GROWTH HORMONE (GH) INFLUENCES ADIPOCYTE SIZE IN MOUSE MODELS WITH VARYING LEVELS OF GH SIGNALING

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GROWTH HORMONE (GH) INFLUENCES ADIPOCYTE SIZE IN MOUSE MODELS WITH VARYING LEVELS OF GH SIGNALING (87 pp.)

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Adipose tissue plays a critical role in producing (lipogenesis) and releasing (lipolysis) triacylglycerols to meet the body’s energy needs. Growth hormone (GH) has repeatedly been shown to decrease adipose tissue mass by reducing lipogenesis and promoting lipolysis, which likely alters the size of individual fat cells (adipocytes) within the tissue. In this study, three mouse lines with altered GH signaling were used to evaluate the effect of GH on adipocyte cell size. The results demonstrate that bovine GH antagonist expressing mice (GHA) with reduced GH signaling and GH receptor knockout mice (GHR-/-) with no GH signaling had enlarged adipocytes as compared to their littermate controls. In contrast, bovine GH expressing mice (bGH) with increased GH signaling had a decreased adipocyte cell size. Therefore, GH decreased the size of adipocytes. In contrast, decrease or absence of GH signaling leads to increased adipocyte size.

Approved:

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Table of Contents

Page

Abstract ............................................................................................................................... 3

Acknowledgments ............................................................................................................... 4

List of Tables ...................................................................................................................... 8

List of Figures ..................................................................................................................... 9

CHAPTER 1 INTRODUCTION ...................................................................................... 10

Purpose/Significance of Study ............................................................................................ 12

Research Questions .......................................................................................................... 13

Delimitations .................................................................................................................... 14

Limitations ....................................................................................................................... 14

Definition of Terms .......................................................................................................... 15

CHAPTER 2 REVIEW OF LITERATURE ..................................................................... 18

Adipose Tissue .................................................................................................................. 18

Functions of WAT .......................................................................................................... 19

Lipogenesis and Lipolysis within WAT ............................................................................. 20

Expansion of Adipose Tissue ......................................................................................... 24

Regional Adipose Tissue Distribution ............................................................................ 25

Methods for Adipocyte Sizing ......................................................................................... 26

Growth Hormone ............................................................................................................ 29

GH Receptors/Binding Protein ......................................................................................... 29

IGF-1 ............................................................................................................................... 31

Functions of GH ............................................................................................................. 31
Effects of GH on Adipose Tissue ............................................................. 32
Transgenic Mouse Models with Altered Growth Hormone Signaling ........... 38
GHR -/- Mice ............................................................................................ 39
GHA Transgenic Mice.............................................................................. 40
bGH Transgenic Mice............................................................................... 41
The Size of Adipose Tissue Mass............................................................. 42
Summary ............................................................................................................... 43

CHAPTER 3 METHODOLOGY ..................................................................................... 44
Mice ...................................................................................................................... 44
Adipose Tissue Preparation ............................................................................. 46
Whole Adipose Tissue .............................................................................. 46
Isolated Adipocytes................................................................................... 46
Measurement of Cell Size..................................................................................... 46
Statistics ................................................................................................................ 47

CHAPTER 4 RESULTS................................................................................................... 49

CHAPTER 5 DISCUSSION ............................................................................................ 57
Adipocyte Cell Size .............................................................................................. 57
Methods for Cell Sizing................................................................................... 59
Perspectives........................................................................................................... 61

References......................................................................................................................... 64

