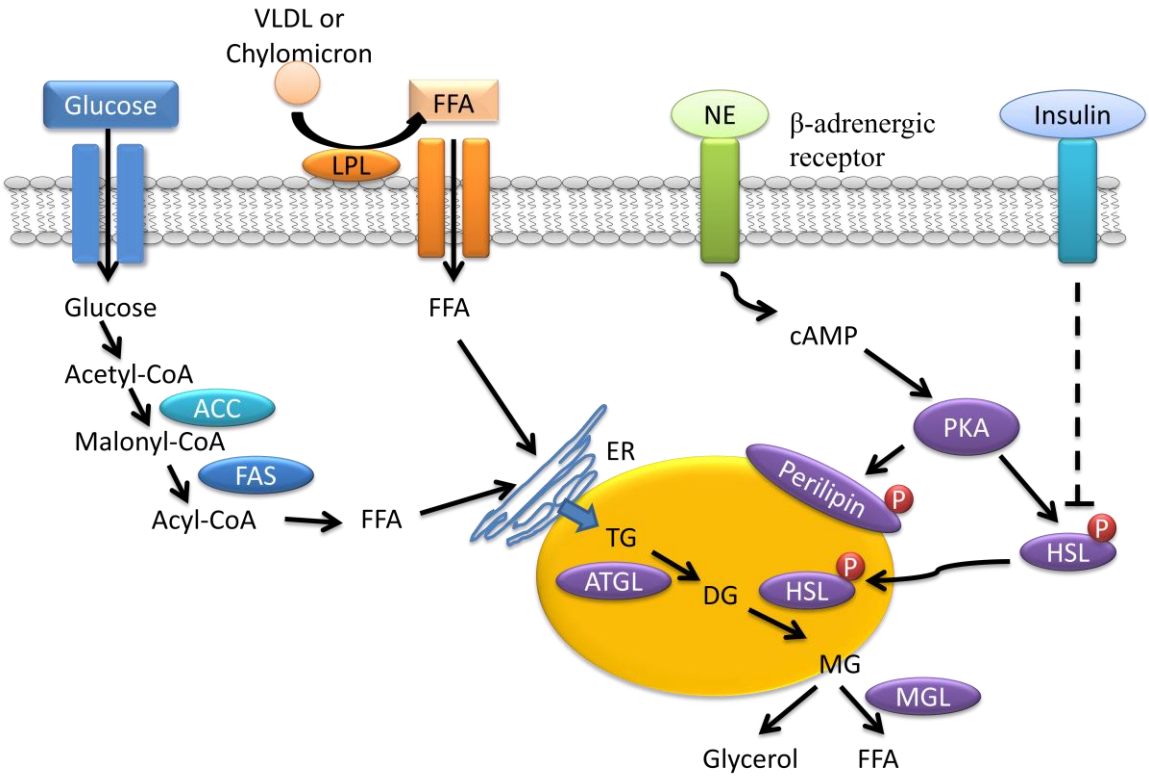

Figure 1.3 Lipolysis and lipogenesis in white adipocytes. Lipid droplets are coated by PAT family proteins, mainly perilipin. The rate of lipolysis is regulated by endocrine factors including catecholamines (epinephrine and nonepinephrine, NE) and insulin, which regulates the phosphorylation of perilipin and hormone sensitive lipase (HSL). The phosphorylated perilipin allows HSL to access lipids and hydrolyze the triglyceride (TG). Free fatty acids (FFA) for lipogenesis are released from chylomicron or very low density lipoprotein (VLDL) by lipoprotein lipase (LPL). Alternatively, FFA could be synthesized by acyl-CoA from glycolysis. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthesis; ATGL, adipose triglyceride lipase; MGL, monoglyceride lipase; PKA, protein kinase A.

Hormonal control of lipogenesis

Insulin has both antilipolitic and lipogenic effects on adipose tissue. Mechanisms of insulin stimulating lipogenesis include increasing the uptake of glucose in the adipocytes and activating lipogenic and glycolytic enzymes (Kersten, 2001). Glucose transporter 4 (GLUT4) is an insulin responsive transporter expressed mainly in muscle and adipose tissue (Gould and Holman, 1993). The translocation of GLUT4 from intracellular storage vesicles to the plasma membrane is mediated by the insulin signaling. One of the Akt substrates, AS160, connects insulin signaling and GLUT4 translocation (Rowland et al., 2011). AS160 confines GLUT4 within the cell. After insulin binding to its receptor, AS160 is phosphorylated by Akt and recruits protein 14-3-3. Then AS160-GAP is replaced by AS160-GTP and this activates Rab protein on GLUT4 storage vesicles (GSV). As a result, GSVs translocate to and fuse with the plasma membrane (Rowland et al., 2011). In addition, insulin increases both activity and expression of lipogenic enzymes FAS and ACC in rat adipose tissue (Assimacopoulosjeannet et al., 1995). LPL located on the cell surface of the vascular endothelium is also activated by insulin, therefore, fatty acids are released from chylomicron and VLDL and taken by adipocytes. (Shi and Burn, 2004). Insulin has been shown to stimulate the gene expression of sterol regulatory element binding protein-1 (SREBP-1) in adipocytes (Kim et al., 1998), which as a transcription factor induces lipogenic gene expression.

Another major hormone involved in lipogenesis is leptin. Leptin controls fat tissue growth by inhibiting food intake and nutrient partitioning. The latter effect is achieved by promoting lipid oxidation and protein synthesis and by inhibiting lipogenesis. The inhibitory effect of leptin on lipogenesis is mediated through down-regulation of the

expression of genes related to fatty acid and triglyceride synthesis. Leptin has been found to significantly inhibit mRNA expression of FAS in rats and up-regulate mRNA expression of PPARα, a fatty acid oxidation related transcription factor (Wang et al., 1999). Additionally, central infusion of leptin suppressed the uptake of circulating free fatty acid into adipose tissue (Buettner et al., 2008).

Nutritional control of lipogenesis

High carbohydrate diets stimulate lipogenesis both in adipose tissue and liver. On high carbohydrate diets, the rate of fatty acid synthesis in human adipose tissue increases by 80 fold and the rate of triglyceride synthesis increases by 3 fold compared to on the low carbohydrate diet (Chascione et al., 1987). High carbohydrate intake increases plasma glucose, which is a substrate for lipogenesis. In rats, the rate of lipogenesis is responsive to high glucose (Fukuda et al., 1999). In hepatocytes, glucose stimulates the transpription of FAS (Hillgartner and Charron, 1998; Semenkovich et al., 1993). Induction of lipogenic gene expression by glucose depends on the transcriptional factor SREBP-1. It has been reported that SREBP-1 promoter is responsive to glucose and SREBP-1 mRNA expression was up-regulated by glucose (Hasty et al., 2000). Additionally, re-feeding a high carbohydrate diet to fasted mice significantly increases nuclear SREBP-1 in hepatocytes (Horton et al., 1998).

During fasting, lipogenesis is suppressed in adipose tissue while lipolysis is increased. However, in the liver, the lipogenesis is increased due to the presence of large amounts of fatty acids from adipose tissue (Kersten, 2001). Post-transcriptional LPL activity is extensively regulated by fasting and refeeding. Fasting causes a decrease in LPL activity, whereas feeding results in an increase in its activity in adipose tissue

(Lithell et al., 1978). Both LPL activity and LPL mass decreased during an overnight fasting, and the mass ratio between inactive and active LPL increased by more than four times (Bergo et al., 1996). The decrease in LPL activity was recently shown to be regulated by angiopoietin-like protein 4 (Angptl-4), since the expression of Angptl-4 increased during fasting (Yoon et al., 2000). The coiled-coil domain of Angptl-4 interacts with LPL to catalyze a conformation change from active dimers to catalytically inactive monomers (Sukonina et al., 2006). When rats were fasted, Angpl-4 mRNA expression increased accompanied by decreased LPL activity and vice versa (Sukonina et al., 2006). The result indicates that Angpl-4 acts as a prime controller of LPL activity during fasting and feeding.

GROWTH HORMONE AND FAT TISSUE GRWOTH

Growth hormone (GH) is a poly-peptide hormone produced primarily by the anterior pituitary. It is the major hormone not only controlling growth but also exerting profound effects on body composition. High levels of GH are usually found during adolescence. It promotes the growth of tissue and bone, protein deposition and the breakdown of fat.

GH structure, production and function

Several isoforms of GH are generated by both posttranscriptional and posttranslational modifications. In the human and the bovine, the major form of GH is a single chain polypeptide containing 191 amino acids with the molecular weight of 22 kDa and two disulfide bonds. The 20 kDa GH is the second most abundant form of GH

which accounts for about 10% of total GH in the pituitary (Lewis et al., 1978). This form arises from alternative splicing within exon 3 of the GH gene and the protein is missing the residues 32-46 of 22 kDa GH (Cooke et al., 1988). A higher molecular weight (about 24 kDa) form was also found in the human blood after exercise (Kohler et al., 2008).

Secretion of GH is regulated by both stimulatory and inhibitory hypothalamic factors (Figure 1.4). The GH hormone control area is in the anterior hypothalamus and the anterior periventricular nucleus. Growth hormone releasing hormone (GHRH) stimulates GH release, and the major site of GHRH production is located in the arcuate nucleus (Martin and Millard, 1986; Spiess et al., 1983). Somatostatin exerts its inhibitory effects on GH release, and is widely distributed throughout the hypothalamus (Brazeau et al., 1973; Martin and Millard, 1986). A burst secretion of GH gives a short feedback to hypothalamus to inhibit GHRH secretion and stimulates somatostatin secretion. GH secretion is also regulated by long negative feedback. GH induces hepatic secretion of IGF-1, which in turn inhibits GH secretion directly or by stimulatory effects on somatostatin. Ghrelin is a growth hormone secretagogue found both in the gastrointestinal tract and the central nervous system with the highest concentrations in stomach. Ghrelin exerts a strong stimulatory effect on GH secretion and the effect is greater than GHRH. Ghrelin produced by the pituitary can regulate GH release in an autocrine or paracrine manner (Kamegai et al., 2004).

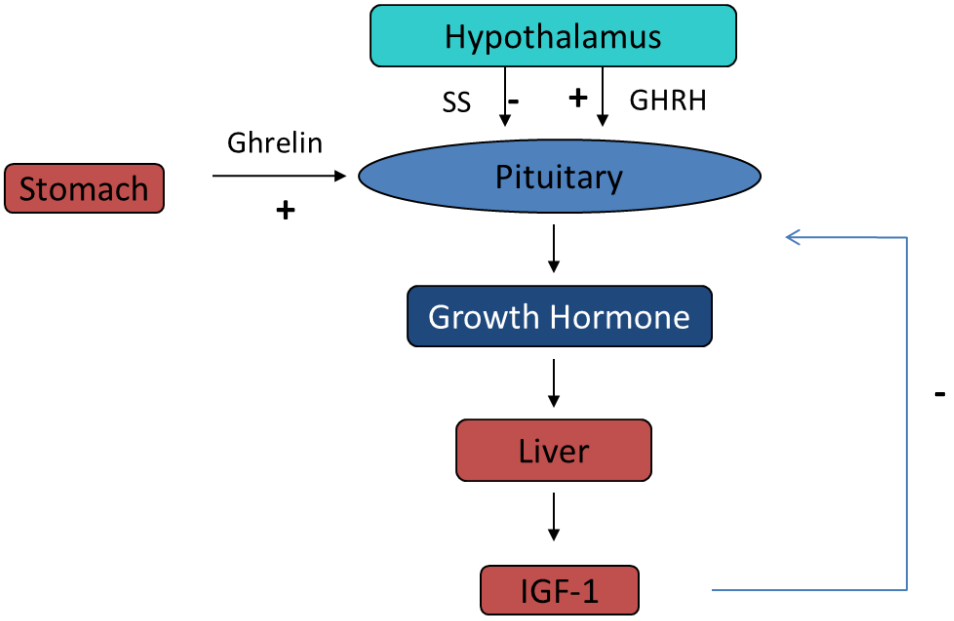

Figure 1.4. Secretion of growth hormone. GH is secreted by the pituitary under the control of GHRH and SS from hypothalamus and ghrelin from stomach. GH stimulates IGF-1 secretion in liver, which in turn inhibits GH production. SS: somatotatin; GHRH: growth hormone releasing hormone; IGF-1: insulin-like growth factor-1.

One major function for GH is to stimulate growth. Deficiency of GH or GH signaling results in growth retardation. GH-deficient children have a slower rate of growth and are much shorter than the others at the same age and gender with delayed bone age, reduced bone density, excess adiposity, and reduced lean tissue mass (Cuneo et al., 1992). On the other hand, excessive GH causes acromegaly in the adult and gigantism in children (Nabarro, 1987; Sotos et al., 1964). GH has both direct and indirect effects on target tissues. The indirect effect of GH is predominantly mediated via IGF-1 produced by liver and peripheral target tissues. The liver is the major target organ of GH to produce

IGF-1. IGF-1 stimulates mitosis and differentiation of fibroblasts, prechondrocytes and other progenitor cells (Madsen et al., 1983; Tsukazaki et al., 1994).

Growth hormone also has metabolic functions. The metabolic effects of GH can be summarized as increasing the rate of protein synthesis and lipid mobilization and decreasing the rate of glucose mobilization. GH has been shown to acutely stimulate forearm muscle protein synthesis in human (Fryburg et al., 1991). GH possibly increases protein synthesis through activating the mammalian target of rapamycin (mTOR) signaling pathway (Hayashi and Proud, 2007). It is well documented that GH induces insulin resistance. There are three possible mechanisms for this effect. First, GH stimulates the free fatty acid release from adipose tissue, which inhibits glucose utilization by other tissues and reduces their sensitivity to insulin (Vijayakumar et al., 2010). Second, SOCS proteins, especially SOCS-1 and SOCS-3, whose expression is induced by GH, are involved in insulin resistance. Third, GH stimulates the expression of p85α regulatory subunits of PI3K, and excessive p85α forms monomers and competitively binds with IRS to block the insulin signaling (Vijayakumar et al., 2010). The effect of GH on lipid mobilization will be discussed below.

GH receptor and signaling

Growth hormone exerts its effects by binding to its receptors. Growth hormone receptor (GHR) belongs to the cytokine receptor super-family, which contains a single transmembrane domain, an extracellular domain and a cytoplasmic domain (Bazan, 1990). The cytoplasmic domain comprises proline-rich Box 1 and Box 2 motifs. Box1 binds to tyrosine kinase JAK2, and Box2 contains binding sites for SH2 domain proteins (Brooks

et al., 2008). The processes of GHR activation includes GH induced dimerization, aggregation and conformational change. It was believed that GH-induced GHR dimerization results in activation of JAKs (Fuh et al., 1992). However, dimerization alone is insufficient to activate full-length GHR, and GHR has been found as a dimer on the cell surface without GH binding (Brown et al., 2005; Gent et al., 2002). In this new model, GH binds to the constitutively dimerized GHR which induces the rotation of intracellular domain, resulting in the signal transduction. GHR is expressed in many tissues including liver, muscle, adipose tissue, kidney, heart, prostate, fibroblasts and lymphocytes with the greatest expression in liver followed by adipose tissue and muscle (Ballesteros et al., 2000). In adipose tissue, GHR is expressed both in preadipocytes and mature adipocytes (Nam and Lobie, 2000). Preadipocytes only express low levels of GHR, after stimulation of differentiation, GHR mRNA abundance is dramatically increased indicating a direct effect of GH on adipocytes differentiation (Zou et al., 1997).

Janus Kinase 2 (JAK2) is a tyrosine kinase associated with GHR. Dimerization of GHR brings two JAK2 protein molecules close enough to phosphorylate each other, which is the key step in initiating GH signaling. The activated JAK2 initiates three major transcription signaling pathways (Figure 1.5): (1) phosphoinositide kinase-3 (PI3K) pathway; (2) mitogen activated protein kinase (MAPK) pathway; (3) signal transducer and activator of transcription (STAT) pathway.

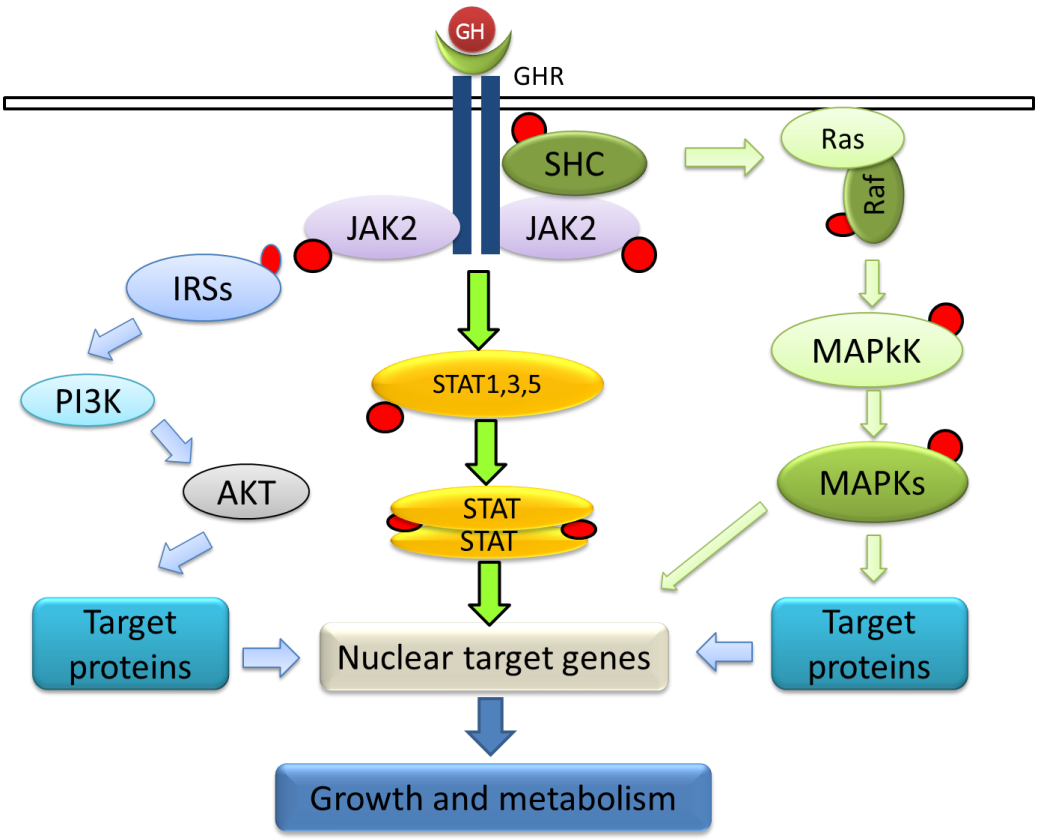

Figure 1.5. Major growth hormone signaling pathways. The signaling pathways are initiated by GH binding to its receptor, which activates JAK2. The activated JAK2 initiates three major signaling pathways: phosphoinositide kinase-3 (PI3K) pathway; mitogen activated protein kinase (MAPK) pathway; signal transducer and activator of transcription (STAT) pathway.

PI3K and MAPK pathways

GH activates PI3K by phosphorylating insulin receptor substrate (IRS) proteins. The tyrosine-phosphorylated IRS-1, IRS-2 and IRS-3 are known to associate with many signaling molecules including p85 subunit of PI3K (Lanning and Carter-Su, 2006). Alternatively, GH might activate PI3K by binding the p85 subunit directly to phosphotyrosine residues in the carboxyl-terminus of the GHR (Lanning and Carter-Su,

2006). The activated PI3K from either pathway activates the serine/threonine kinase Akt/PKB to regulate the down-stream responses (Zhu et al., 2001).

In addition to activating the PI3K pathway, GH also activates the MAPK pathway. The MAPKs are a group of proline-directed serine-threonine protein kinases functioning as mediators activated by a variety of extracellular stimuli. The activation of MAPK needs a signaling cascade including Src homology 2 domain containing transforming protein 1 (Shc), growth factor receptor-bound protein 2 (Grb2), son of sevenless homologes (SOS), Ras, Raf and MAP/ERK kinase (MEK) (Zhu et al., 2001). The adapter protein Shc binds to GHR-JAK2 complex after GH stimulation, which results in the phosphorylation of Shc and its binding to Grb2 and SOS; the complex of Shc-Grb2-SOS activates Ras, Raf and MEK pathway and finally ERK 1 and 2 proteins (Zhu et al., 2001).