Appendix A....................................................................................................................... 83
Isolated Adipocyte Protocol.............................................................................................. 83
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cross-Sectional Areas of Adipocytes</td>
<td>51</td>
</tr>
<tr>
<td>2. Percent Change in Adipocyte Cell Size for GHR -/-, GHA and bGH Mice Compared to Littermate Controls</td>
<td>56</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Adipocyte lipogenesis</td>
<td>21</td>
</tr>
<tr>
<td>2. Adipocyte lipolysis</td>
<td>23</td>
</tr>
<tr>
<td>3. Growth hormone (GH) effects on adipocyte lipolysis</td>
<td>34</td>
</tr>
<tr>
<td>4. Growth hormone (GH) effects on adipocyte lipogenesis</td>
<td>35</td>
</tr>
<tr>
<td>5. Mouse models with varying levels of growth hormone (GH) signaling</td>
<td>39</td>
</tr>
<tr>
<td>6. Distribution of adipocyte size obtained from isolated adipocytes and whole adipose tissue</td>
<td>52</td>
</tr>
<tr>
<td>7. Histology of whole adipose tissue from mouse models with varying levels of GH signaling</td>
<td>53</td>
</tr>
<tr>
<td>8. Mean cross-sectional areas of adipocytes obtained by the whole adipose tissue method</td>
<td>54</td>
</tr>
<tr>
<td>9. Cell size distribution of GHR -/-, GHA, bGH mice, and their littermate controls</td>
<td>55</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Adipose tissue, composed of millions of adipocytes, or fat cells, is a complex organ. In addition to providing protection to the body (Klaus, 2001), adipose tissue works as an endocrine organ, ultimately impacting a wide range of metabolic processes, such as energy regulation and insulin resistance (Kershaw & Flier, 2004). Adipose tissue also plays a critical role in lipid homeostasis, storing and releasing lipid (Louveau & Gondret, 2004). That is, during states of energy excess, extra energy from circulation will be taken up by adipocytes and stored in the form of triacylglycerols (TAG), a process known as lipogenesis. In contrast, during periods of energy deprivation, TAGs stored in adipocytes are released as free fatty acids (FFA) and glycerol, a process known as lipolysis, to meet the body’s energy needs. Both lipogenesis and lipolysis are regulated by several factors, such as lipoprotein lipase (LPL), glucose transporter 4 (GLUT4) and hormone sensitive lipase (HSL) (Large, Peroni, Letexier, Ray, & Beylot, 2004). The balance between lipogenesis and lipolysis regulates the lipid content of an adipocyte, which determines the size of adipocytes and adipose tissue mass (Louveau & Gondret, 2004). Interestingly, adipose tissue from various anatomic regions of the body may have distinct metabolic effects. In particular, an excessive deposition of visceral (or intra-abdominal) fat has been known to be associated with increased risk of metabolic syndrome, which is characterized by a group of metabolic risk factors including
hyperlipidemia, hypertension, diabetes, and cardiovascular disease (Bouchard, Despres, & Mauriege, 1993; Merino-Ibarra et al., 2005; Smith, Elemendorf, David, & Turinsky, 1997).

Growth hormone (GH) is a protein released by the anterior pituitary gland (Anderson, Jefinjia, & Scanes, 2004). GH has diverse effects on a variety of organs and tissues. Normal GH secretion and function contribute to the proper growth of bones and muscles during childhood and adolescence (Le Roith, Bondy, Yakar, Liu, & Butler, 2001; Stewart, 2000). GH also influences nutrient metabolism, regulating protein, carbohydrate and fat utilization by the body (Le Roith et al., 2001). To exert all these effects, GH binds to growth hormone receptors (GHR) on cell surfaces and alters transcription of a variety of GH-responding genes (Brown et al., 2005). One GH-responsive gene, insulin-like growth factor 1 (IGF-1) is a mediator of many of the effects of GH (Berneis & Keller, 1996).

As one of the target organs of GH, adipose tissue is profoundly impacted by GH (Leung et al., 1987) GH decreases adipose tissue mass by simultaneously reducing lipogenesis and increasing lipolysis, which alters the size of individual adipocytes (Davidson, 1987; Richelsen et al., 1994). GH also appears to impact adipose tissue in a depot-specific manner. For example, chronic administration of GH to individuals with growth hormone deficiency (GHD) is associated with a redistribution of adipose tissue from an abdominal to a more peripheral distribution (Rosenbaum, Gertner, & Leibel, 1989).