STAT pathway

The STAT signaling pathway is one of the three major pathways activated by GH. Among STAT family members, GH activates STAT1, STAT3, STAT5a and STAT5b (Zhu et al., 2001). The mechanism of activation differs for STAT1, STAT3 and STAT5 (Paukku and Silvennoinen, 2004). However, they all require GH activation of JAK2. The membrane proximal region of GHR for phosphorylation of JAK2 is sufficient for STAT1 and STAT3 activation (Sotiropoulos et al., 1995; Zhu et al., 2001). There a STAT1-like association motif and STAT3 association motifs on JAK2. Unlike STAT1 and 3, STAT5 needs a C-terminal tyrosine residue in addition to a membrane proximal region (Sotiropoulos et al., 1996). The phosphorylated sites on GHR provide docking sites for

STAT5. Once STAT5 proteins bind to these sites, they can be phosphorylated by JAK2, dimerize, translocate to the nucleus, and act as transcription factors (Lanning and Carter-Su, 2006).

Among these STAT proteins, STAT5 plays a key role in many GH functions. STAT5a and STAT5b share more than 90% of amino acids at the protein level. STAT5a is predominantly expressed in mammary gland and STAT5b is mainly expressed in liver and muscle (Hennighausen and Robinson, 2008). Both STAT5a and STAT5b are found in adipose tissue (Richter et al., 2003). The role of STAT5 in adipogenesis has been already discussed above. The key role of STAT5 in hepatocytes is confined to modulating cellular metabolism and the production of insulin-like growth factor-1 (IGF-1). Loss of STAT5 from mouse liver tissue resulted in a significant reduction of circulating IGF-1 (Cui et al., 2007; Engblom et al., 2007).

Effect of GH on fat tissue growth

GH and body composition

Adipose tissue is an important target tissue for GH. Clearly, GH regulates the body composition by burning fat and sparing protein. The effects of GH increasing lean body mass and reducing fat body mass have been repeatedly demonstrated. Lee and Schaffer (1934) found that pituitary extracts rich in growth promoting activity increased lean body mass at the expense of body fat in rats. Maison et al. (2004) reviewed thirty-seven trials, which were conducted to test GH treatment in adult patients with GH deficiency. They concluded that GH treatment significantly reduced fat mass by 3.1 kg and increased lean body mass by 2.8 kg on average. A recent study showed the results of

two-year body composition analyses of long-lived growth hormone receptor (GHR) null mice, in which the percent of fat mass was markedly elevated in male GHR-/- mice compared to the wild type male mice, and a similar trend was also observed for female mice (Berryman et al., 2010). Consistent with these observations, body composition measurements revealed a GH dose-dependent decrease in fat in obese mice, and subcutaneous and mesenteric fat was the most sensitive to GH treatment (List et al., 2009). This effect of GH was also found in other animal models. In pigs, exogenous GH increased average daily gain (ADG) by 10 to 20%, improved feed efficiency (FE) by 15 to 35 %, increased protein deposition by 50% and decreased fat accretion by 50 to 70% (Etherton et al., 1993; Etherton et al., 1987). The increased protein accretion may relate to increased utilization of absorbed amino acids (Krick et al., 1993). A daily dose of bovine somatotropin (bST) between 16.5 and 33 μg/kg of body weight improved ADG and FE in finishing beef steers and longissimus muscle area increased quadratically (Moseley et al., 1992). As doses increased from 0 to 160 mg/week, the backfat thickness and kidney-pelvic-heart fat accumulation were decreased linearly and marble score was also decreased (Preston et al., 1995). In agreement with those experiments, long-term bST administration from initial body weight of 185 kg to slaughter increased carcass protein and water content and lowered carcass lipid and lipid accretion (Schlegel et al., 2006).

GH and adipogenesis

During the differentiation of adipocytes GHR gene expression is dramatically up-regulated (Zou et al., 1997) indicating the role of GH in adipogenesis. However, the

effect of GH on adipogenesis is controversial when using cell lines and primary cells as models. In undifferentiated cells, GH increased both cell number and ^{3}H-thymidine incorporation, which indicated the stimulatory effect of GH on cell proliferation (Wabitsch et al., 1996). In preadipocyte cell lines such as 3T3-L1, 3T3-F442A, and Ob1771, GH treatment has been demonstrated to induce differentiation from preadipocyte to adipocyte (Corin et al., 1990; Doglio et al., 1986; Morikawa et al., 1982). GH may exert its effects by up-regulating C/EBPβ and C/EBPδ, two transcription factors expressed at early stage of differentiation (Clarkson et al., 1995). GH is also found to stimulate c-*fos* and c-*jun*, which are implicated in cell growth and differentiation (Gurland et al., 1990; Sumantran et al., 1992)

In contrast to its effect on preadipocyte cell lines, GH inhibits differentiation of primary preadipocytes into adipocytes. GH significantly inhibited the fat cell cluster formation during the differentiation of pig stromal vascular cells (Hausman and Martin, 1989). In primary cultured rat adipocyte precursor cells, GH markedly reduced the formation of new fat cells and the glycerol-3-phosphate dehydrogenase (G3PDH) activity, a marker enzyme of adipose differentiation, in a dose-dependent manner (Wabitsch et al., 1996). The mechanisms by which GH regulates adipogenesis are still unclear. It has been reported that GH inhibited PPARγ expression in rat primary adipocyte and reduced the DNA binding activity of PPARγ/retinoid X receptor-α (RXRα) to the binding element of the aP2 gene, and this effect was not dependent on the MAPK pathway (Hansen et al., 1998). Another mechanism may involve Pref-1. Pref-1 is highly expressed in preadipocytes to inhibit adipogenesis and its expression is decreased during

differentiation (Wang et al., 2006). GH treatment prevented the decrease of Pref-1 expression in differentiating preadipocytes (Hansen et al., 1998).

GH and lipolysis

Lipolysis and lipogenesis are two ongoing processes within the adipocytes. Stimulating lipolysis is one of the mechanisms by which GH regulates the fat tissue *in vivo*. Pulsatile delivery of GH instead of constant GH administration increased basal lipolysis in humans (Cersosimo et al., 1996). A significant increase in GH secretion was accompanied by increases in the rate of lipolysis after two days of fasting in humans; blocking GH secretion by growth hormone releasing hormone receptor (GHRH) antagonist decreased the rate of lipolysis (Sakharova et al., 2008). Serum free fatty acids rose after GH exposure, and pharmacological suppression of HSL abrogated this effect of GH (Richelsen, 1997). The amount of glycerol in the culture medium of GH-treated rat primary adipocytes, which indicates the reduction of cellular triglyceride content as a result of lipolysis, was significantly higher than that of cells cultured in the absence of GH (Wabitsch et al., 1996). However, there are also studies showing that GH has no direct effect on lipolysis *in vitro* (Fain et al., 2008; Frigeri et al., 1983). It has been reported that bST did not change basal lipolysis using chronic culture (48 h) of adipose tissue from lactating cows (Lanna and Bauman, 1999), and there was no lipolytic effect of bST over a 3-h incubation as well (Houseknecht et al., 1996).

The mechanisms of GH effects on lipolysis are still unclear. In primary cultures of adipose tissue from STAT5-deleted transgenic mice or wild type mice, GH enhanced lipolysis in control but not in transgenic mice (Fain et al., 1999). This result indicates that

the lipolytic action of GH may depend on STAT5. GH is thought to enhance lipolysis through collaboration with other hormones *in vitro*. Catecholamine is an important stimulator of lipolysis. Previous studies have shown that GH enhanced catecholamine-stimulated lipolysis (Beauville et al., 1992; Marcus et al., 1994; Yang et al., 1996). The increased lipolysis by GH may be partly mediated through increased β-adrenergic receptor function. Yang et al. (2004) examined the effects of GH on different types of β-adrenergic receptors in rat adipocytes and found that GH stimulated lipolysis at the presence of agonists of β1 and β3-adrenergic receptor. Additionally, they also found that GH decreased expression of Gαi protein, a antilipolytic signaling protein present in rat adipocyte membranes. In contrast to these studies, an increase of *in vitro* β-adrenergic-stimulated lipolysis was not found in adipose tissue from lactating cows chronically treated with bST (Lanna et al., 1995). However, a dramatic increase in lipolysis induced by epinephrine in lactating cows treated with bST compared with that of control was observed *in vivo* (Houseknecht et al., 1995). They believed that bST increased epinephrine-induced lipolysis *in vivo* was an indirect consequence of regulating adenosine-related inhibitory signaling (Lanna et al., 1995). GH also stimulated lipolysis in omental adipose tissue explants from obese women in the presence of dexamethasone (Fain et al., 2008). Furthermore, GH may stimulate lipolysis by increasing the expression of HSL mRNA (Yang et al., 2004), and by inducing the HSL activity (Dietz and Schwartz, 1991). HSL activity was increased in adipose tissue from bST-treated lactating cows (Lanna et al., 1995)

GH and lipogenesis

Another mechanism whereby GH may reduce fat mass is the suppression of lipogenesis. Daily administration of porcine somatotropin (pST) for 30 to 60 days decreased lipid accretion in growing pigs, which was due to a marked decrease in glucose transport and lipogenesis (Etherton et al., 1993). LPL is involved in providing free fatty acids from circulating chylomicron and very-low-density lipoprotein triglyceride (VLDL-TG) to adipose tissue for storage (Shi and Burn, 2004). Treatment with GH resulted in up to 50% of reduction of LPL activity in adipose tissue in humans both *in vivo* and *in vitro*; however, the mRNA level of LPL was not affected by GH (Richelsen, 1999). Fatty acid synthase (FAS) is the key lipogenic enzyme that catalyzes the conversion of acetyl-CoA and malonyl-CoA to palmitic acid. The activity of FAS was reduced in bST treated lactating cows (Lanna et al., 1995). Both activity and mRNA levels of FAS were decreased in adipose tissue of GH-treated pigs (Donkin et al., 1996). In line with this experiment, daily injection of 12 mg/kg of body weight of pST for 11 days decreased the basal rate of lipogenesis in growing pigs which was corresponded to a 79% decrease in ACC and a 67% decrease in FAS activity (Harris et al., 1993). Decreased mRNA expression of FAS by GH was also found in 3T3-F442A cells (Yin et al., 1998). The signaling pathways by which GH inhibits mRNA expression of FAS was conducted by Yin et al. (2001), they found that protein kinase A, protein kinase C and JAK2 do not mediate the effect of GH on regulation of FAS mRNA expression. Down-regulation of FAS expression in adipose tissue may be a result of decreased insulin sensitivity by GH (Yin et al., 1998). Decreased insulin sensitivity caused by GH was found in adipose tissue, meanwhile the insulin sensitivity was relatively unchanged in skeletal muscle (Etherton and Bauman, 1998). This tissue specific insulin regulation redirected glucose

which is used for lipid synthesis to the skeletal muscle to facilitate nutrient repartitioning (Etherton, 2001).

CONCLUSION AND PERSPECTIVES

Adipose tissue is a loose connective tissue scattered throughout the body. It functions as energy storage, insulator/cushion of the body, and an endocrine organ. Adipose tissue growth involves hyperplasia and hypertrophy. Adipocytes are considered to originate from fibroblast-like precursor cells. The process of adipogenesis is regulated by many transcription factors including CEBPα and PPARγ. The size of an adipocyte is primarily determined by lipid accumulation in it, which is the result of lipolysis and lipogenesis. Fat tissue growth is also regulated by many extrinsic factors including nutrients and hormones. Growth hormone is one of the hormones that regulate fat tissue growth. GH administration reduces body fat mass in a variety of animal models and humans. *In vitro* studies have shown that GH inhibits adipogenesis in primary preadipocytes but stimulates adipogenesis in preadipocyte cell lines. Therefore, whether GH inhibits adipose tissue growth through its effect on adipogenesis remains unclear. GH clearly has lipolytic effects. However, whether GH causes lipolysis by direct or indirect action on adipose tissue is also unclear.

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Chapter II

Growth hormone stimulates lipolysis in mice through an indirect mechanism independent of adrenergic receptors

ABSTRACT

It has been long known that growth hormone (GH) inhibits fat tissue growth in various animals. However, the underlying mechanism remains poorly understood. The objective of this study was to determine if GH inhibits fat tissue growth by stimulating lipolysis. We addressed this question using the GH-deficient growth hormone releasing hormone receptor (*Ghrhr*) homozygous mutant mice (i.e., *lit/lit* mice). The *lit/lit* mice weighted less but had more subcutaneous fat and larger adipocytes compared to *Ghrhr* heterozygous mutant (*lit/+)* mice at the same ages. GH treatment to *lit/lit* mice for four weeks partially reversed these differences. This indicates that GH inhibits subcutaneous adipose tissue growth in mice at least in part by stimulating lipolysis. To determine if GH has a direct effect on lipolysis, subcutaneous fat tissue explants and adipocytes differentiated from stromal vascular fraction from *lit/lit* mice were cultured in the presence or absence of 100 ng/ml GH. GH had no effect on glycerol release into the culture medium within 4 h or 24 h. This suggests that GH had no direct lipolytic effect on mouse adipose tissue or adipocytes. To investigate if GH stimulates lipolysis by enhancing the lipolytic response of adipose tissue to epinephrine or norepinephrine, we compared mRNA expression of β adrenergic receptors between *lit/lit* and *lit/+* mouse fat. β1 and β3-adrenergic receptor mRNAs were lower in *lit/lit* than in *lit/+* mouse subcutaneous fat, and 6-h GH treatment increased β1 and β2-adrenergic receptor mRNA

expression in *lit/lit* mice. However, no differences in isoproterenol-induced lipolysis were observed between *lit/lit* and *lit/+* mice and between subcutaneous fat from *lit/lit* and *lit/+* mice. We also observed no difference in isoproterenol-induced phosphorylation of hormone sensitive lipase between *lit/lit* and *lit/+* mouse subcutaneous adipose tissue. These results argue against the hypothesis that GH stimulates lipolysis by enhancing the direct lipolytic effect of epinephrine and norepinephrine in mice.

Keywords: Growth hormone; Adipose tissue; *lit/lit* mice; Lipolysis; Adipogenesis.

INTRODUCTION

Growth hormone (GH) is a polypeptide hormone secreted by the anterior pituitary. It is an important regulator of somatic growth, metabolism and body composition. Numerous studies have demonstrated the effects of GH on body composition including increased muscle growth and reduced adipose tissue growth (Berryman et al., 2010; Etherton et al., 1993; Rosen et al., 1993). In humans and mice, GH deficiency resulted in increased body fat, and GH replacement reversed the change (Bengtsson et al., 1993; Berryman et al., 2010; Maison et al., 2004). It was believed that GH decreased body fat mass by both reducing lipogenesis and enhancing lipolysis (Richelsen et al., 1994; Rosenbaum et al., 1989).

Increased serum free fatty acid or glycerol concentrations are indicators of increased lipolysis, after GH exposure in mice (Chen et al., 2011), rats (Yin et al., 1998), bovine (Gong et al., 1991), and humans (Moller and Jorgensen, 2009; Zhao et al., 2011). Different models including cell lines, isolated adipocytes, and adipose tissue explants have been used to elucidate the mechanisms by which GH stimulates lipolysis. In adipocytes derived from 3T3-F442A preadipocytes, GH stimulated the release of glycerol after 24 to 48 h in a dose-dependent maner (Dietz and Schwartz, 1991). In epididymal fat explants from rats, GH treatment for 1 h significantly increased glycerol released into the medium (Yip and Goodman, 1999). These results indicate that GH may directly act on adipocytes to stimulate lipolysis. However, GH was not lipolytic when added to cultures of differentiated 3T3-L1 adipocytes (Frigeri et al., 1983). The stimulatory effect of GH on lipolysis was undetectable in the absence of glucocorticoids in human omental adipose tissue explants and in adipocytes from rat parametrial adipose tissue (Fain et al., 2008;

Fain et al., 1965). Additionally, no lipolytic effect of GH was observed in isolated adipocytes from healthy adults during 30 min to 6 h incubation (Marcus et al., 1994). Catecholamine is a strong lipolytic agent, and it stimulates lipolysis through β-adrenergic receptors. These receptors are G-protein coupled receptors which activate adenylate cyclase, which is positively regulated by the stimulatory guanine nucleotide-binding protein (Gs protein) and negatively regulated by Gi protein α subunits (Spiegel et al., 1992). Yang et al., (2004) found that the function of β1 and β3 but not β2- adrenergic receptor was increased, and the expression of Gαi protein was decreased by GH in rat adipocytes. Six months of GH administration to GH-deficient adults significantly improved the lipolytic response of isolated adipocytes to epinephrine (Beauville et al., 1992). The result was confirmed in rat adipocytes (Yang et al., 1996). This effect of GH was considered to be mediated by a stimulatory effect on β-adrenergic receptors or by an inhibitory effect on the inhibitory G protein (Gi protein) (Marcus et al., 1994; Yang et al., 1996).

Because previous studies on the lipolytic effect of GH have generated largely conflicting results, we decided to revisit this action of GH in GH-deficient mice. Specifically, we wanted to determine whether GH has direct lipolytic effect on adipose tissue and the mechanism that mediates the direct or indirect effect of GH on lipolysis.

MATERIALS AND METHODS

Animal and tissue collection:

Breeding pairs of C57BL/6J-*Ghrhr*^{lit} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The *lit/lit* mouse contains a mutation in growth hormone releasing hormone receptor (*Ghrhr*) gene, as a result, GH is deficient in homozygous *lit/lit* mice (Godfrey et al., 1993). The mice were housed on 12 h light/dark cycle at 23 ℃ with free access to food and water. Male *lit/lit* mice and male littermates heterozygous for the *lit* mutation (*lit/+*) were used in this study. In a first experiment, 9-week-old mice were given daily subcutaneous injections of recombinant bovine GH (2 μg/g BW; The National Hormone and Peptide Program, Torrance, CA) or an equal volume of 0.01 M $NaHCO_3$ (vehicle for GH) for 4 weeks. Body weights were taken weekly. At the end of this experiment, mice were euthanized for isolation of inguinal subcutaneous fat pads. In a second experiment, 17-week-old *lit/lit* mice were given a subcutaneous injection of recombinant GH or $NaHCO_3$ and samples were taken as described above. In a third mouse experiment, subcutaneous fat was taken from 13-week-old mice for explant culture. The stromal vascular fractions were isolated from 13-week-old mice and differentiated for lipolysis assay. In a fourth mouse experiment, 13-week-old *lit/lit* mice were subcutaneously injected with GH or $NaHCO_3$ as described above for 6 h. The subcutaneous fat was taken for RNA extraction. In a fifth mouse experiment, mice (13 week) were intraperitoneally injected with isoproterenol (10 μg/g BW, Sigma-Aldrich, St. Louis, MO), and 15 min later they were euthanized for tissue collection. Blood samples were taken from retro-orbital sinus under isoflurane anesthesia. All procedures

involving animals were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Histology

The inguinal subcutaneous fat pads were fixed in 10% of phosphate buffered formalin (Fisher Scientific, Pittsburg, PA) for 18 h, dehydrated and embedded in paraffin for sectioning. 5-μm-thick tissue sections were stained with hematoxylin and eosin (HE, Fisher Scientific, Pittsburgh, PA) and photographed at 10× of magnification. Areas of adipocytes were measured using the ImageJ software (NIH, Bethesda, Maryland). At least 200 adipocytes were counted from each section and 3 sections from each mouse.

Culture of adipose tissue explants and adipocytes

Freshly isolated subcutaneous fat pads were cut into pieces of 5-10 mg. About 50 mg of tissue explants in one well of 24-well plate were incubated in Krebs Ringer bicarbonate buffer (pH 7.4) supplemented with 4% of bovine serum albumin (BSA, Fisher Scientific, Pittsburg, PA), 1 mg/ml of glucose (Fisher Scientific, Pittsburgh, PA), and 100 ng/ml of recombinant bovine GH or equal volume of PBS at 37 ℃ under an atmosphere of 5% CO_2 at 90% of humidity. Medium samples were collected at 4 h and 24 h after initiation of incubation. Glycerol concentrations in culture medium were measured using a glycerol assay kit (Cayman chemical, Ann Arbor, Michigan), following the manufacturer's instructions. Intra-assay and inter-assay coefficients of variability were 6.5% and 8.0%, respectively.