In this proposal, three different mouse models developed previously will be used. These three mouse models include growth hormone receptor/binding protein gene-
disrupted (GHR-/-) mice (Zhou et al., 1997), bovine GH antagonist transgenic (GHA) mice, and bovine GH transgenic (bGH) mice (Chen et al., 1991b). These mouse models have very distinct differences in GH signaling and body composition. GHR-/- mice have no GH signaling and GHA mice have significantly reduced GH signaling (Coschigano et al., 2003). Both mouse models are dwarf and obese. In addition, GHR-/- and GHA mice have reduced IGF-1 levels, yet only GHR-/- mice have improved insulin sensitivity and increased lifespan (Berryman et al., 2004; Coschigano et al., 2003). In contrast, bGH mice have dramatically elevated GH signaling, resulting in increased body size, reduced fat body mass, severe insulin resistance, as well as short lifespan (Berryman et al., 2004; Chen et al., 1991a; Coschigano, Clemmons, Bellush, & Kopchick, 2000; Coschigano et al., 2003; Higashimori et al., 2003). Overall, these mouse models with varying GH levels are a useful system to explore mechanistically how adipose tissue is influenced by different levels of GH function.

Purpose/Significance of Study

Overweight and obesity are leading health indicators in the United States (U.S. Department of Human and Health Services, 2000). Data from the National Health and Nutrition Examination Survey (NHANES) indicated that 66% of adults aged 20 years and over are overweight (body mass index ≥ 25) and 33% of adults are obese (body mass index ≥ 30) in the United States (Center of Disease Control and Prevention). As obesity is associated with a number of diseases such as dyslipidemia, cardiovascular disease, hypertension, type 2 diabetes, and certain types of cancers, to establish an efficient therapy for obesity is very important (Lawrence & Kopelman, 2004).
Obesity has a close relationship to GH. A reduced GH level has been reported in obese subjects (Munzer et al., 2001; Veldhuis et al., 1991). Therefore, use of GH has been suggested as a therapeutic tool for weight reduction and redistribution of body tissue from adipose tissue to muscle mass in obese individuals (Scacchi, Pincelli, & Cavagnini, 1999; Shadid & Jensen, 2003). For GH therapy to be considered for obesity treatment, a more profound understanding of the mechanism of how GH influences adipose tissue, such as how GH affects adipocyte size, is required. Since the study of human models is limited due to ethical reasons, animal models with extremes of GH signaling ranging from absence to excess would be extremely useful to understand the relationship between GH and adipose tissue.

GHR -/-, GHA and bGH mice are good animal models to study the human conditions with altered GH signaling. These mouse models have been well characterized previously with respect to growth, glucose metabolism, obesity and lifespan. However, no research has been done to examine adipocyte size within adipose tissue of these three mouse models. Accurate data of adipocyte size of each adipose depot from different mouse models will lead to a further understanding of the mechanism of GH effects on adipose tissue. Therefore, the purpose of this study is to evaluate the effects of GH on adipocyte size in various adipose depots using mouse models with varying levels of GH signaling.

Research Questions

Hypotheses: The level of GH function affects adipocyte size and the impact on cell size varies depending on the location of adipose tissue. The following research questions will be addressed in this study:
1. Is the whole tissue method adapted from Chen and Farese (2002) for measuring the cross-sectional area of adipocytes comparable to standard methods utilizing isolated adipocytes?

2. For each depot, do the adipocyte sizes differ between GHR-/-, GHA, and bGH mice and their corresponding littermate controls?

3. Does GH affect adipocyte size in a regional specific manner? If yes, how are the sizes of adipocytes from epididymal, retroperitoneal and subcutaneous fat pads altered in GHR-/-, GHA and bGH mice?

**Delimitations**

1. As mice of each genotype used in this study are not housed singly until the age of 2½ months, a hierarchy system could have developed. Dominant mice of each cage may consume more food compared to the rest of the mice, which could impact adipose tissue mass.