To isolate preadipocytes, freshly collected inguinal subcutaneous fat pads were minced and then digested by 1 mg/ml collagenase D (Roche, Indianapolis, IN), in HEPEs

buffer (0.1 M HEPES, 0.12 M NaCl, 0.05 M KCl, 5 mM glucose, 1.5% BSA, and 1 mM $CaCl_2$, pH 7.4) at 37 ℃ with shaking at 115 rpm for 1 h. The digested solution was filtered using 240 μm mesh filter followed by 40 μm mesh filter. The cell suspension passed through mesh filter was centrifuged at 400 g for 5 min, and the pellet was washed twice by HEPEs buffer and re-suspended and plated into 6-cm dish for proliferation. After cells reached 80% confluency, they were trypsinized and plated into 24-well plate at density of 25,000 cells/cm^2. The preadipocytes at 100% confluency were induced to differentiate into adipocytes in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (DMEM/F12, Mediatech, Manassas, VA) containing 5% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 1% of antibiotics-antimycotics (ABAM; Mediatech, Manassas, VA), 2 mM of L-glutamine (Mediatech, Manassas, VA), 17 nM insulin, 0.1 μM dexamethasone, 250 μM 3-Isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St. Louis, MO), and 60 μM indomethacin MP Biomedical, Solon, OH) for the first 48 h, then in DMEM/F12 supplemented with 10% FBS, 1% ABAM, 2 mM L-gutamine, and 17 mM insulin for another 48 h, last in DMEM/F12 supplemented with 10% FBS, 1% ABAM, 2mM L-gutamine for 4 d as described before (Hausman et al., 2008). On d 8 of differentiation, cells were treated with or without 100 ng/ml GH for 4 h and medium were collected for glycerol release assays as described above.

RNA extraction and real time PCR

Total RNA from adipose tissue or adipocytes was extracted using TRI reagent (Invitrogen, Grand island, NY, USA) according to the manufacturer's instruction. RNA was reverse-transcribed into cDNA using Promega ImProm-II™ Reverse Transcription system (Promega, Fitchburg, Wisconsin) according to the manufacture's instruction**.**

Real-time PCR was performed using Fast SYBRGreen Master Mix (Applied Biosystems, Foster City, CA) on an Applied Biosystems 7500 Real-Time PCR machine. Sequences of primers for PCR are described in Table 2.1. Mouse 18s rRNA was used as an internal control since, based on the Ct values, its expression was not affected by the treatments in this study ($P > 0.1$). The relative quantification of mRNA was obtained using the comparative threshold cycle (ΔΔCt) method.

Protein isolation and Western blot analysis

Adipose tissue (200-300 mg) was homogenized in 1 ml of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with protease inhibitors and phosphatase inhibitors (Roche, Indianapolis, IN). The homogenates were centrifuged at 10,000 ×g for 20 min at 4 ℃. The clear phase below the fat cake but above the pellet was transferred to another tube and centrifuged again at 13,000 ×g for 10 min. Protein concentrations in the supernatant were measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Equal amounts of protein samples were separated by 8% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes. The membranes were immunoblotted first with anti-phospho-hormone sensitive lipase (HSL) (at 1:1000 dilution, Cell Signaling, Danvers, MA) and then with anti-HSL (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected by 1:1000 horseradish peroxidase-coupled IgG (Santa Cruz Biotechnology, Santa Cruz, CA) followed by Pierce ECL substrate (Thermo Scientific, Rockford, IL). Chemiluminescent signals were detected by exposing the membrane to a CL-Xposure film (Thermo Scientific, Rockford, IL).

Statistical analysis

ANOVA was used to compare multiple means followed by Tukey's test. T-test was used to compare two means. These analyses were performed using the General Linear Model of JMP (SAS Institute Inc., Cary, NC). The individual mouse was the experimental unit for all analyses. In each treatment 4 or 5 mice were used. In the explant culture and adipocyte culture experiments, samples were measured in duplicate and each experiment was repeated at least 4 times, each time using tissue or cells from different mice. In analyzing the data from the third experiment, the statistical model included genotype, culture time, and genotype × time. In analyzing the NEFA data from the fifth experiment, the model included genotype, injection time, and genotype × time. All data are expressed as the mean ± SEM (standard error of the mean). $P < 0.05$ indicated statistical significance.

RESULTS

lit/lit mice had greater body fat percentage and larger adipocytes than lit/+ mice

The body weight and adipose tissue weight of *lit/+* mice and *lit/lit* mice were measured before (at 9 weeks of age) and after 4 weeks of experiment. At the beginning of the experiment, the body weight of *lit/lit* mice was only 50% of that of *lit/+* controls ($P < 0.05$, Figure 2.1A). There were no changes of body weights during 4 weeks of experiment either in *lit/lit* mice or *lit/+* mice injected with 0.01 M $NaHCO_3$ (Figure 2.1A). GH injection increased the body weight of *lit/lit* mice by approximately 3.3 g ($P < 0.05$, Figure 2.1A). Weight and body percentage of inguinal subcutaneous fat were both greater in *lit/lit* mice than in *lit/+* mice, and were both decreased by GH injection ($P < 0.05$, Figures 2.1B and C)

Tissue sections of inguinal subcutaneous fat pad with HE staining are shown in Figure 2.2. The *lit/lit* mice had larger adipocytes than *lit/+* mice at the same age. The size of adipocytes was decreased in the *lit/lit* mice treated with GH for 4 weeks (Figure 2.2A). The average area of adipocytes in *lit/lit* mice was greater than that in *lit/+* mice at the same age, and GH injection decreased the area of adipocytes in *lit/lit* mice ($P < 0.05$, Figure 2.2B). That *lit/lit* mice had larger adipocytes than *lit/+* mice suggests that GH inhibits adipocyte hypertrophy in mice.

GH injection reduced body fat percentage and adipocyte size in lit/lit mice

To determine if GH inhibits adipocyte growth by inducing lipolysis, we compared the size of adipocytes in *lit/lit* mice before (~ 17 weeks of age) and 4 weeks after GH injection. In this experiment, one group of mice was weighed and killed at the beginning of the experiment as the 0-wk control, and two groups were injected with GH (2 μg/g BW) and equal volume of $NaHCO_3$ (0.01 M) for 4 weeks. The body weight of *lit/lit* mice injected with $NaHCO_3$ was unchanged, but the body weight of *lit/lit* mice injected with GH was increased compared to that of 0-week controls ($P < 0.05$, Figure 2.3A). Consistent with the data shown in Figure 2.1, GH injection decreased both absolute and percentage fat weight in *lit/lit* mice ($P < 0.05$, Figures 2.3B and 2.3C). As can be seen in Figures 2.4A and 2.4B, *lit/lit* mice injected with GH for 4 weeks had smaller adipocytes than *lit/lit* mice 4 weeks ago and *lit/lit* mice injected with $NaHCO_3$ for 4 weeks ($P < 0.05$). This result indicates that GH reduced subcutaneous fat weight at least in part by inducing lipolysis.

GH did not induce lipolysis in cultured adipose tissue explants or adipocytes

To determine whether GH induces lipolysis by direct action on adipocytes, we cultured adipose tissue explants from *lit/lit* mice with or without 100 ng/ml GH for 4 h or 24 h. Addition of GH did not change glycerol release to the medium during either period of time (Figure 2.5A). To confirm this result, we isolated stromal vascular cells from *lit/lit* mice subcutaneous fat, induced them to differentiate into adipocytes, and then incubated these adipocytes with GH for 4 h or 24 h. Again, GH had no effect on glycerol release from adipoctyes (Figure 2.5B). These data suggested that GH has no direct effect on lipolysis in mouse subcutaneous fat.

GH stimulated mRNA expression of β receptors in mouse adipose tissue

Epinephrine or nonepinephrine have strong lipolytic effects by binding to β adrenergic receptors (Arner, 1999). GH has been found to increase the lipolytic sensitivity of epinephrine in human and rat adipose tissue (Marcus et al., 1994; Yang et al., 1996). Here we tested the effect of GH on adrenergic receptor mRNA expression. β1 and β3 adrenergic receptor mRNAs were decreased in the subcutaneous fat of *lit/lit* mice compared to *lit/+* mice ($P < 0.05$), but β2 receptor mRNA expression was not different (Figure 2.6A). 6-h GH injection increased mRNA expression of β2 adrenergic receptor ($P < 0.05$), and tended to increase mRNA expression of β1 adrenergic receptor ($P < 0.1$), but had no effect on β3 receptor mRNA expression in *lit/lit* mice (Figure 2.6B). This result indicates that GH may regulate lipolysis by stimulating adrenergic receptor expression in adipose tissue.

GH did not change isoproterenol-induced lipolysis in vitro

To test whether GH potentiates epinephrine or norepinephrine-induced lipolysis in mice, subcutaneous fat explants from *lit/+* and *lit/lit* mice were cultured with isoproterenol or isoproterenol and GH. In this study we chose 10 μM isoproterenol for tissue explant culture and 1 μM for adipocyte culture according to previous studies (Allen and Quesenberry, 1988; Kosteli, et al., 2010) Isoproterenol increased glycerol release from adipose explants of both *lit/+* and *lit/lit* mice, but this release was not different between the two types of fat (Figure 2.7A). GH did not change the isoproterenol-induced glycerol from either type of fat (Figure 2.7A). We also isolated stromal vascular cells from *lit/lit* mouse subcutaneous fat, and induced them to

differentiate into adipocytes, and treated these adipocytes with isoproterenol. Isoproterenol stimulated glycerol release from these cells, but this effect was not changed at the presence of GH (Figure 2.7B). Data from both explant culture and adipocyte culture suggested that GH did not affect epinephrine or norepinephrine-induced lipolysis.

lit/lit mice did not differ from lit/+ mice in isoproterenol-induced lipolysis in vivo

In our next study, we compared the lipolytic response of *lit/+* and *lit/lit* mice to isoproterenol. Mice were intraperitoneally injected with isoproterenol (10 μg/g BW), and blood sample was collect immediately before and 15 min after injection. Concentration of free fatty acids in the serum was significantly increased after isoproterenol injection in both *lit/+* and *lit/lit* mice ($P < 0.05$, Figure 8A). However, there was no difference in the increase between two types of mice. Hormone sensitive lipase (HSL) is a key enzyme for lipolysis, and its activity is induced by epinephrine or norepinephrine through phosphorylation. We compared the phosphorylation levels of HSL in the subcutaneous fat of *lit/lit* and *lit/+* mice. The western blot analysis showed that there was no difference in the abundance of phosphorylated HSL between *lit/+* and *lit/lit* mice (Figure 2.8B and 2.8C), which indicated that the subcutaneous adipose tissue of these mice did not differ in the response to epinephrine or norepinephrine.

DISCUSSION

It has been long known that GH plays a critical role in regulating fat tissue homeostasis. Studies have consistently shown that GH-deficient mice had a higher percent of fat mass than normal mice (Berryman et al., 2004; Berryman et al., 2010; Egecioglu et al., 2006). In the present experiments, the *lit/lit* mice containing a mutation in *Ghrhr* gene were used to study the effect of GH on fat tissue growth. At birth, the body size of *lit/lit* mice was similar to their heterozygous littermates. But by the time of weaning, *lit/lit* mice were obviously smaller than *lit/+* mice, and the body weight of *lit/lit* mice was approximately 50% that of *lit/+*mice. *lit/lit* mice also showed marked increases in subcutaneous fat mass. GH replacement increased body weight and decreased the subcutaneous fat weight. The results are consistent with previously reported data that GH receptor-deficient mice had more subcutaneous fat (Berryman et al., 2004; Flint et al., 2003), and that anti-GH treatment resulted in a significant increase in subcutaneous fat in rats (Flint and Gardner, 1993). We measured epididymal fat weight as well but found that it was not different between *lit/+* and *lit/lit* mice (data not shown). Consistent with our result, Li et al. (2003) reported that epididymal fat weight of GH receptor-disrupted mice was not different from their littermate controls. Therefore, subcutaneous fat growth appears to be more sensitive to GH deficiency than epididymal fat growth in mice.

Since adipose tissue growth can be the result of increased cell size (hypertrophy) and/or increased cell number (hyperplasia), we further determined the effect of GH deficiency or replacement on the size of adipocytes in mice. We found that GH-deficient *lit/lit* mice had much larger adipocytes in subcutaneous fat than *lit/+* controls, and that GH injection decreased the size of adipocyte in *lit/lit* mice. In line with our result,

patients with GH deficiency had enlarged fat cells and treatment with GH resulted in decreased cell volume (Ukropec et al., 2008; Wabitsch and Heinze, 1993). Similar result was also found in GH receptor null mice (Li, 2006). Although the number of mature adipocytes was not determined in this study, the enlargement of adipocytes was at least partially responsible for the increase of fat mass in *lit/lit* mice. Similarly, the decrease in adipocyte size, i.e., lipolysis, is at least partially responsible for GH injection-induced decrease of fat mass in *lit/lit* mice.

Stimulating lipolysis was previously considered to be one of the mechanisms by which GH regulates fat tissue growth (Snyder et al., 1988; Zhao et al., 2011). However, it has been unclear whether GH stimulates lipolysis through direct action on adipocytes. In this study, we treated both adipose tissue explants and adipocytes from *lit/lit* mice with GH *in vitro* and found that GH had no direct effect on glycerol release, indicating that GH did not directly cause lipolysis. This result is consistent with the previous observations that GH was not lipolytic in differentiated 3T3-L1 adipocytes (Frigeri et al., 1983), and that GH alone had no effect on lipolysis in human fat explants or isolated adipocytes (Fain et al., 2008; Marcus et al., 1994). However, there have been studies showing that GH directly stimulates lipolysis *in vitro* (Ottosson et al., 2000; Yip and Goodman, 1999). The conflicts between these results cannot be attributed to different culture conditions used by different studies because similar culture conditions and GH treatments were used between our study, and the studies conducted by Fain et al. (2008) and Marcus et al. (1994), and the study by Yip and Goodman (1999).

Epinephrine and norepinephrine are strong lipolytic hormones. Previous studies have shown that GH enhanced lipolysis induced by these hormones in isolated rat

adipocytes (Ottosson et al., 2000), and that GH did so partly by increasing both β1 and β3 adrenergic receptor function (Yang et al., 2004). We presumed that GH stimulates lipolysis in mice through upregulation of β adrenergic receptors. Therefore, we measured the mRNA expression of β-adrenergic receptors in *lit/lit* and *lit/+* mouse adipose tissue. We found that subcutaneous fat expression of β1 and β3 adrenergic receptor mRNAs was decreased in *lit/lit* mice compared to *lit/+* mice and that GH injection increased β1 and β2 receptor mRNA expression. This result supported the assumption that GH may increase lipolysis by increasing the expression of adrenergic receptors in the adipose tissue. However, adipose tissue and adipocytes from *lit/+* and *lit/lit* mice did not differ in isoproterenol-induced lipolysis. We also did not detect a difference in isoproterenol-induced lipolysis between *lit/+* and *lit/lit* mice. HSL is one of the key enzymes catalyzing the hydrolysis of triglyceride (Yeaman, 1990), and it is activated by epinephrine and norepinephrine upon binding to their β-receptors (Holm, 2003). Our data showed no difference in isoproterenol-induced phosphorylation of HSL in subcutaneous fat between *lit/+* and *lit/lit* mice. These results together argue against the assumption that GH stimulates lipolysis by enhancing the expression of or signaling from β adrenergic receptors. It has been reported that GH can markedly increase adipose tissue's sensitivity to catecholamines without changing maximal lipolysis (Marcus et al., 1994). This is in accordance with our observation that GH did not affect isoproterenol-induced lipolysis despite increasing the expression of β receptors. It has been shown previously that there were "spare" β-adrenergic receptors in rat white adipocyte membranes (Lacasa et al., 1984). Thus increased expression of adrenergic receptors may not lead to increased stimulation of adenylate cyclase and cyclic AMP accumulation. This may explain why

isoproterenol-induced phosphorylation of HSL was unchanged by GH deficiency in this study.

In summary, GH deficiency results in increased subcutaneous fat tissue growth in mice and the increase is partially reversed by GH administration. GH inhibits adipose tissue growth in mice at least in part by stimulating lipolysis. The effect of GH on lipolysis is likely mediated by indirect mechanism, and this indirect mechanism does not involve the adrenergic system.

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Table 2.1. **Primers for RT-PCR analyses**

Target name[1]		Primer sequence (5'to 3')	GenBank #	Amplicon size (bp)
ADRβ1	Forward	GCTGATCTGGTCATGGGATT	NM_007419.2	100
	Reverse	AAGTCCAGAGCTCGCAGAAG		
ADRβ2	Forward	TTCGAAAACCTATGGGAACG	NM_007420	185
	Reverse	CTTGGGAGTCAACGCTAAGG		
ADRβ3	Forward	CCTTCCGTCGTCTTCTGTGT	NM_013462.3	120
	Reverse	AGCCATCAAACCTGTTGAGC		
18S	Forward	TTAAGAGGGACGGCCGGGGG	NR_003278.1	77
	Reverse	CTCTGGTCCGTCTTGCGCCG		

[1]*ADR* = Adrenergic receptor; *18s* = 18s ribosomal RNA

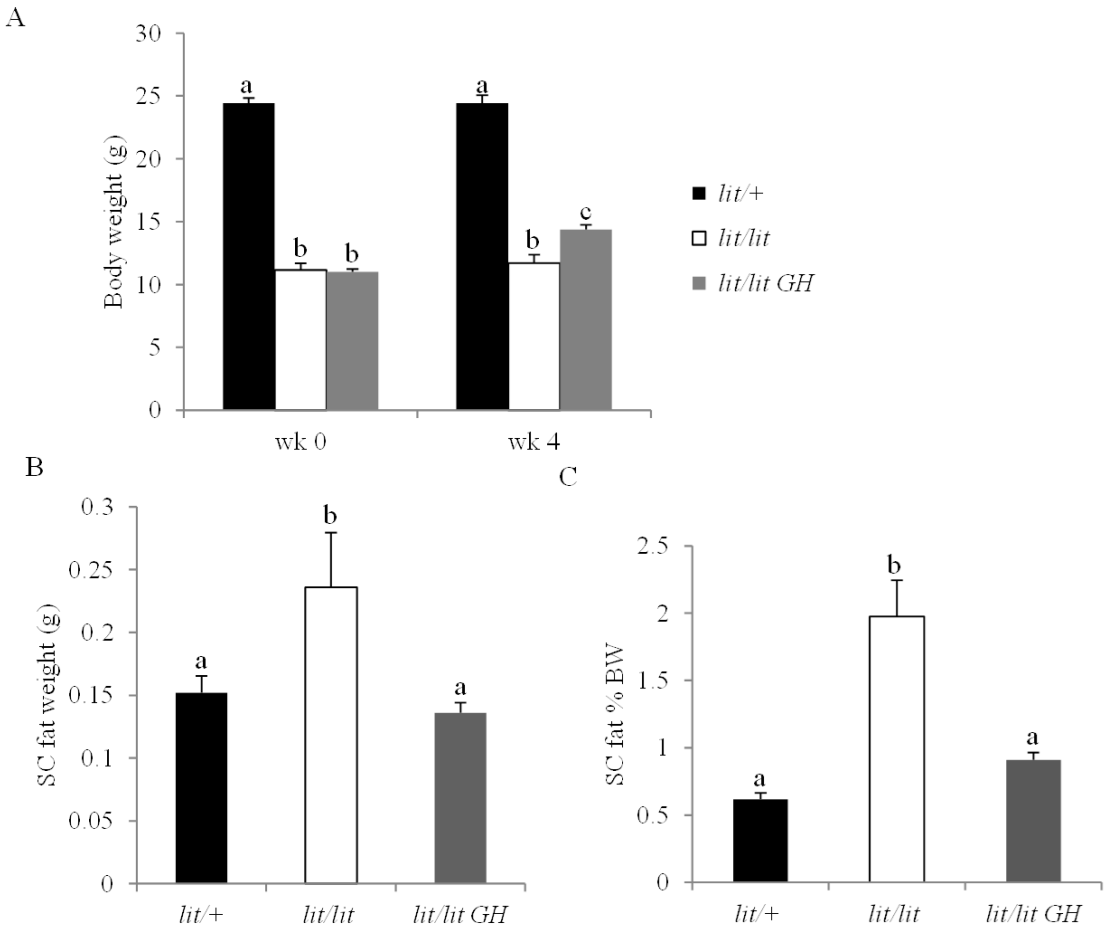


Figure 2.1. Body and fat weights of *lit/+*, *lit/lit*, and *lit/lit* mice injected with GH.