2. Mouse models can not fully represent the human condition; thus, the result may not apply to humans.

**Limitations**

1. Only three animals are used for each genotype. This limited number of mice could impact the results of cell size. However, for each genotype, mice are being compared to control mice from exactly the same genetic background and at the same age when sacrificed. The feeding and housing methods are also identical for each animal. Moreover, measuring a large number of adipocytes from each mouse will reduce the distortion of adipocyte size.
2. To take digital images of whole adipose tissue, three non-overlapping microscope fields will be selected. Bias of field selection might exist during this process. To eliminate the bias, microscope fields with the most intact adipocytes will be selected.

3. The use of methods using isolated adipocytes and whole adipose tissue in this study to determine the cell size requires an assumption that all cells are cut through their equators, therefore, revealing their maximum cross-sectional areas. Although the mean size of adipocytes might be different than the true value, all mouse models examined will have the same type of bias introduced into the measurements of cell size.

4. Distortion of cell size may exist due to cell damage during adipose tissue sample preparation for the whole adipose tissue method. However, if a large number of adipocytes are measured and all samples are treated in a similar manner, the error related to this variable will be minimal.

**Definition of Terms**

*Adipose tissue.* Adipose tissue, also know as fat tissue, is mainly composed of adipocytes or fat cells. Adipose tissue is mainly located under skin (subcutaneous) and around internal organs (visceral or intra-abdominal). The main function of adipose tissue is to provide protection to the body, store energy, and work as an endocrine organ (Klaus, 2001).

*Bovine GH (bGH) mice.* bGH transgenic mice were generated by injection of a gene fusion between a mouse metallothionein transcriptional regulatory element and a bGH cDNA containing the first intron into the pronucleus of mice embryos (Berryman et al., 2004). bGH mice have dramatically elevated GH signaling, resulting in a increased
body size, reduced fat body mass and reduced lifespan (Berryman et al., 2004; Chen et al., 1991a; Coschigano et al., 2000; Coschigano et al., 2003).

*Growth hormone (GH).* GH is a protein released by the anterior pituitary gland. GH exerts its effect either by binding to GH receptors within the tissues or via circulating insulin-like growth factor 1 (IGF-1). The actions of GH include postnatal growth stimulation and macronutrient (carbohydrate, lipid and protein) metabolism (Le Roith et al., 2001).

*GH antagonist (GHA) mice.* GHA transgenic mice were originally generated by pronuclear injection of a bovine GH minigene with a mutation resulting in glycine 119 being replaced by lysine in the third helix. The mutated protein can bind GHR but cannot transmit a signal. Due to the decreased GH signaling, GHA mice are dwarf and obese (Berryman et al., 2004; Chen et al., 1991a).

*GH deficiency syndrome (GHD).* GHD is induced by several means, such as pituitary tumors and genetics. In children, GHD leads to short stature and low growth velocity for age. In adults, GHD is associated with reduced skeletal muscle, reduced lean body mass and increased fat mass, particularly in the abdominal area (Ayuk & Sheppard, 2006).

*GH receptor/binding protein gene disrupted (GHR-/-) mice.* GHR-/- mice were generated by disruption of the GH receptor/binding protein gene through homologous recombination. GHR-/- mice have no functional GHR. Therefore GHR-/- mice have a smaller body size but increased adipose tissue (Berryman et al., 2004). In addition, GHR-/- mice have reduced IGF-1 level, improved insulin sensitivity and increased lifespan (Coschigano et al., 2003; Liu et al., 2004; Zhou et al., 1997).
Lipogenesis. Lipogenesis refers to the process of adipocytes taking up energy and synthesizing TAGs when energy expenditure is lower than energy intake. Promotion of lipogenesis increases lipid accumulation within adipocytes. Lipogenesis is promoted by multiple factors, such as insulin and LPL (Kersten, 2001).

Lipolysis. Lipolysis is the process of breaking down TAG and release of FFAs and glycerol from adipose tissue when energy expenditure is greater than energy intake. The rate of lipolysis is promoted by several factors including the phosphorylation of HSL, and perilipins (Yang, Mulder, Holm, & Eden, 2004).