About 9-week-old male *lit/+* mice were injected with 0.01 M $NaHCO_3$ or 2µg/g BW of recombinant bovine GH for four weeks. Body weight and weight of inguinal subcutaneous fat pads were recorded at the end of this 4-week experiment. (A) Body weight. (B) Weight of inguinal subcutaneous fat pads. (C) Percentage of inguinal subcutaneous fat based on body weight. Data are expressed as means ± SEM (n=5 mice). Means labeled with different letters are statistically different ($P < 0.05$).

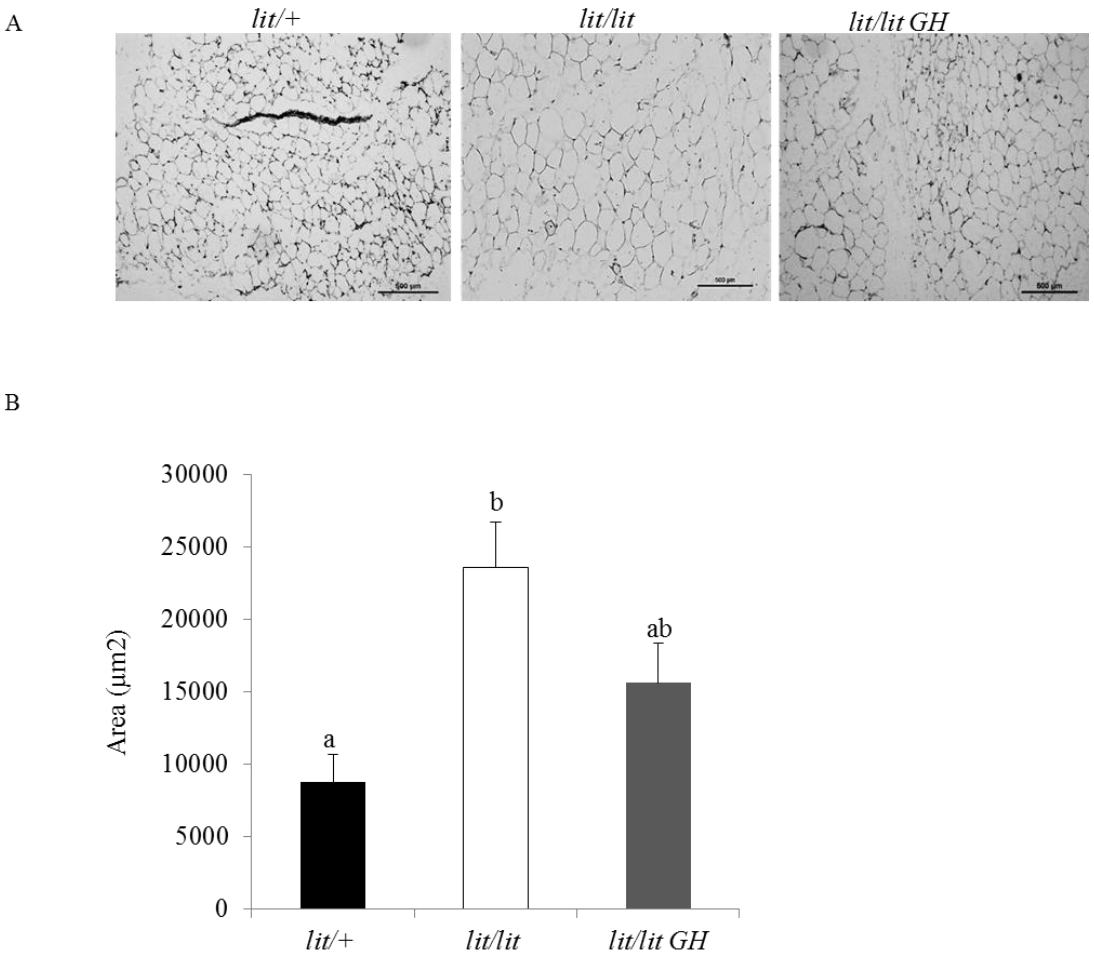


Figure 2.2. Adipocyte size of inguinal subcutaneous fat in *lit/+*mice, *lit/lit* mice, and *lit/lit* mice injected with GH. The inguinal subcutaneous fat pads collected at the end of 4-week injection experiment (see Figure 1 for details about the injections) were sectioned and stained with hematoxylin and eosin. The area of adipocytes was measured by image J. (A) Representative photomicrographs of sections of subcutaneous fat from *lit/+* mice, *lit/lit* mice and *lit/lit* mice injected with GH. (B) Average areas of adipocytes in *lit/+* mice, *lit/lit* mice and *lit/lit* mice injected with GH. Data are expressed as means ± SEM (n=4 mice). Means labeled with different letters are statistically different ($P < 0.05$).

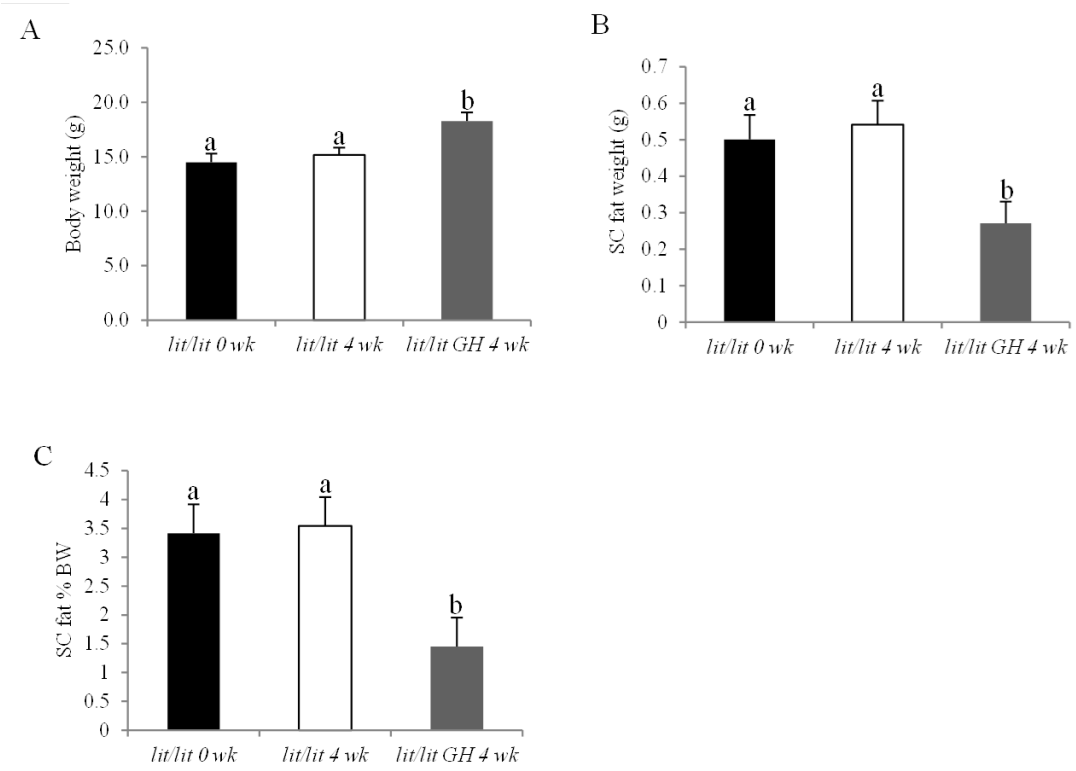


Figure 2.3. Body weight and inguinal subcutaneous fat weight of *lit/lit* mice before and after GH injection. Body weight and inguinal subcutaneous fat pads weight were taken from a group of *lit/lit* mice at 17 weeks of age (indicated as *lit/lit* 0 wk in the graph). Two other groups of lit/lit mice at similar ages were injected with buffer (0.01 M NaHCO3) or GH (2 μg/g BW) for 4 weeks (indicated as *lit/lit* 4 wk and *lit/lit* GH 4 wk, respectively in the graph). Body weight and subcutaneous fat pads weight were recorded from them at the end of four weeks. (A) Body weight. (B) Inguinal subcutaneous fat weight. (C) Inguinal subcutaneous fat percentage of body weight. Data are expressed as means ± SEM (n=4 mice). Means labeled with different letters are statistically different ($P < 0.05$).

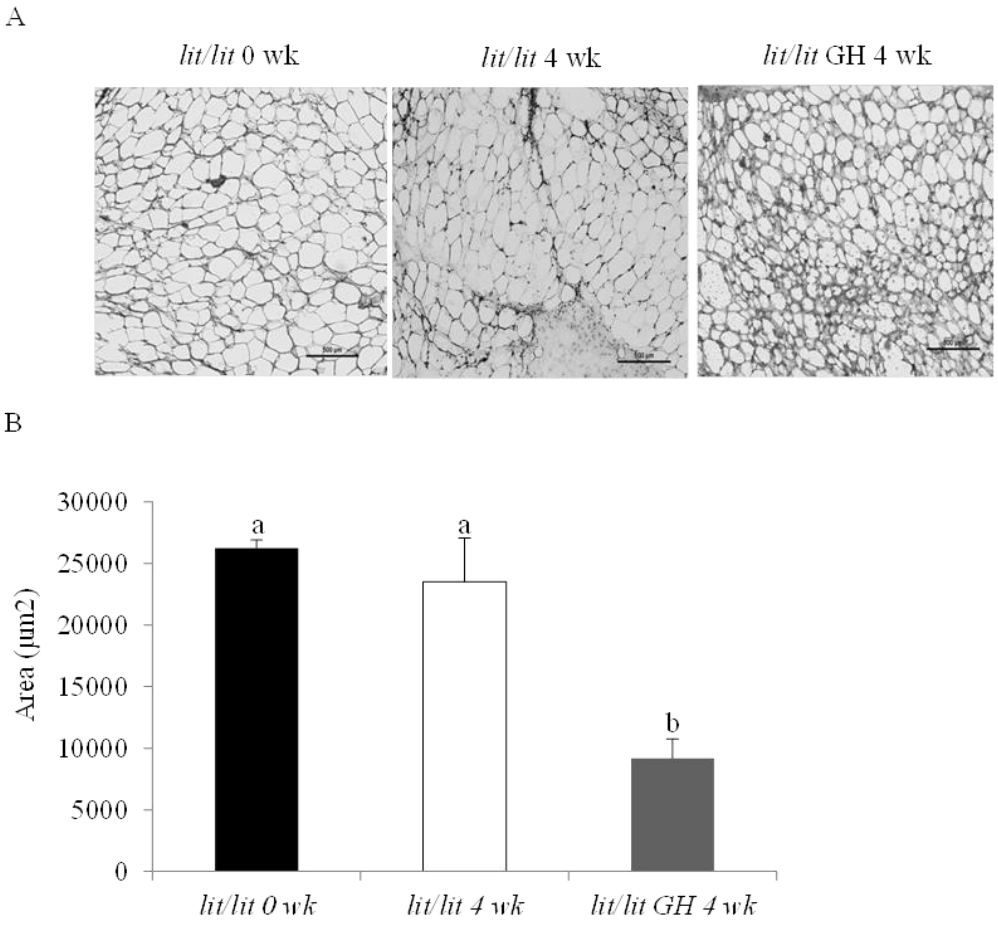


Figure 2.4. Adipocyte size of *lit/lit* mice before and after GH injection. Inguinal subcutaneous fat was taken from *lit/lit* mice at 17 weeks of age (indicated as *lit/lit* 0 wk in the graph), *lit/lit* mice at 21 weeks of age that had received daily injection of buffer (0.01 M NaHCO3) or GH (2 µg/g BW) for four weeks (indicated as *lit/lit* 4 wk and *lit/lit* GH 4 wk in the graph, respectively). (A) Representative photomicrographs of adipose tissue sections stained with hematoxylin and eosin. (B) Average area of adipocytes. Data are expressed as means ± SEM (n=4 mice). Means labeled with different letters are statistically different ($P < 0.05$).

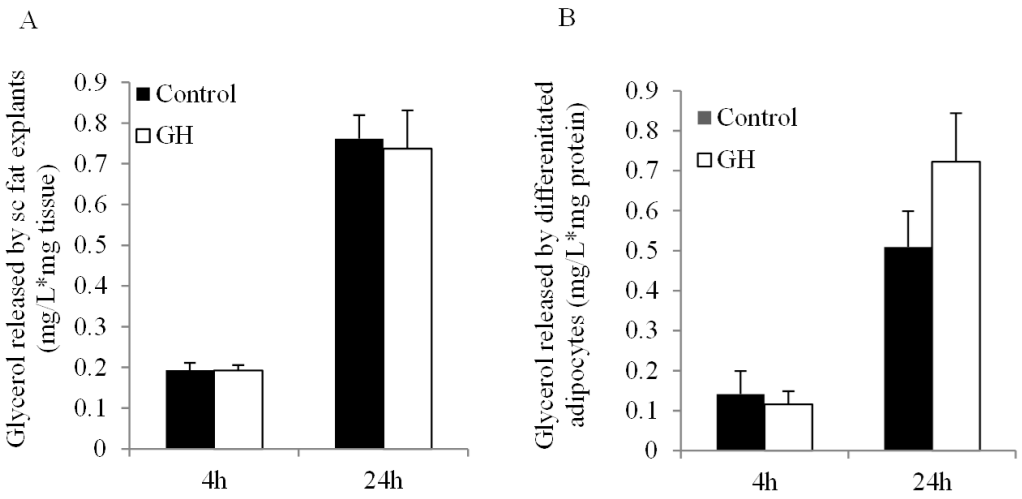


Figure 2.5. Effect of GH on lipolysis of subcutaneous fat explants and adipocytes from *lit/lit* mice. (A) Inguinal subcutaneous fat explants from *lit/lit* mice were cultured with or without (i.e., control) 100 ng/ml GH for 4 h and 24 h, and glycerol released into the medium was measured. (B) Stromal vascular cells from subcutaneous fat of *lit/lit* mice were isolated and differentiated into adipocytes. These adipocytes were cultured with or without 100 ng/ml GH for 4 h and 24 h, and glycerol in the medium was measured. Data are expressed as means ± SEM (n=5 independent culture experiments). Both 4-h and 24-h GH treatment had no effect on glycerol release from either adipose tissue or adipocytes. sc fat: subcutaneous fat.

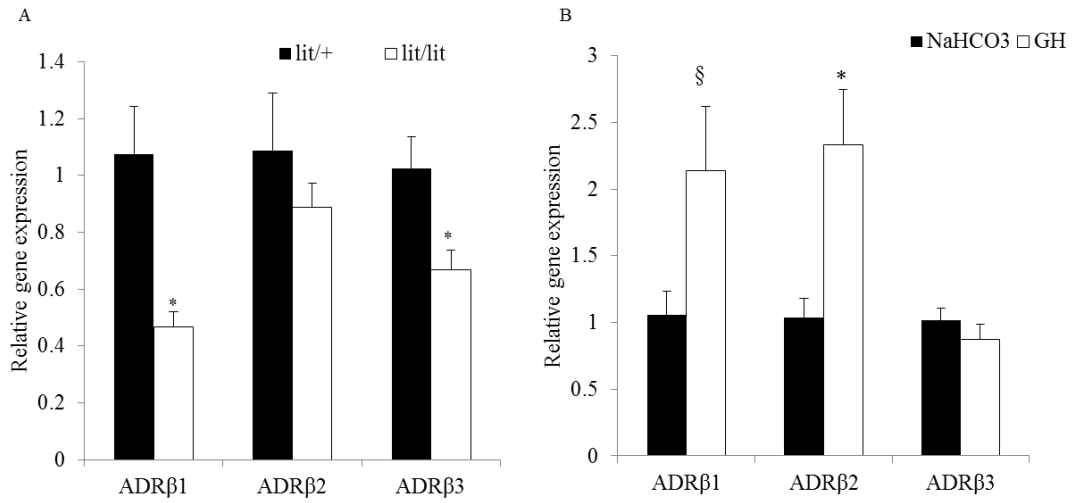
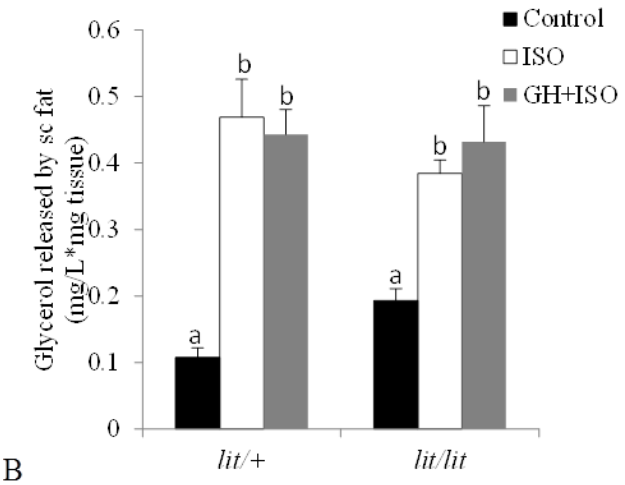


Figure 2.6. mRNA expressions of adrenergic receptors in inguinal subcutaneous fat from *lit/lit* and *lit/+* mice. (A) Subcutaneous fat was taken from 13-week-old *lit/lit* or *lit/+* mice, and mRNA expressions of β1, β2 and β3- adrenergic receptors were measured by real-time PCR. (B) 13-week-old *lit/lit* mice were injected with buffer (0.01 M $NaHCO_3$) or GH (2 μg/g BW). Six hours later subcutaneous fat was taken and subjected to mRNA expression analysis by real-time PCR. 18S was used as an internal control. Data are expressed as means ± SEM (n=5 mice). **P* < 0.05 and §*P* < 0.1 within genes. *ADR*: adrenergic receptor.

A



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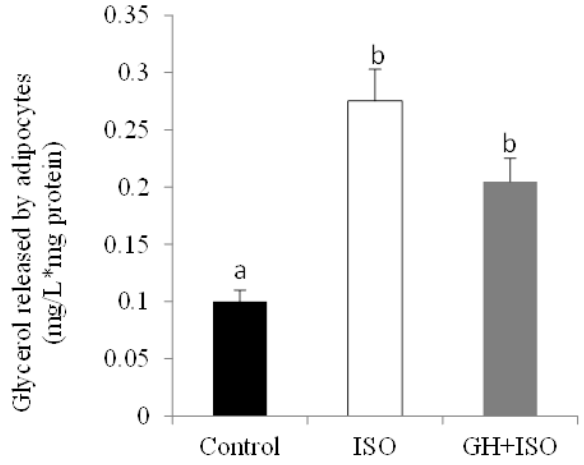

Figure 2.7. Isoproterenol-induced lipolysis in inguinal subcutaneous fat explants and adipocytes from *lit/lit* and *lit/+* mice. (A) Subcutaneous fat explants from *lit/+* and *lit/lit* mice were cultured in the absence (control) or presence of 10 μM of ISO, or 100 ng/ml GH + 10 μM ISO for 4 h, and glycerol released into the medium was measured. (B) Adipocytes differentiated from stromal vascular cells from subcutaneous fat in *lit/lit* mice were cultured in the absence (control) or presence of 1 μM of ISO or 100 ng/ml GH + 1

μM ISO for 4 h. Glycerol in the medium was measured. Data are expressed as means ± SEM (n=5 mice or independent adipocyte cultures). Means labeled with different letters are statistically different ($P < 0.05$). sc fat: subcutaneous fat; ISO: isoproterenol.

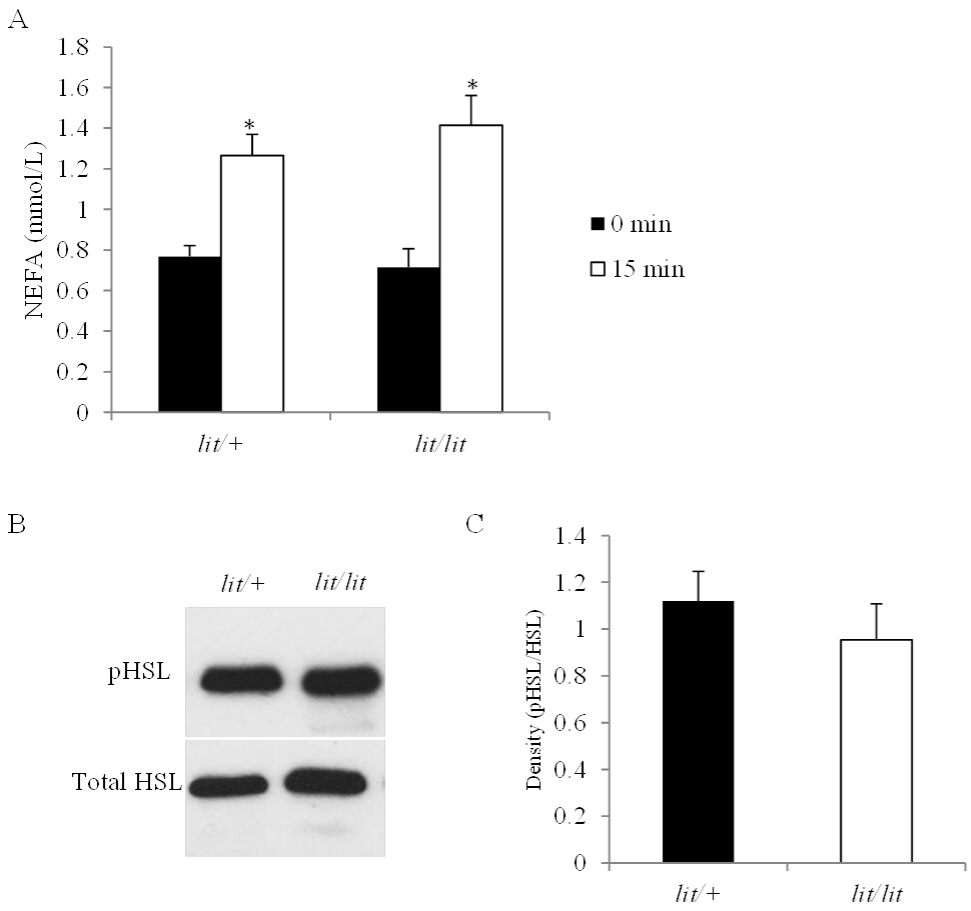

Figure 2.8. Isoproterenol-induced lipolysis in *lit/+* and *lit/lit* mice. Mice were intraperitoneally injected with isoproterenol (10 μg/g BW), and blood sample was collected immediately before and 15 min after the injection. Subcutaneous adipose tissue was taken 15 min after the injection. (A) Serum concentrations of non-esterified fatty acids (NEFA). (B) Representative images of Western blot analyses of phosphorylated hormone-sensitive lipase (pHSL) and total HSL in adipose tissue. (C) Density ratio of pHSL to total HSL. Data are expressed as means ± SEM (n=4 mice). *$P < 0.05$ from 0 time. NEFA concentration and pHSL/HSL are not different between *lit/+* and *lit/lit* mice.

Chapter III

Growth hormone deficiency increases the number of preadipocytes in mouse subcutaneous fat

ABSTRACT

The inhibitory effect of GH on adipose tissue growth is well established, but the underlying mechanism is not fully understood. Here we compared the body composition of growth hormone releasing hormone receptor (*Ghrhr*) mutant *lit/lit* mice (GH deficient mice) with heterozygous lit mutant (*lit/+)* mice. The *lit/lit* mice accumulated more inguinal subcutaneous fat than the *lit/+* mice. There was no difference in epididymal fat accumulation between them. Stromal vascular fraction (SVF) cells from subcutaneous fat of *lit/lit* mice showed greater adipogenic potential in culture than those from *lit/+* mice, as evidenced by Oil Red O staining and expression of adipocyte marker genes including CCAAT/enhancer binding protein α (*Cebpa*), peroxisome proliferator activated receptor γ (*Pparg*), hormone sensitive lipase (*Lipe*) and perillipin-1 (*Plin-1*). Furthermore, SVF cells from *lit/lit* mice expressed higher levels of preadipocyte markers *CD34*, *CD29*, *Sca-1*, and *Pref-1* than those from *lit/+* mice. These differences suggest that GH inhibits adipogenesis in mice at least in part through inhibition of the formation of preadipocytes.

Keywords: Growth hormone; Adipogenesis; Stromal vascular fraction; Mice.

INTRODUCTION

Increase in adipose tissue mass is the result of hyperplasia (increase in cell number) and/or hypertrophy (increase in cell size) (Lane and Tang, 2005). During the development of obesity, hypertrophy usually is observed before hyperplasia (Bjorntorp et al., 1982; Faust et al., 1978). However, the size of adipocytes cannot increase unlimitedly. When adipocytes reach a critical size, they trigger a mechanism to increase the number of adipocytes (Faust et al., 1978). Hyperplasia occurs by proliferation and differentiation of preadipocytes (i.e., adipogenesis), which accounts for the turnover of adipocytes and increased demand of energy storage (Prins and O'Rahilly, 1997). Spalding et al. (2008) compared the adipocyte turnover in obese and lean adults, and found that there was no significant difference in death rate per year between two groups, but obese individuals had a higher production rate per year than lean individuals.

The increase of fat mass is regulated by many hormones acting in endocrine, paracrine or autocrine manners. Growth hormone (GH) is especially important in regulation of growth and metabolism. The inhibitory effect of GH on fat tissue growth has been demonstrated in multiple species (Berryman et al., 2010; Wabitsch and Heinze, 1993; Wiegand et al., 2001). Adipogenic activity is decreased in GH transgenic mice (Chen et al., 2001). In primary cultured rat adipocyte precursor cells, GH markedly reduced the formation of adipocytes (Deslex et al., 1987; Hansen et al., 1998; Wabitsch et al., 1996). However, extensive studies in the 3T3 preadipocyte cell line showed that GH stimulated the differentiation from preadipocytes to adipocytes (Kawai et al., 2007; Morikawa et al., 1982; Yarwood et al., 1999).

Because of these conflicting results, it is unclear if inhibition of adipogenesis is one of the reasons that GH inhibits fat tissue growth in animals. In this study, we aimed to determine if GH inhibits adipogenesis. We conducted the study in the growth hormone releasing hormone receptor (*Ghrhr*) mutant *lit/lit* mice.

MATERIALS AND METHODS

Animals and tissue collection

Breeding pairs of C57BL/6J-Ghrhrlit mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Homozygous *lit/lit* mice have been described previously (Godfrey et al., 1993). The mice were housed on an automatically timed 12 h light/dark cycle at 23 ℃ with free access to standard rodent food and water. All animal-related procedures were approved by the Institutional Animal Care and Use Committee of Virginia Tech.

Isolation and differentiation of stromal vascular fraction of adipose tissue

Mice were euthanized at age of 13-wk-old for collection of subcutaneous fat pads. The stromal vascular fractions (SVF) were isolated as previously described with minor modifications (Hausman et al., 2008). Subcutaneous fat pads were minced and digested using 1 mg/ml of collagenase D (Roche, Indianapolis, IN) in HEPES buffer (0.1 M HEPES, 0.12 M NaCl, 0.05 M KCl, 0.0005 M glucose, 1.5% bovine serum albumin, and 1 mM $CaCl_2$, , pH 7.4) at 37 ℃ with shaking at 115 rpm for 1 h. After centrifugation at 400 g for 10 min, the SVF cell pellet was collected and washed twice with DMEM/F12 (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 2.5 mM of L-glutamine (Mediatech, Manassas, VA), 1% of antibiotics-antimycotics (ABAM; Mediatech, Manassas, VA) (Growth medium). The SVF cells were plated into 6-cm dish and cultured in growth medium. Four or five days later, cells were trypsinized and re-seeded in 6- or 24-well plates at a density of 25,000/cm^2. The cells at 100% confluency were induced to differentiate using the previously

described method (Hausman et al., 2008). This method included initially culturing the cells in DMEM/F12 medium supplemented with 5% FBS, 17 nM insulin, 0.1 μM dexamethasone, 250 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO), and 60 μM indomethacin (MP Biomedical, Solon, OH) for two days, then in DMEM/F12 medium supplemented with 17 nM insulin and 10% FBS for another two days, and lastly in DMEM/F12 supplemented with 10% FBS for four days.

Oil Red O staining

The differentiated cells were washed with phosphate buffered saline (PBS) twice and fixed with 10% phosphate buffered formalin (Fisher Scientific, Pittsburg, PA) for 1 h. Cells were stained with 60% of Oil Red O solution made from 3.5 mg/ml of stock in isopropanol (Fisher Scientific, Pittsburg, PA) for 1 h, and washed with running tap water and photographed, as previously described (Moon et al., 2007).

RNA isolation and real-time PCR

Total RNA from adipocytes was isolated using TRI reagent (Invitrogen, Grand island, NY, USA) according to the manufacture's instruction. Total RNA was reverse-transcribed into cDNA using ImProm-II™ Reverse Transcription system (Promega, Fitchburg, Wisconsin). The cDNA was amplified with primers (Table 1) and Fast SYBRGreen Master Mix (Applied Biosystems, Foster City, CA) on an Applied Biosystems 7500 Real-Time PCR machine.

Statistical analysis

ANOVA was used to compare multiple means followed by Tukey's test. T-test was used to compare two means. These analyses were performed using the General Linear Model of JMP (SAS Institute Inc., Cary, NC). The mouse was the experiment unit, and 4 mice were used in each treatment. Each sample was assayed in duplicate. The model for PPARγ, C/EBPα, HSL, and Perilipin-1 mRNA expression analysis included genotype, day, and genotype ×day interaction. All data are expressed as the mean ± SEM (standard error of the mean). $P < 0.05$ was considered significant.

RESULTS

GH-deficient mice had greater body fat percentages than normal mice

Body weight, and fat tissue weight were compared between 13-wk-old male *lit/lit* and *lit/+* mice. As expected, *lit/lit* mice were 40% lighter than *lit/+* mice (Figure 3.1A, $P < 0.05$). However, *lit/lit* mice had 67% more inguinal subcutaneous fat than *lit/+* mice of the same age (Figure 3.1B). *lit/lit* and *lit/+* mice did not differ in the weight of epididymal fat (Figure 3.1B). When the inguinal subcutaneous fat was compared between the two types of mice as percentage of body weight, the difference was more dramatic, with the former being four times that of the latter (Figure 3.1C, $P < 0.05$). The percentage of epididymal fat was also greater in *lit/lit* mice than in *lit/+* mice (Figure 3.1C, $P < 0.05$). As the homozygous *lit* mutation causes GH deficiency (Godfrey et al., 1993), these data suggested that GH deficiency resulted in greater accumulation of fat, in particular, of subcutaneous fat, in these mice.

Stromal vascular cells from GH-deficient mice had greater adipogenic potential than those from normal mice

The stromal vascular fraction (SVF) from male *lit/+* and *lit/lit* mice at approximately the same age (13 week) were isolated, and induced to differentiate in culture. During the course of differentiation, the SVF cells from both *lit/lit* and *lit/+* subcutaneous fat underwent a morphology change; the cells changed from fibroblast-like morphology to spherical shapes and they gradually accumulated lipids (Figure 3.2). However, a greater percentage of the SVF cells from *lit/lit* mice transformed into adipocyte-like cells than from *lit/+* mice at d 2 and d 4 of differentiation (Figure 3.2). Oil

Red O staining showed that at d 8 of differentiation, more SVF cells from *lit/lit* mice had developed into adipocytes with large lipid droplets than SVF cells from *lit/+* mice (Figure 3.2).

To further assess the adipogenic status of differentiating SVF cells from *lit/lit* and *lit/+* mice, mRNA levels of several adipocyte markers were quantified by real-time RT-PCR. These markers included CCAAT/enhancer-binding protein α (*Cebpa*), peroxisome proliferator activated receptor γ (*Pparg*), hormone sensitive lipase (*Lipe*), and perlipin-1 (*Plin1*) genes. At day 2, 4, and 8 of differentiation, all of these genes were expressed at much higher levels in SVF cells from *lit/lit* mice than in those from *lit/+* mice (Figure 3.3, $P < 0.05$). The mRNA levels of *Cebpa* and *Pparg*, which are two master transcriptional regulators of adipogenesis, were approximately 10 times higher in differentiating SVF cells from *lit/lit* mice than in those from *lit/+* mice (Figure 3.3). The mRNA levels of *Lipe* and *Plin1*, two genes that are involved in lipid metabolism in adipocytes (Yeaman, 1990), were more than a hundred times higher in SVF cells from *lit/lit* mice than from lit/+ control mice (Figure 3.3, $P < 0.01$). These observations together demonstrated that the SVF cells from *lit/lit* subcutaneous fat had greater potential to differentiate into adipocytes than those from *lit/+* mice.

SVF cells from GH-deficient mice had more adipogenic precursor cells than those from normal mice

The SVF cells are believed to contain endothelial precursor cells, T regulatory cells, macrophages, preadipocytes, and adipocyte stem cells that are capable of proliferating and differentiating into mature adipocytes (Riordan et al., 2009). These

adipocyte stem cells express the following markers on their cell surface: CD29, CD34, Sca-1, and CD24 (Rodeheffer et al., 2008). We next determined the possibility that SVF cells from *lit/lit* mouse fat contain more adipocyte stem cells and therefore have greater adipogenic capacity than those from *lit/+* mice. We analyzed the expression of *CD29*, *CD34*, *Sca-1*, and *CD24* mRNAs in SVF cells from *lit/lit* and *lit/+* mice. The result showed that *CD29*, *CD34* and *Sca-1* mRNAs were expressed at higher levels in *lit/lit* than *lit/+* SVF cells (Figure 3.4, $P < 0.05$); there was a trend that *lit/lit* SVF cells expressed more *CD24* mRNA than *lit/+* SVF cells (Figure 3.4, $P < 0.1$). We also measured mRNA expression of preadipocyte factor -1 (*Dlk-1*), a widely considered preadipocyte marker (Smas and Sul, 1993; Wang et al., 2006). *Dlk-1* mRNA was also expressed at higher levels in *lit/lit* than *lit/+* SVF cells (Figure 3.4, $P < 0.05$). These data indicated that the SVF cells from *lit/lit* mice contained more adipogenic precursor cells.

DISCUSSION

Inhibitory effect of GH on fat tissue growth has been consistently demonstrated (Berryman et al., 2010; Wabitsch and Heinze, 1993; Wiegand et al., 2001), but the underlying mechanism is not fully understood. In this study, we aimed to gain new understanding of this mechanism in the GH deficient *lit/lit* mice. As expected, these GH-deficient mice were growth retarded but had more subcutaneous fat compared to normal mice at the same age. These phenotypes of GH-deficient mice are consistent with those of growth hormone receptor knockout (Berryman et al., 2004). Similar changes in body weight and composition were also found in growth hormone-deficient humans (Rosen et al., 1993; Wabitsch and Heinze, 1993). In our study and a previous study by Berryman et al. (2004), epididymal fat weight in GH- or GH receptor-deficient mice was not significantly different from controls. Although the percentage of the epididymal fat was higher in *lit/lit* mice than in *lit/+* mice, the difference was not as much as that of subcutaneous fat. These results indicate that the effect of GH on adipose tissue growth is depot specific, with subcutaneous fat being more sensitive to GH action.

During the development of adult obesity, hypertrophy is considered to be the initial event (Fajas, 2003). However, the existing adipocytes cannot enlarge infinitely, therefore the increased adipocyte number accounts for much of the increase of adipose tissue mass. The adipose tissue contains not only adipocytes but also a stromal vascular fraction, which contains adipocyte progenitors (Tang et al., 2008). We compared the differentiation ability of SVF cells from GH deficient *lit/lit* mice and *lit/+* control. This result showed that SVF cells in subcutaneous fat from *lit/lit* mice had greater potential to differentiate into adipocytes than those from *lit/+* mice. This indicates that after a long-

term GH deficiency, either more preadipocytes are recruited in the adipose tissue or the existing preadipocytes become more sensitive to adigogenic hormones. Consequently, more adipocytes are generated in *lit/lit* mice.

Adipocyes are derived from mesenchymal stem cells, which also give rise to muscle and cartilage precursor cells depending on the conditions (Dicker et al., 2005). Two phases are used to describe adipogenesis from mesenchymal stem cells to adipocytes (Rosen and MacDougald, 2006): one is determination which results in the conversion of stem cells into preadipocytes; the other is terminal differentiation which results in the differentiation of preadipocytes into adipocytes. Mesenchymal stem cells express certain types of markers on their surface. In adipose tissue, the Lin^- :$CD29^+$:$CD34^+$:$Sca\text{-}1^+$:$CD24^+$ subpopulation in SVF was identified to be capable of differentiating into adipocytes both *in vitro* and *in vivo* (Rodeheffer et al., 2008). In this study, we measured mRNA expression of those markers in SVF from *lit/lit* and *lit/+* mice. The result showed that almost all of these markers were expressed at higher levels in SVF cells from *lit/lit* mice than from *lit/+* mice. This suggested that there might be more adipocyte stem cells in SVF of *lit/lit* mouse fat than in that of *lit/+* mouse fat. *Dlk-1* is expressed in many mouse embryonic tissues, but becomes restricted to certain types of cells, such as preadipocytes, after birth (Wang et al., 2006). *Dlk-1* is considered as an excellent preadipocyte marker because it is highly expressed in preadipocytes and silenced during differentiation into adipocytes (Smas and Sul, 1993). In this study we found that the mRNA level of *Dlk-1* was significantly higher in *lit/lit* SVF cells than in *lit/+* SVF cells. This again indicates that the *lit/lit* mouse fat contains more preadipocytes

than the *lit/+* mouse fat. These results suggest that GH participates in the regulation of commitment of adipose stem cells to preadipocytes.

In summary, the present study shows that GH deficiency increases subcutaneous fat accumulation in mice and the number of adipogenic precursor cells in subcutaneous fat. These results suggest that GH may inhibit adipose tissue growth in part by inhibiting the formation of preadipocytes from adipose stem cells.

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Table 3.1. Primers for RT-PCR analyses.

Target name[1]	Primer sequence (5'to 3')	GenBank #	Amplicon size (bp)
Pparg	Forward: TTCAGAAGTGCCTTGCTGTG Reverse: CCAACAGCTTCTCCTTCTCG	NM_001127330.1	84
Cebpa	Forward: AGCAACGAGTACCGGGTACG Reverse: TGTTTGGCTTTATCTCGGCTC	NM_007678.3	71
Lipe	Forward: TCGCTGTTCCTCAGAGACCT Reverse: CTGCCTCAGACACACTCCTG	NM_001039507	151
Plin-1	Forward**:** AAGGATCCTGCACCTCACAC Reverse: CCTCTGCTGAAGGGTTATCG	NM_175640.2	191
CD24	Forward:ATGCCGCTATTGAATCTGCTGGAG Reverse:TGCACTATGGCCTTATCGGTCAGA	NM_009846	210
CD29	Forward: CAATGGCGTGTGCAGGTGTC Reverse: ACGCCAAGGCAGGTCTGAC	NM_010578.2	207
CD34	Forward: GCAGGTCCACAGGGACACGC Reverse: TGGCTGGTACTTCCAGGGATGCT	NM_001111059.1	198
Sca-1	Forward: GGGACTGGAGTGTTACCAGTGCTA Reverse: AGGAGGGCAGATGGGTAAGCAA	NM_010738.2	166
Dlk-1	Forward: GCGTGGACCTGGAGAAAGGCCA Reverse: GGAAGTCACCCCCGATGTCGGT	NM_010052.5	276
18s	Forward:TTAAGAGGGACGGCCGGGGG Reverse: CTCTGGTCCGTCTTGCGCCG	NR_003278.1	77

[1]*Pparg* = Peroxisome proliferator activated receptor γ; *Cebpa* = CCAAT/enhancer binding protein α; *lipe* = Hormone sensitive lipase; *CD* = cluster of differentiation molecule; *Sca-1* = Stem cell antigen-1; *Dlk-1* = preadipocyte factor-1; *18s* = 18s ribosomal RNA.