Intra-abdominal adipose tissue. Intra-abdominal adipose tissue is also known as “visceral” adipose tissue, though visceral adipose tissue sometimes specifically refers to the fat mass drainages through the portal vein to the liver. In this study, intra-abdominal adipose tissue refers to the adipose tissue located in the abdominal cavities, including epididymal and retroperitoneal adipose tissue. Epididymal adipose tissue is located above the epididymus (DiGirolamo, Fine, Tagra, & Rossmanith, 1998) while retroperitoneal adipose tissue is located on the border between the spine and the posterior abdominal wall behind the kidneys (Cinti, 2005).

Subcutaneous adipose tissue. Subcutaneous adipose tissue is defined as the adipose tissue layer found between the dermis and the aponeuroses and fasciae of the muscles (Shen et al., 2003). Rodents have two main subcutaneous adipose tissues, one anterior and one posterior. The inguinal adipose tissue used for whole adipose tissue method in this paper is part of the posterior subcutaneous adipose tissue (Cinti, 2005).
Adipose tissue, which is also known as fat tissue, is a type of connective tissue consisting mainly of adipocytes or fat cells. Adipose tissue is mainly located either subcutaneously under the skin or intra-abdominally (or viscerally) around internal organs. In addition, adipocytes also infiltrate organs such as skin, parathyroid, synovia, bone marrow and pancreas (Cinti, 2001). In mammals, there are two different types of adipose tissue: brown adipose tissue (BAT) and white adipose tissue (WAT). These types of tissues are differentiated by their anatomical locations, structure and functions (Tiraby & Langin, 2003). In this review, only WAT, the most abundant tissue in fat mass, will be addressed.

WAT is composed of millions of white adipocytes. Besides adipocytes, there are a few stromal-vascular cells, such as fibroblasts, histiocytes and mast cells (Cinti, 2001). Histologically, each white adipocyte contains a large lipid droplet, surrounded by a class of proteins called perilipins (Tansey et al., 2003). Due to this large lipid content, adipose tissue has a lower water content as compared to other tissues in the body (Sherwood, 2001).
Functions of WAT

A major function of WAT is to store and release TAG. When energy intake exceeds energy expenditure, the extra energy is taken up by adipose tissue and stored as TAGs (Boone, Mourot, Gregoire, & Remacle, 2000). Therefore, up to 90% of adipocyte volume is occupied by a droplet of TAG. In contrast, during starvation or exercise, TAG stored in adipocytes is released as FFAs and glycerol to fulfill the body’s energy needs (Boone et al., 2000). Adipose tissue is considered the largest energy reservoir of the body. An adult with 15 kg adipose tissue mass has more than 460 MJ (110,000 kcal) of energy in storage, which could provide 2000 kcal daily for up to 2 months (Jensen, 1997). The details regarding the storage and release of energy from adipose tissue will be discussed in subsequent sections.

In addition to working as an energy reservoir, adipose tissue also provides protection to the body. In cold weather, subcutaneous adipose tissue, which is located directly below the skin, provides additional insulation against cold weather by reducing heat loss. Furthermore, the intra-abdominal adipose tissue around internal organs serves as a mechanical cushion against external forces (Klaus, 2001).

A more recently recognized function of adipose tissue is its endocrine function. Adipose tissue synthesizes a wide range of molecules, known as adipokines (Kershaw & Flier, 2004). These adipokines, including hormones, growth factors and cytokines, act at both the local (autocrine/paracrine) and the systemic (endocrine) level. Adipocytes also express numerous receptors, allowing the peripheral signals, such as catecholamines and prostaglandins, to modulate the cellular function of adipocytes (Kershaw & Flier, 2004). Through this interactive endocrine network, adipose tissue is involved in a number of