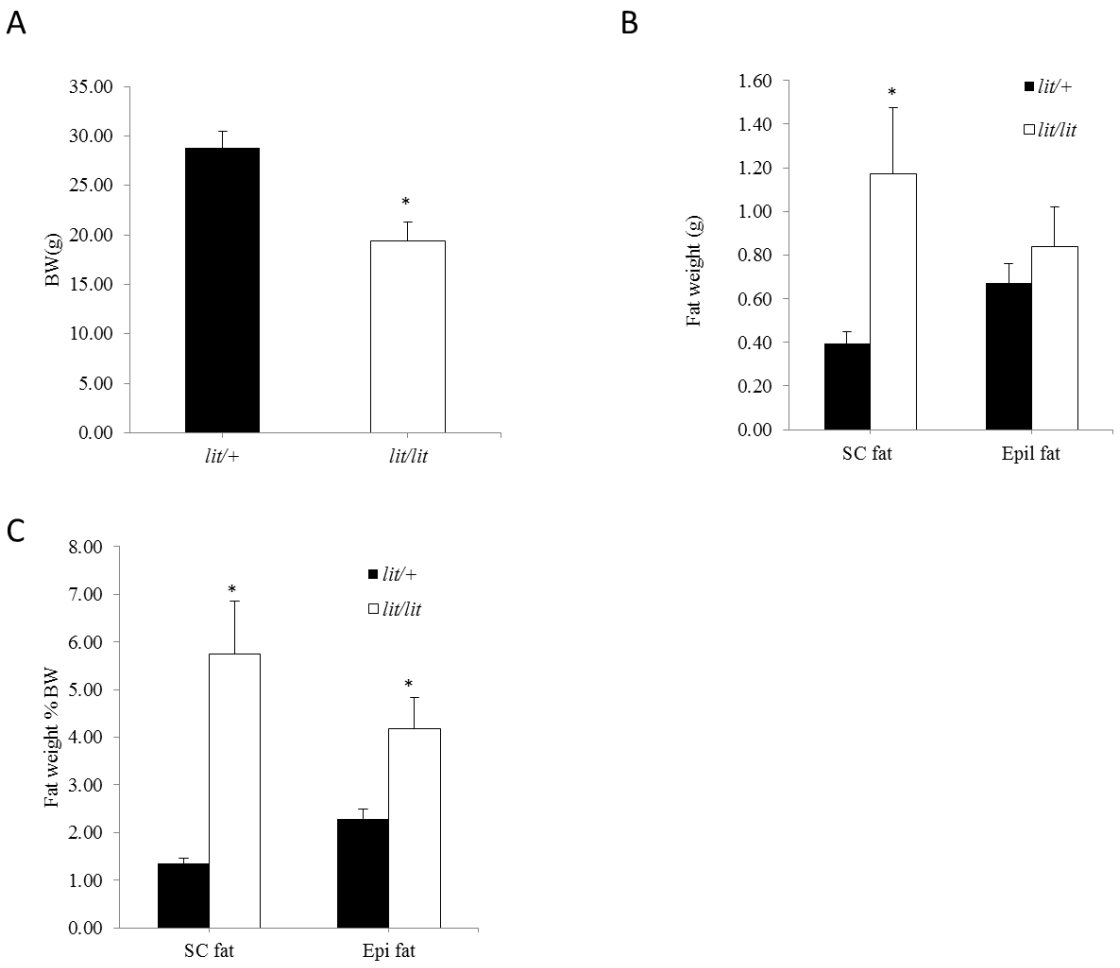

Figure 3.1. Body composition of male *lit/+* and *lit/lit* mice (A) Body weight. (B) subcutaneous fat, and epididymal fat weight. (C) Percentage of subcutaneous and epididymal fat of body weight. Mice of 13-week-old were analyzed. Data are expressed as means ± SEM (n=4 mice per group). *$P < 0.05$ vs. *lit/+*. BW: body weight; SC fat: subcutaneous fat; Epi fat: epididymal fat.

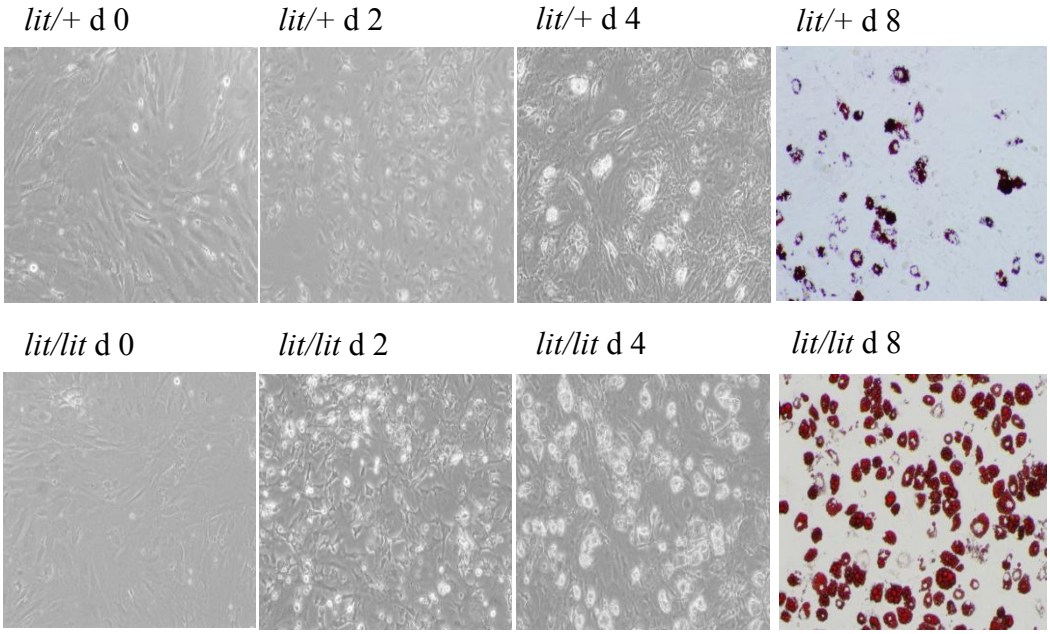

Figure 3.2. Differentiation of stromal vascular fraction cells of subcutaneous fat from *lit/+* and *lit/lit* mice. Stromal vascular fraction was isolated from inguinal subcutaneous fat, expanded in growth medium for 5 to 7 days, and then induced to differentiate into adipocytes using standard protocols. Microscopy pictures were taken on d 0 (right before induction of differentiation), d 2, d4, and d 8 of induced differentiation at 10x magnification. Adipocytes on d 8 of differentiation were stained with Oil red O. Pictures are representatives of 4 independent experiments.

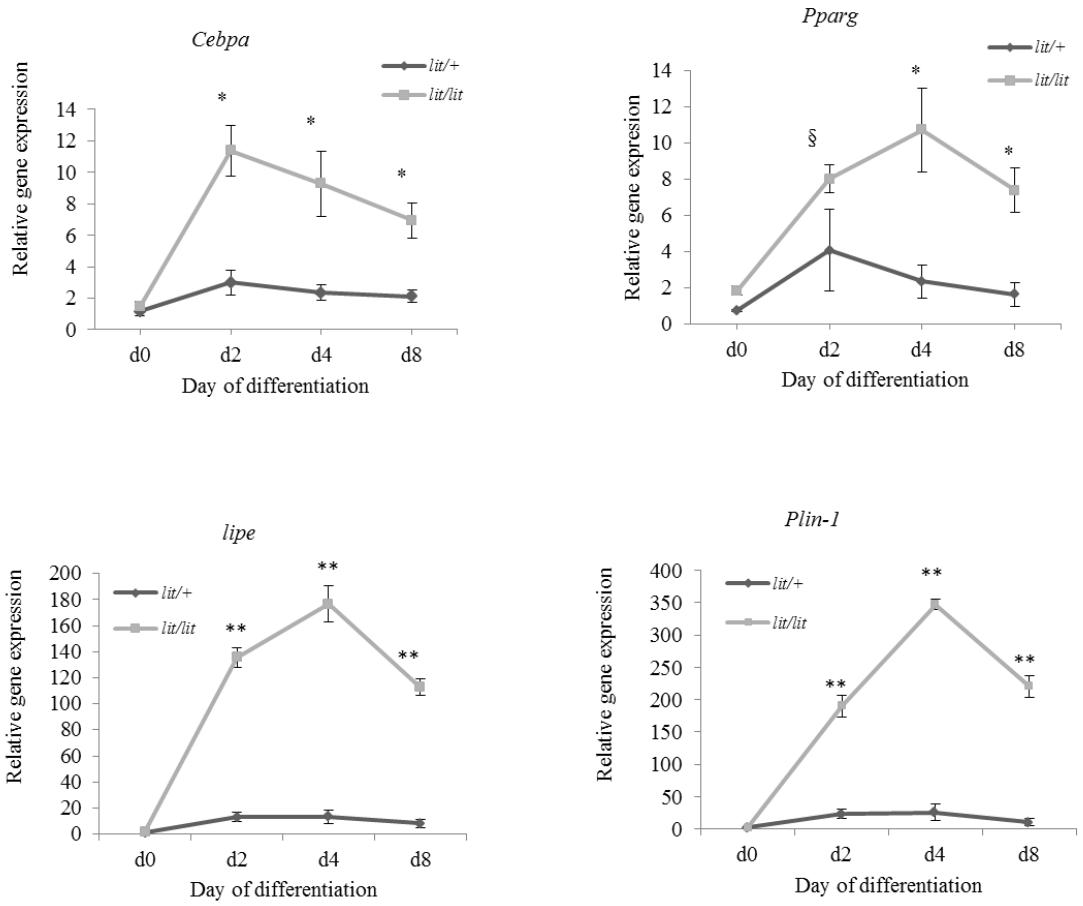

Figure 3.3. Expression of *Cebpa, Pparg, lipe* and *Plin-1* mRNAs in differentiating SVF cells from subcutaneous fat of *lit/lit* and *lit/+* mice. Stromal vascular fraction was isolated from inguinal subcutaneous fat, expanded in growth medium for 5 to 7 days, and then induced to differentiate into adipocytes using standard protocols.Cells were collected for RNA extraction at indicated time points. mRNA was quantified by real-time PCR, using 18s as an internal control. Cebpa: C/EBPα; Pparg: PPARγ; *lipe*: HSL; *Plin-1*: perilipin-1. Data are expressed as means ± SEM (n = 4 independent cell culture experiments). **P* < 0.05, ***P* < 0.01, and §*P* < 0.1 vs. *lit/+*.

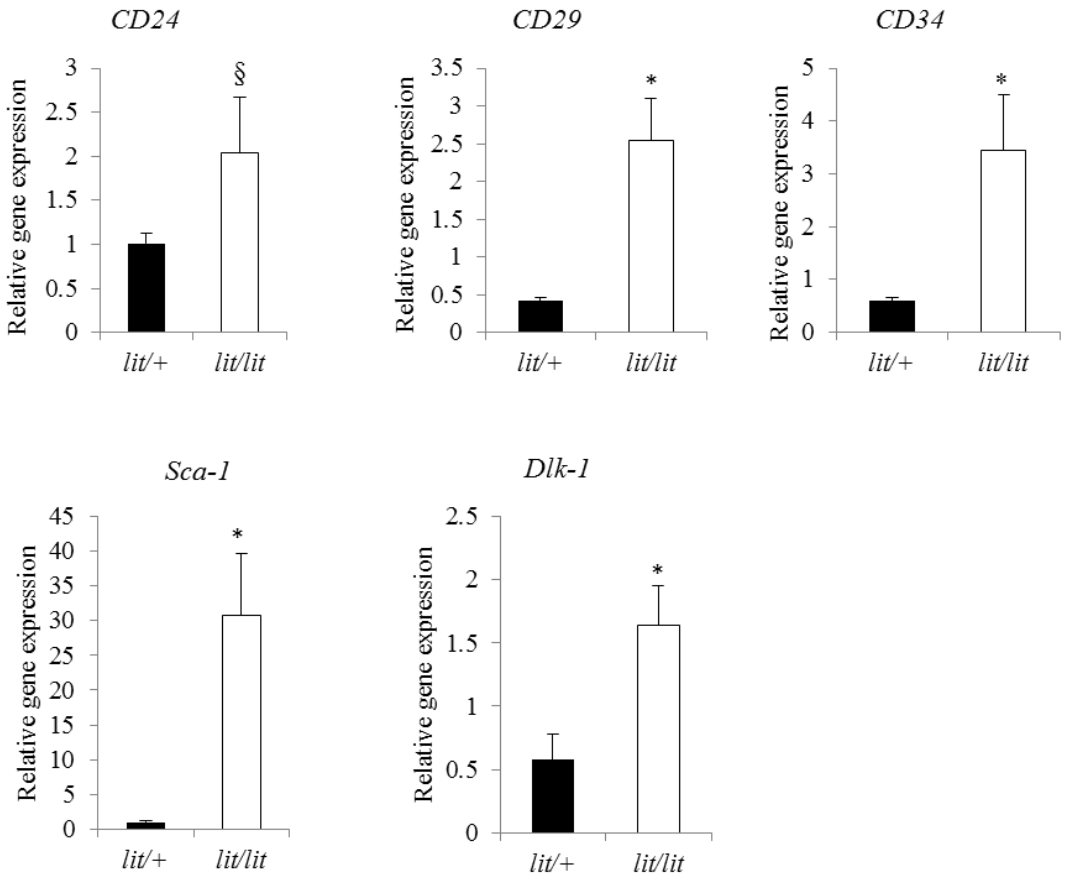

Figure 3.4. mRNA levels of adipocyte stem cell markers in SVF cells from subcutaneous fat of *lit/lit* and *lit/+* mice. Stromal vascular fraction was isolated from inguinal subcutaneous fat, and incubated in growth medium for 24 h. Total RNA was extracted from attached cells and subjected to real-time PCR analysis. mRNA abundance was normalized to that of 18s rRNA. Data are expressed as means ± SEM (n = 4 independent cell culture experiments). *$P < 0.05$ vs. *lit/+*; §$P < 0.1$ vs. *lit/+*.

Chapter IV

Growth hormone inhibits differentiation of bovine preadipocytes into adipocytes partly through signal transducer and activator of transcription 5

ABSTRACT

In this work, we determined the effect of growth hormone (GH) on the differentiation of primary bovine preadipocytes into adipocytes and the role of signal transducer and activator of transcription 5 (STAT5) in this effect. Preadipocytes were derived from bovine adipose tissue explants in culture and were induced to differentiate into adipocytes in the absence or presence of recombinant bovine GH or adenovirus expressing constitutively active STAT5b (STAT5bCA). Differentiation status of preadipocytes was assessed by Oil Red O staining and by measuring glycerol-3-phosphate dehydrogenase (G3PDH) activity and the rate of acetate incorporation. Fewer preadipocytes became adipocytes in the presence of GH. Adipocytes treated with GH had lower G3PDH activity and lower rate of acetate incorporation, and lower expression of CCAAT/enhancer binding protein α (*C/EBPα*) and peroxisome proliferator-activated receptor γ (*PPARγ*) mRNAs. Adipocytes cultured in the presence of STAT5bCA adenovirus formed fewer adipocytes and had lower G3PDH activity, and lower acetate incorporation rate, compared to those cultured in the presence of control adenovirus. STAT5bCA overexpression reduced the expression of *C/EBPα* mRNA but not that of *PPARγ* in adipocytes. Addition of the synthetic STAT5 inhibitor N-((4-Oxo-4H-chromen-3-yl) methylene) nicotinohydrazide blocked GH-induced phosphorylation of

STAT5 but not GH-induced decrease in *PPARγ* mRNA expression. Taken together, these results suggest that GH inhibits differentiation of primary bovine preadipocytes into adipocytes by inhibiting *C/EBPα* and *PPARγ* expression and that STAT5 is involved in GH inhibition of *C/EBPα* expression but not in that of *PPARγ* expression.

Keywords: Growth hormone; STAT5; Bovine; Preadipocytes.

INTRODUCTION

Adipogenesis is the process of differentiation of preadipocytes into lipids containing mature adipocytes. Two transcription factors, C/AAAT enhancer binding protein α (C/EBPα) and peroxisome proliferators activated receptor γ (PPARγ) play a central role in this process (Rangwala and Lazar, 2000). PPARγ initiates the adipogenic program (Tontonoz et al., 1994). In late differentiation, PPARγ also induces *C/EBPα* expression (Rangwala and Lazar, 2000), and C/EBPα and PPARγ together maintain adipocyte differentiation (Rosen and MacDougald, 2006). Two splicing variants of PPARγ, γ1 and γ2, are expressed; PPARγ2 is considered to be adipose tissue specific (Rangwala and Lazar, 2000).

Growth hormone (GH) is a polypeptide hormone produced by the anterior pituitary. Besides stimulating body growth, GH is also known to exert metabolic effects, especially on glucose and lipid metabolism. GH deficiency causes overweight or obesity (Berryman et al., 2006; Rosen et al., 1993; Wabitsch and Heinze, 1993). GH treatment is effective in decreasing body fat in GH-deficient subjects (Bengtsson et al., 1993; Miller et al., 2010; Ottosson et al., 2000). The mass of adipose tissue is determined by adipogenesis, lipogenesis, and lipolysis. GH may have an effect on each of these processes. GH stimulates lipolysis in various species, although the underlying mechanism is unclear (Fain et al., 1999; Marcus et al., 1994a; Marcus et al., 1994b). Studies in pigs and sheep have demonstrated an inhibitory effect of GH on lipogenesis (Borland et al., 1994; Donkin et al., 1996). Compared to the effects of GH on lipolysis and lipogenesis, that of GH on adipogenesis is less clear. Whereas studies using preadipocyete cell lines suggest a stimulatory effect of GH on adipogenesis, those using primary preadipocytes

indicate the opposite (Gerfault et al., 1999; Hansen et al., 1998; Kawai et al., 2007; Yarwood et al., 1999). Clearly, an inhibitory effect of GH on adipogenesis would fit with its inhibitory effect on adipose tissue mass, but this needs to be substantiated with additional studies because, compared to the extensive studies on preadipocyte cell lines, limited research has been conducted to determine the effect of GH on the differentiation of primary preadipocytes (Gerfault et al., 1999; Hansen et al., 1998; Wabitsch et al., 1996; Yang et al., 2012). Furthermore, little is known about the mechanism of the inhibitory effect of GH on the differentiation of primary preadipocytes.

The objective of this study was, therefore, to determine the effect of GH on differentiation of primary preadipocytes and the underlying mechanism. In this study, we used bovine preadipocytes as the primary preadipocyte model because they represent a species in which the effect of GH on adipogenesis has not been previously tested. In studying the mechanism, we focused on the role of signal transducer and activator of transcription 5 (STAT5) because this transcription factor plays a central role in mediating the action of GH at the cellular level (Lanning and Carter-Su, 2006).

MATERIALS AND METHODS

Bovine preadipocytes preparation and culture

Bovine preadipocytes were prepared as previously described by Lengi and Corl (2010). Briefly, subcutaneous fat was collected from steers when slaughtered and transported in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Manassas, VA) to the lab in less than 30 minutes. The tissue was minced into small pieces and then cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 1% of antibiotics-antimycotics (ABAM; Mediatech, Manassas, VA) and 2 mM of L-glutamine (Mediatech, Manassas, VA) at 37 ℃ and 5% of CO_2. Medium was changed every 4 days. After 10 days, cells derived from the explants were collected and plated into 6-well plates or 24-well plates at a density of $2.5 \times 10^4/cm^2$. When cells reached 100% confluency, they were induced to differentiate into adipocytes. In the first 2 days of differentiation, the cells were cultured in serum-free DMEM/F12 (1:1 vol/vol, Sigma-Aldrich, St. Louis, MO) supplemented with 10 μg/ml insulin (Sigma-Aldrich, St. Louis, MO), 0.25 μM dexamethasone (Sigma-Aldrich, St. Louis, MO), 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St. Louis, MO), and 1 μM rosiglitazone (Cayman Chemical, Ann Arbor, MI). Thereafter, cells were cultured in serum-free DMEM/F12 (1:1 vol/vol) containing 10 μg/ml insulin and 1 μM rosiglitazone. The medium was refreshed every other day during differentiation and treated with GH (The National Hormone and Peptide Program, Torrance, CA), adenovirus or STAT5 inhibitor (Santa Cruz Biotechnology, Santa Cruz, CA).

Oil Red O staining

Adipocytes were stained with Oil Red O (Fisher Scientific, Pittsburg, PA) on day 6 of differentiation. Cells were washed twice with phosphate buffered saline (PBS) and subsequently fixed with 10% formalin in PBS for 1 h at room temperature. Following fixation, cells were washed twice with PBS and subsequently stained with 60% Oil Red O solution for 1 h. Cells were then washed with running tap water and photographed.

Gycerol-3-phosphate dehydrogenase activity assay

Gycerol-3-phosphate dehydrogenase (G3PDH*)* activity was measured following a previously described protocol (Wise and Green, 1979). Adipocytes in 6-well plates were washed twice with PBS and were then scraped in 150 µL of extraction buffer (50 mM Tris-HCl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 7.5). The cell suspension briefly sonicated on ice using a Sonic Dismembrator (Model 100, Fisher Scientific, Pittsburg, PA). The cell lysate was centrifuged at 10,000 ×g for 30 min at 4 ℃. The supernatant was collected and added to 100 µl reaction buffer consisting of 100 mM triethanolamine-HCl (pH 7.5; Fisher Scientific, Pittsburg, PA), 2.5 mM EDTA, 0.12 mM NADH (Sigma-Aldrich, St. Louis, MO), 0.2 mM dihydroxy acetone phosphate (Sigma-Aldrich, St. Louis, MO), and 0.1 mM β-mercaptoethanol. The OD values at 340 nm were measured 20 times within 5 min at room temperature. The slope of OD change with time (ΔOD/min) was used to calculate the total activity of G3PDH (Wise and Green, 1979). Total protein concentration in cell lysate was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL). All treatments were tested in duplicate. The G3PDH activity was normalized to the protein concentration in the same cell lysate to control for well-to-well variation in cell numbers.

Acetate incorporation assay

Acetate incorporation assay was conducted as previously described (Lengi and Corl, 2010). In brief, the medium of differentiating adipocytes was replaced with fresh differentiation medium supplemented with 1 μCi ^{14}C-acetic acid (ARC Inc., St. Louis, MO). The incubation was continued for another 4 h at 37 ˚C and 5% of CO_2. Cells were washed twice with PBS and lysed in 0.1% SDS. The lysate was extracted with hexane:isopropanol (3:2). The organic phase was transferred to the scintillation vials and subjected to scintillation counting. Each sample was counted in duplicate.

Preparation of whole cell lysates and Western blot analysis

Cells were washed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxychlolate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitors and phosphatase inhibitors (Roche, Indianapolis, IN), and sonicated as described above. The whole cell lysates were centrifuged at 13,000 ×g for 15 min at 4 ℃. Protein concentrations were measured as described above. Equal amounts of protein samples were separated by 10% SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes. Membranes were immunoblotted with mouse anti-STAT5 (at 1:1,000 dilution), anti-phosphorylated STAT5 (1:1,000), or anti β-actin antibody (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA). Primary antibodies were detected by 1:1,000 diluted anti-mouse horseradish peroxidase-coupled IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody was detected by incubating the membrane with ECL substrate (Thermo Scientific, Rockford, IL).

Total RNA extraction and real-time PCR

Total RNA from the cells was isolated using TRI reagent (MRC, Cincinnati, OH), according to the manufacturer's instruction. cDNA was generated using the ImProm-II™ Reverse Transcription System (Promega, Fitchburg, Wisconsin), according to the manufacturer's instruction. PPARγ, PPARγ1, PPARγ2, and C/EBPα mRNAs were quantified by real-time PCR using the *Power* SYBR® Green PCR Fast Master Mix (Applied Biosystems, Foster City, CA). Sequences of primers are shown in Table 1. Each sample was quantified in duplicate. 18s rRNA was used as the internal control. Based on the Ct values, 18s rRNA had a consistent expression across the samples ($P > 0.1$). The relative quantification of mRNA was obtained using the comparative threshold cycle (ΔΔCt) method.

STAT5bCA and LacZ adenovirus generation

cDNA encoding constitutively active STAT5b (STAT5bCA) was kindly provided by Dr. Peter Rotwein (Oregon Health and Science University). This cDNA contained a substitution of histidine for asparagine at position 642 compared to the natural STAT5b (Woelfle et al., 2003). Adenovirus expressing STAT5bCA was generated using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA), according to the manufacturer's instruction. Briefly, STAT5bCA cDNA was inserted into the adenoviral expression vector pAd/CMV/V5-DEST to generate pAD/CMV/STAT5bCA. This plasmid was confirmed by sequencing and transfected into 293A cells using FuGENE6 (Promega, Fitchburg, Wisconsin). Cells were harvested at the time of 70-80% plaque formation, followed by 3 cycles of freezing (-80 ℃) and thawing (37 ℃). Crude virus in the supernatant was collected by centrifuging at 3,000 ×g for 15 min at 4 ℃. The crude virus was amplified twice in 293A cells and then purified using a cesium chloride (CsCl)

density gradient combined with ultracentrifugation. We also constructed a LacZ expression adenovirus using the construct pAd/CMV/V5-GW/lacZ from Invitrogen. The virus was titered using the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA). The ability of the STAT5bCA adenoviruses to express constitutively active STAT5 was confirmed by an electrophoretic mobility shift assay, in which the expressed protein was able to bind to a known STAT5 binding DNA element (data not shown).

Statistical analysis

Data were statistically analyzed using General Linear Model of JMP (SAS Inst. Inc., Cary, NC). ANOVA was used to compare treatments. When the effects of treatments were significant, treatment means were separated using LSD comparison. T-test was used to compare two means. Differences were considered significant when $P < 0.05$. An individual steer was considered experiment unit. Each assay was repeated at least 4 times in duplicated wells. Samples taken from each well were measured in duplicate for G3PDH activity, acetate incorporation, and mRNA expression assay.

RESULTS

GH inhibited differentiation of bovine preadipocytes into adipocytes

To investigate the effect of GH on adipogenesis, preadipocytes derived from bovine adipose tissue explants in culture were induced to differentiate in the absence or presence of 100 ng/ml recombinant bovine GH for 6 days. On day 0, the cells had a fibroblast-like appearance with no visible lipid droplets when observed with a microscope. At day 6 of differentiation, many of the cells acquired a round shape, and lipid droplets became visible in the cytoplasm. Lipid accumulation in adipocytes was confirmed by Oil Red O staining. Fewer preadipocyes cultured in the presence of GH formed adipocytes than those cultured without GH (Figure 4.1A). To further determine the effect of GH on differentiation of preadipocytes into adipocytes, we measured the activity of G3PDH in differentiating preadipocytes. G3PDH catalyzes the formation of glycerol and is considered the rate-limiting enzyme for triglyceride synthesis in adipocytes (Wise and Green, 1979). As can be seen in Figure 4.1B, preadipocytes that were induced to differentiate in the presence of GH had less G3PDH activity than those in the absence of GH ($P < 0.05$). In ruminant adipose tissue, acetate is the primary source for *de novo* fatty acid synthesis (Chilliard, 1993). We measured the rate of acetate incorporation into lipids as another index of the differentiation status of bovine preadipocyets. As can be seen in Figure 4.1C, the rate of acetate incorporation in preadipocytes induced to differentiate in the presence of GH was 40% lower than those without GH ($P < 0.05$).

Effects of GH on C/EBPα and PPARγ mRNA expression in bovine preadipocytes

Differentiation of preadipocytes into adipocytes is primarily driven by the two transcription factors PPARγ and C/EBPα. To determine the possibility that GH inhibits differentiation of preadipocytes by inhibiting the expression of PPARγ and/or C/EBPα, we quantified the mRNA levels of these two genes in bovine preadipocytes differentiated in the presence or absence of GH. As can be seen from Figure 4.2A, preadipocytes induced to differentiate for 6 days in the presence of GH expressed less C/EBPα, total PPARγ, PPARγ1, and PPARγ2 mRNAs than those differentiated without GH (Figure 4.2A).

To test the possibility that GH inhibits differentiation of preadipoctyes by directly inhibiting the expression of PPARγ and/or C/EBPα, we determined the effect of short-term GH treatment on PPARγ and C/EBPα expression in bovine preadipoctyes before and during induced differentiation. Treating preadipocytes before differentiation with 100 ng/mL GH for 6 hours had no effect on the mRNA level of PPARγ or C/EBPα (data not shown). The same GH treatment caused a 58% reduction in total PPARγ, and a 64% reduction in PPARγ1 mRNA expressions in differentiating preadipocyes compared to the control ($P < 0.05$, Figure 4.2B). The 6-h GH treatment tended to reduce PPARγ2 mRNA expression in differentiating preadipocytes ($P = 0.1$, Figure 4.2B).

Effect of STAT5 overexpression on differentiation of bovine preadipocytes

STAT5 is a transcription factor activated by GH through phosphorylation and plays a central role in mediating the effect of GH on gene expression (Lanning and Carter-Su, 2006; Piwien-Pilipuk et al., 2002). To investigate the role of STAT5 in GH inhibition of preadipocyte differentiation, we determined if overexpression of

constitutively active STAT5b (STATbCA) would mimic the effect of GH on preadipocyte differentiation. Overexpression of STAT5bCA was achieved through adenoviral transduction. Increased expression of STAT5bCA was confirmed by Western blot analysis (Figure 4.3A). The Oil Red O staining showed that both LacZ (adenoviral control) and STAT5bCA adenoviruses inhibited differentiation of bovine preadipocytes compared to the no-virus control. However, the number of Oil Red O-stained cells transduced with the STAT5bCA adenovirus was less than that with the LacZ adenovirus (Figure 4.3B), indicating that STAT5bCA overexpression inhibited bovine preadipocyte differentiation. The inhibition of LacZ adenovirus on preadipocyte differentiation was probably a side effect of adenoviral transduction.

We also determined the effects of STAT5bCA overexpression on the activity of G3PDH and the rate of acetate incorporation in differentiating preadipocytes. As shown in Figure 4.3C and 4.3D, STAT5bCA adenovirus inhibited both the G3PDH activity and the rate of acetate incorporation compared with the no-viral control or the LacZ adenovirus ($P < 0.05$). These data indicated that the inhibitory effect of GH on preadipocyte differentiation might be mediated in part by STAT5.

Effects of STAT5 overexpression on PPARγ and C/EBPα gene expression

To further determine the role of STAT5 in mediating the effect of GH on preadipocyte differentiation, we measured *PPARγ* and *C/EBPα* mRNA levels in differentiating preadipocytes infected with STAT5bCA adenovirus, LacZ adenovirus, or not infected by either type of adenovirus. The STAT5bCA adenovirus reduced expression of *C/EBPα* mRNA in preadipocytes at day 6 of differentiation compared with the LacZ

adenovirus or the no-adenovirus control ($P < 0.05$). However, the STAT5bCA adenovirus did not change the expression of total *PPARγ, PPARγ1,* or *PPARγ2* mRNA (Figure 4.4). These data indicated that STAT5 might mediate the inhibitory effect of GH action on *C/EBPα* but not on *PPARγ* expression in bovine preadipocytes.

Effects of STAT5 inhibitor on GH-induced inhibition of PPARγ and C/EBPα gene expression

To further investigate the role of STAT5 in mediating the inhibitory effects of GH on *PPARγ* and *C/EBPα* gene expression, we treated the differentiating bovine preadipocytes (day 2 of differentiation) with 100 ng/ml GH, 100 μM STAT5 inhibitor N-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide, or a combination of them. N-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide is a cell permeable molecule that suppresses STAT5 phosphorylation via binding to the SH2 domain (Muller et al., 2008). We first determined if this inhibitor was effective in blocking GH-induced phosphorylation of STAT5 in bovine preadipocytes. Based on Western blot analyses, there was no detectable phosphorylation of STAT5 in untreated cells or cells treated with the STAT5 inhibitor alone (Figure 4.5A). GH action induced phosphorylation of STAT5 in these cells, and the induced phosphorylation was almost completely blocked by the inhibitor (Figure 4.5A).

Consistent with our earlier observation (Figure 4.2), 6-h GH treatment inhibited the expression of total *PPARγ,* and *PPARγ1* mRNAs and tend to inhibited the expression of *PPARγ2* mRNA but not that of *C/EBPα* mRNA in differentiating preadipocyes (Figure 4.5B). This GH-induced inhibition of total *PPARγ,* and *PPARγ1,* mRNA was, however,

not blocked by the STAT5 inhibitor (Figure 4.5B). These data suggested that GH action-induced inhibition of *PPARγ* mRNA expression in differentiating bovine preadipocytes was not mediated through STAT5.

DISCUSSION

The effect of GH on adipogenesis has been studied in both preadipocyte cell lines and primary preadipocytes, with conflicting results. Studies in preadipocytes showed a stimulatory effect of GH on the formation of adipocytes from preadipocytes (Morikawa et al., 1982; Nanbu-Wakao et al., 2002), whereas those in primary adipocytes indicated the opposite (Richter et al., 2003; Wabitsch et al., 1996). In this study, we examined the effect of GH on differentiation of primary bovine preadipocytes. The hallmark of differentiation of preadipocytes into adipocytes is accumulation of lipids. In this study, we found that addition of GH to the differentiation medium for bovine preadipocytes inhibited the formation of lipid-containing adipocytes, as measured by Oil Red O staining. During the process of differentiation of preadipoctyes into adipocytes, the activity of many lipogenic enzymes increases (Wise and Green, 1979). The backbone of triglyceride comes from glucose-derived glycerol-β-phosphate, which is formed from dihydroxyacetone phosphate catalyzed by G3PDH. The activity of G3PDH increases several hundred fold from preadipocytes to adipocytes (Kuri-Harcuch et al., 1978). Therefore, G3PDH is widely considered as a marker of adipogenesis. In this study, we found that adipocytes differentiated in the presence of GH had less G3PDH activity than those in the absence of GH. This result again supports that GH inhibits differentiation of bovine adipocytes and suggests that GH may do so by inhibiting the G3PDH activity, a mechanism previously suggested by the study on rat adipocytes (Wabitsch et al., 1996). Acetate is the predominant carbon source for *de novo* fatty acid synthesis in ruminant adipose tissue (Chilliard, 1993). In this study, GH reduced the rate of acetate

incorporation into lipids in bovine adipocytes. This provides a third line of evidence for an inhibitory effect of GH on adipocytes differentiation.

Differentiation of preadipocytes into adipocytes is driven primarily by PPARγ and C/EBPα (Rosen et al., 2000). PPARγ is considered the master regulator of adipogenesis because PPARγ-deficient mice failed to form any types of adipose tissue (Barak et al., 1999). GH inhibits *PPARγ* expression in primary rat adipocytes (Hansen et al., 1998). Our data showed that GH inhibited *PPARγ* mRNA expression in primary bovine adipocytes. Therefore, a common mechanism by which GH inhibits adipogenesis in different species may involve inhibiting *PPARγ* gene expression. In this study, besides inhibiting *PPARγ* expression, GH also reduced *C/EBPα* expression in bovine adipocytes, suggesting that the reduction in *C/EBPα* expression may also contribute to GH inhibition of differentiation of those cells. However, the GH-induced reduction in *C/EBPα* expression appeared to be delayed compared to that in *PPARγ* expression. Since *PPARγ* and *C/EBPα* can stimulate each other's expression in adipocytes (Rosen and MacDougald, 2006; Rosen et al., 2000), the reduction in *C/EBPα* expression may be secondary to the GH-induced reduction in *PPARγ* expression.

At the cellular level, GH exerts its function through various intracellular signaling pathways including phosphatidyl-inositol 3 kinase (PI3K), mitogen activated protein kinase (MAPK), and signal transducers and activators of transcription (STAT) pathways (Piwien-Pilipuk et al., 2002). Among these pathways, the JAK2-STAT5 pathway plays a major role in mediating GH regulation of gene transcription. In human adipose tissue, the JAK2-STAT5 pathway is activated both by short time GH treatment (i.e. 30 min) and constant GH infusion (Jorgensen et al., 2006; Moller et al., 2009; Nielsen et al., 2008). In

undifferentiated and differentiating 3T3-L1 cells, GH increased both STAT5a and 5b activity (Fleenor et al., 2006), and the stimulatory effect of GH on adipogenesis in 3T3-L1 cells was mediated through the STAT5-PPARγ pathway (Kawai et al., 2007). Therefore, in this study we examined the possibility that GH inhibits differentiation of bovine adipocytes through STAT5-mediated reduction in *PPARγ* and/or *C/EBPα* gene expression. We found that GH does activate JAK2-STAT5 signaling in bovine adipoctyes because GH induced STAT5 phosphorylation. Overexpression of STAT5bCA in differentiating adipoctyes inhibited the G3PDH activity, the rate of acetate incorporation, and the percentage of adipocytes formed. These effects of STAT5bCA overexpression are similar to those caused by GH, supporting the possibility that GH inhibits differentiation of bovine adipocytes through STAT5 activation.

How does STAT5 mediate GH inhibition of preadipocyte differentiation into adipocytes? GH inhibited the expression of both *PPARγ* and *C/EBPα* mRNAs. STAT5bCA overexpression did not alter *PPARγ* expression, but it reduced *C/EBPα* expression. Therefore, STAT5 may mediate the inhibitory effect of GH on preadipocyte differentiation by inhibiting the expression of *C/EBPα*. STAT5 is in general considered a transcriptional activator, and upon activation, it binds to the interferon gamma-activated sequence (GAS) element, TTCNNNGAA, to activate gene transcription (Horvath et al., 1995; Soldaini et al., 2000). The bovine *C/EBPα* promoter does not contain a GAS element (data not shown). Therefore, the inhibitory effects of STAT5bCA and GH on *C/EBPα* expression in bovine adipocytes are unlikely the result of direct binding of STAT5 to the *C/EBPα* gene. This notion is also supported by the observations that long-term (6 days) but not short-term (6 hours) GH action reduced *C/EBPα* expression in those

cells. We speculate that GH-activated STAT5 directly upregulates the expression of a transcriptional inhibitor, which directly or indirectly inhibits the expression of C/EBPα, which in turn leads to reduced differentiation of preadipocytes (Figure 4.6). Although GH also inhibits PPARγ gene expression in bovine adipocytes, this inhibition is clearly not mediated by STAT5 because STAT5bCA overexpression did not alter *PPARγ* expression and because GH-induced reduction in *PPARγ* expression was not blocked by the STAT5 inhibitor. Because 6-h GH action is sufficient to reduce *PPARγ* expression, we speculate that GH inhibition of *PPARγ* expression is mediated directly by a non-STAT5 transcription factor (Figure 4.6).

In summary, multiple lines of evidence from this study suggest that GH inhibits differentiation of primary bovine preadipocytes. This inhibition may be mediated by two parallel signaling pathways from the GH receptor (GHR). In one pathway, GH binding to the GHR activates STAT5 through phosphorylation, and phosphorylated STAT5 activates the transcription of a yet-to-be-identified inhibitory transcription factor that directly or indirectly inhibits the transcription of the *C/EBPα* gene. In the other pathway, GH binding to the GHR activates a non-STAT5 transcription factor, and this factor directly or indirectly represses the transcription of the *PPARγ* gene (Figure 4.6).

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Table 4.1. Primers for RT-PCR analyses

Target name[1]	Primer sequence (5'to 3')	GenBank #	Amplicon size (bp)
PPARγ	Forward: GATCTTGACGGGAAAGACGA Reverse: GGGGACTGATGTGCTTGAAC	NM_181024.2	228
PPARγ1	Forward: ACAAGAGGGACGTTTCCGTA Reverse: GATGTCAAAGGCATGGGAGT	Y12419.1	150
PPARγ2	Forward: CAGTGTCTGCAAGGACCTCA Reverse: ATAGTGCGGAGTGGAAATGC	Y12420.1	176
C/EBPα	Forward: ATCGACATCAGCGCCTACAT Reverse: CGGGTAGTCAAAGTCGTTGC	NM_176784.2	138
18s	Forward: AAACGGCTACCACATCCAAG Reverse: CCTCCAATGGATCCTCGTTA	DQ222453	155

[1]*PPARγ* = Peroxidase proliferator activated receptor γ; *C/EBPα* = CCAAT/enhancer binding protein α; *18s* = 18s ribosomal RNA.

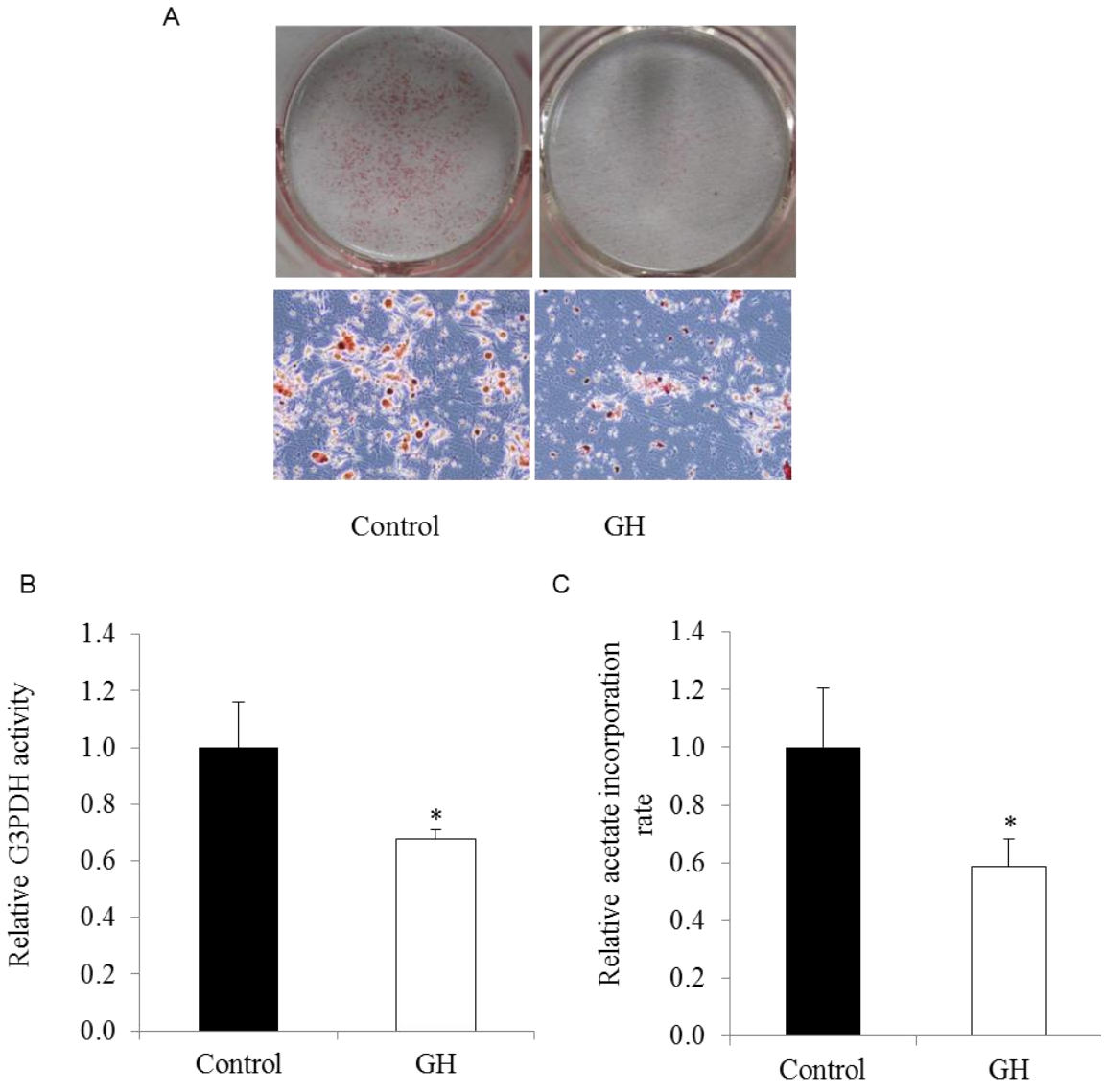

Figure 4.1. Effects of GH on differentiation of bovine preadipocytes. Preadipocytes grown out of bovine adipose tissue explants were induced to differentiate in medium containing no GH (Control) or 100 ng/ml recombinant bovine GH for 6 days. (A) Oil Red O staining. (B) G3PDH activity. (C) Rate of acetate incorporation. Oil Red O staining, G3PDH activity, and rate of acetate incorporation were measured at day 6 of differentiation. Data are expressed as mean ± SEM (n=4 independent experiments). *$P <$ 0.05.

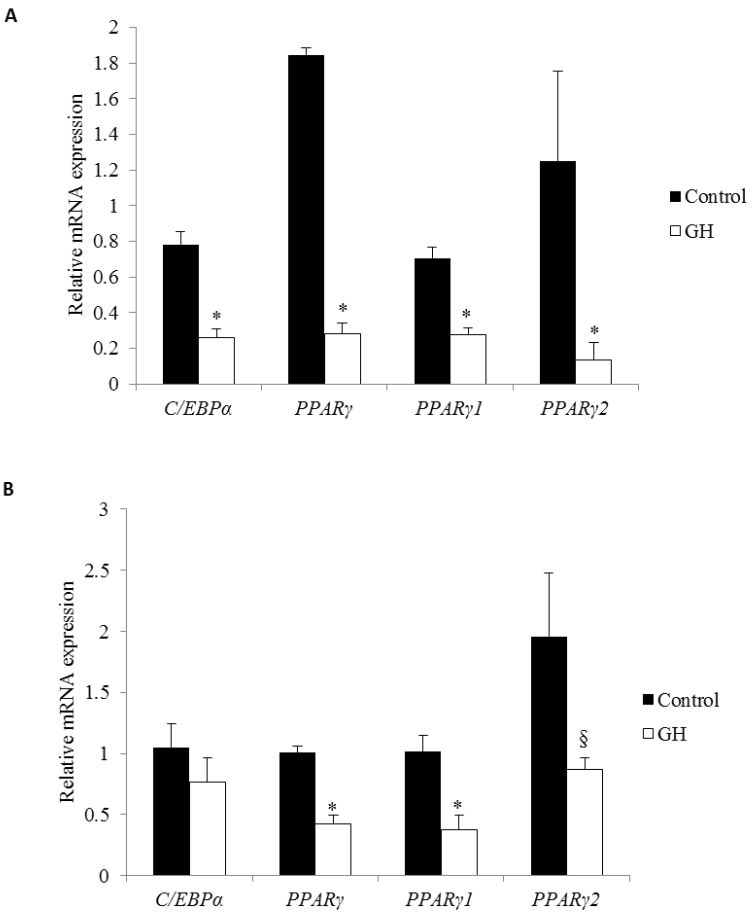

Figure 4.2. Effects of GH on the expressions of *C/EBPα*, total *PPARγ*, *PPARγ1*, and *PPARγ2* mRNAs in bovine preadipocytes. (A) Long-term effect of GH. Preadipocytes were induced to differentiate in medium containing no GH (Control) or 100 ng/ml GH. mRNA expression was measured at day 6 of differentiation. (B) Short-term effect of GH. Preadipocytes were induced to differentiate for 2 days without GH and then treated with no GH (Control) or 100 ng/ml GH for 6 hours before mRNA expression was measured. Data are expressed as mean ± SEM for 4 independent experiments with duplicates (n=4). *$P < 0.05$, and $^{§}P = 0.1$.

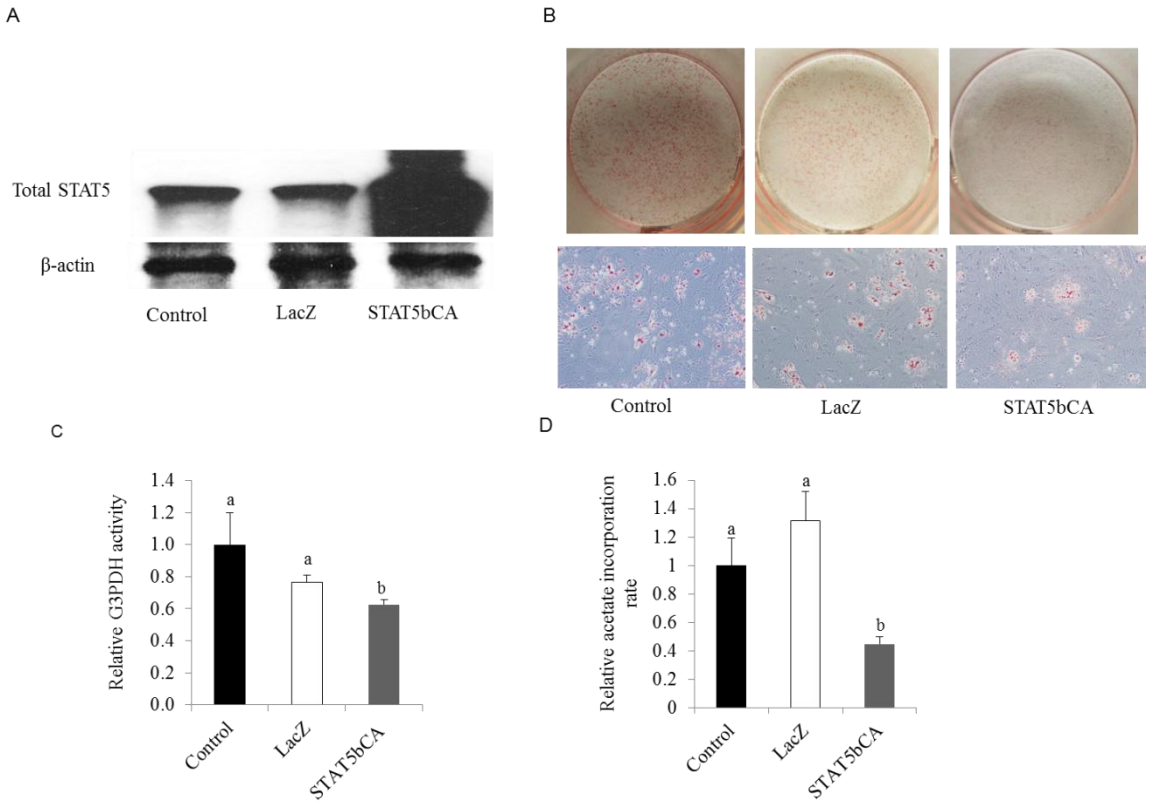

Figure 4.3. Effects of STAT5bCA overexpression on differentiation of bovine preadipocytes. Preadipocytes were induced to differentiate in the absence (Control) or presence of 100 MOI LacZ adenovirus (LacZ), or 100 MOI STAT5bCA adenovirus (STAT5bCA). Stat5 protein, Oil Red O staining, G3PDH activity, and rate of acetate incorporation were measured at day 6 of differentiation. (A) STAT5 protein expression detected by western blotting. β-actin served as a loading control. (B) Oil Red O staining. (C) G3PDH activity. (D) Rate of acetate incorporation. Data are presented as mean ± SEM (n=6 independent experiments). Means labeled with different letters are statistically different ($P < 0.05$).

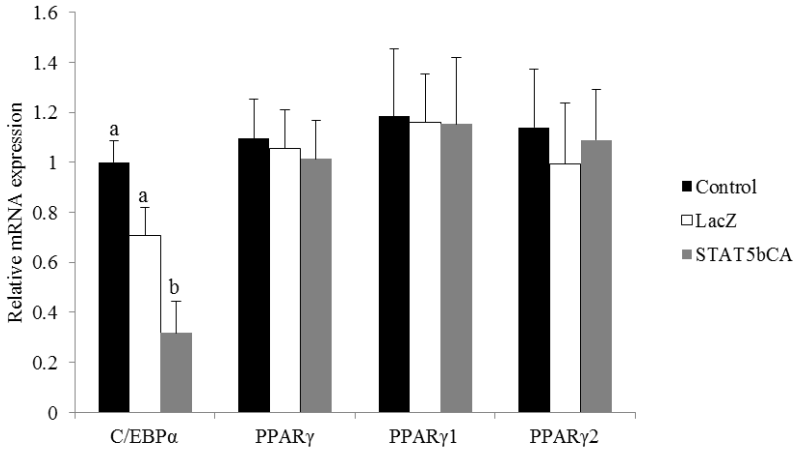

Figure 4.4. Effects of STAT5bCA overexpression on the expression of *C/EBPα*, total *PPARγ*, *PPARγ1*, and *PPARγ2* mRNAs in bovine preadipocytes. Preadipocytes were induced to differentiate in the absence (Control) or presence of 100 MOI LacZ adenovirus (LacZ) or 100 MOI STAT5bCA adenovirus (STAT5bCA). mRNA expression was measured at day 6 of differentiation. Data are presented as mean ± SEM (n=7 independent experiments). Means labeled with different letters are statistically different ($P < 0.05$).

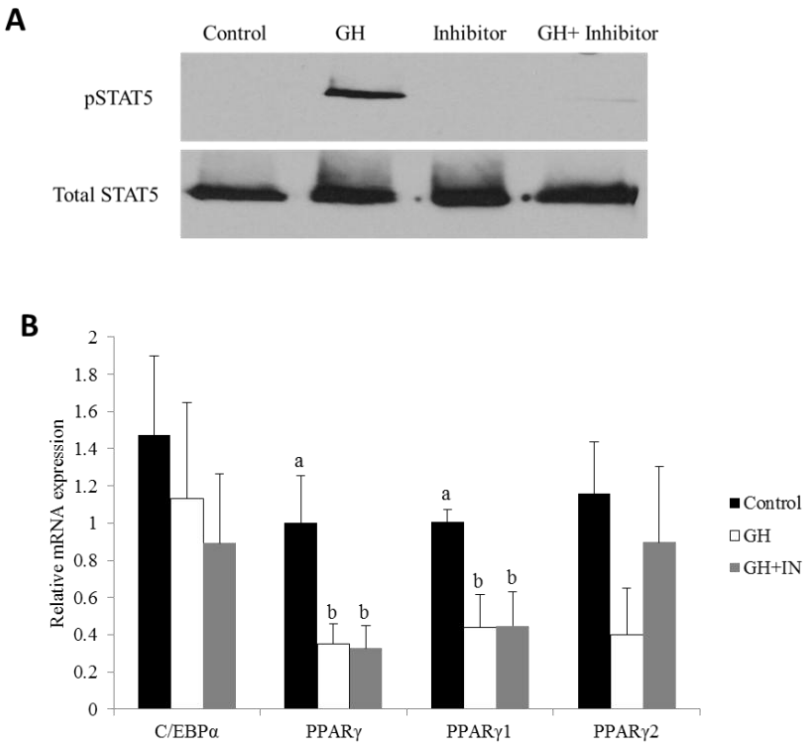

Figure 4.5. Effects of the STAT5 inhibitor N-((4-Oxo-4H-chromen-3-yl) methylene) nicotinohydrazide on GH-induced phosphorylation of STAT5 and reduction in *C/EBPα* mRNA expression in bovine preadipocytes. (A) Effect of the inhibitor on GH-induced phosphorylation of STAT5. Preadipocytes were induced to differentiate for 2 days, then treated with or without 100 μM inhibitor for 3 hours, followed by 100 ng/ml GH treatment for 30 min. Phosphorylated STAT5 (pSTAT5) and total STAT5 were detected by Western blot analyses. (B) Effects of the inhibitor on GH inhibition of *C/EBPα* mRNA expression. Preadipocytes were induced to differentiate for 2 days, and treated with the inhibitor for 3 hours, followed by GH treatment for 6 hours. Data are presented as mean ± SEM (n=4 independent experiments). Means labeled with different letters are statistically different ($P < 0.05$).

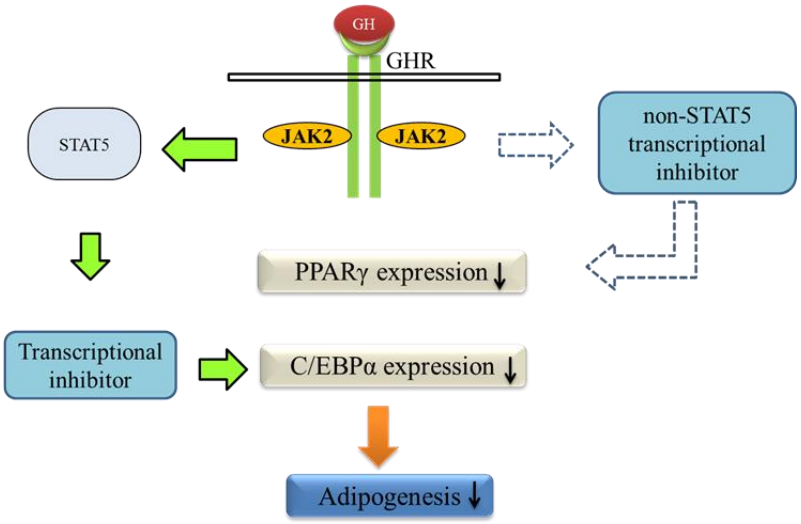

Figure 4.6. Putative signaling mechanism of GH inhibition of differentiation of primary bovine preadipocytes. In one signaling pathway, GH activates STAT5 through Janus kinase 2 (JAK2)-mediated phosphorylation, and activated STAT5 induces the expression of an inhibitory transcription factor that inhibits *C/EBPα* gene transcription. In the other signaling pathway, GH activates a non-STAT5 inhibitory transcription factor that inhibits *PPARγ* gene transcription, thereby inhibiting preadipocyte differentiation.

Chapter V

Future Directions

Role of GH in lipolysis

In my dissertation research, I focused on the effect of chronic GH injection on subcutaneous fat growth and adipocyte size. The serum concentrations of free fatty acids during GH treatment need to be monitored to help understand changes in lipolysis. The short-term effect of GH on lipolysis still needs to be studied. I showed that GH had no direct effect on lipolysis *in vitro*, and its lipolytic effect was not mediated through catecholamines. Therefore, the pathways by which GH stimulates lipolysis need to be further investigated. There are other hormones that may collaborate with GH to regulate lipolysis including dexamethasone and insulin. Although GH did not change isoproterenol-induced lipolysis, mRNA expression of β adrenergic receptors was increased by GH. The protein level of those receptors and their downstream pathways in subcutaneous fat may need to be investigated to elucidate the functions of those receptors during GH treatment.

Role of GH in adipogenesis

In my mouse adipogenesis experiments, more preadipocyte markers were expressed in stromal vascular cells from *lit/lit* mice than from *lit/+* mice. Since stromal vascular cells contain other cell types besides adipocyte progenitors, those stem cells bearing preadipocyte markers need to be separated by flow cytometry and cell numbers need to be compared between two types of mice. A genome-wide RNA sequencing to

compare RNA profile in subcutaneous fat between *lit/lit* mice and *lit/+* mice may help us to understand both lipolytic effect of GH and adipogenesis-inhibiting effect of GH.

In my bovine adipogenesis experiments, I found that GH inhibited *PPARγ* mRNA expression, which did not appear to be mediated through the STAT5 signaling pathway. The other two signaling pathways, MAPK and PI3K, may be involved in GH inhibiting *PPARγ* mRNA expression. This possibility can be tested by inhibiting those two pathways by corresponding inhibitors. I also found putative STAT5 binding sites on the promoter region of *PPARγ*. The functions of these binding sites for STAT5 need to be studied. The inhibition of *C/EBPα* mRNA expression by GH was mediated indirectly by STAT5. Therefore, the direct transcriptional inhibitor of *C/EBPα* gene transcription involved in this process needs to be investigated.