*Lipogenesis and Lipolysis within WAT*

**Lipogenesis.** Adipose tissue is the major site where the body stores lipid. The lipid content of adipose tissue is regulated by the balance between fat accumulation and fat release (Farnier et al., 2003; Louveau & Gondret, 2004). Fat accumulation in adipose tissue largely depends on efficient FFA synthesis and subsequent TAG synthesis (Kersten, 2001), which is known as lipogenesis (see Figure 1). Lipogenesis increases lipid accumulation within the adipocyte, therefore, the size of adipocytes is typically increased under conditions that promote lipogenesis. The TAGs in the circulation are mainly carried by the TAG-rich lipoproteins, very-low-density lipoproteins (VLDL) and chylomicrons (Deshaies & Despres, 2000). TAGs cannot cross biological membranes and, thus, cannot be taken up by adipocytes directly. In order to be used for TAG synthesis inside adipocytes, TAGs must first be hydrolyzed into FFAs and glycerol by an enzyme known as LPL at the surface of adipocytes before crossing the cell membrane. Once inside the cell, the hydrolyzed products, FFAs, are re-esterified and stored in the form of TAG in lipid droplets. In addition to TAGs from lipoproteins, circulating glucose is also a potential substrate for TAG synthesis in adipocytes. That is, FFAs can also be synthesized from the glucose product, acetyl coenzyme A (CoA), following glucose uptake via glucose transporters, especially GLUT 4. This pathway is called de novo lipogenesis and is normally thought to play a minor role in lipogenesis (Hellerstein et al., 1991).
Figure 1. Adipocyte lipogenesis. The process of adipocytes taking up energy including triacylglycerol (TAG) and glucose, and synthesizing TAG during positive energy status. LPL = lipoprotein lipase, GLUT4 = glucose transporter 4, Glucose-6-P = glucose-6-phosphate, CoA = coenzyme A, FFA = free fatty acid.

In the lipogenic pathway, LPL plays an essential role in regulating the rate of TAG uptake. Fried and DiGirolamo (1986) reported that larger adipocytes had nearly three times higher cellular LPL activity compared with smaller cells. These data suggest that LPL may determine the influx of FFAs and the amount of fat deposited. In other words, a decrease of LPL activity inhibits TAG uptake and fat deposition; over-expression of LPL promotes TAG influx and fat accumulation in adipocytes. Therefore, LPL is considered a rate-limiting enzyme of lipogenesis (Zechner et al., 2000).

Lipolysis. Opposite to lipogenesis, lipolysis is the process of breaking down stored TAG and releasing FFAs and glycerol from adipose tissue (see Figure 2). When lipolysis is increased, more TAGs stored in adipocytes are released, resulting in a
reduction in adipocyte size. During periods of increased energy demand, lipolysis is stimulated by lipolytic hormones, such as catecholamines. Catecholamines stimulate lipolysis by binding to β-adrenergic receptors at the cell surface, activating stimulating guanine-nucleotide regulator protein (Gs), which in turn stimulates adenylate cyclase to catalyze the formation of cyclic adenosine monophosphate (cAMP). The increased concentration of cAMP then activates the cAMP-dependent protein kinase A (PKA) and induces phosphorylation of (HSL). The phosphorylation of HSL consequently results in the activation and translocation of HSL to the lipid droplet, where TAG hydrolysis occurs (Yang et al., 2004). Following complete hydrolysis, FFAs and glycerols are released into circulation to meet the body’s energy needs.
Figure 2. Adipocyte lipolysis. The process of breaking down triacylglycerol (TAG) and release of FFAs and glycerol from adipose tissue during negative energy status. Gs = stimulating guanine-nucleotide regulator protein, Gi = inhibitory guanine-nucleotide regulator protein, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, HSL = hormone sensitive lipase, ATGL = adipose triglyceride lipase.

Traditionally, HSL phosphorylation was considered the exclusive rate-limiting step in the lipolytic pathway. However, recent data also suggest that other proteins appear critical in regulating lipolysis. For example, perilipins, proteins that coat the lipid droplet surface, are also phosphorylated in response to increased intracellular cAMP concentration and function as critical docking proteins for intracellular lipases (Tansey et al., 2003; Wang et al., 2003b). Recently, a novel adipocyte triglyceride lipase has been identified in mice, namely adipose triglyceride lipase (ATGL) (Zimmermann et al.,
2004). Study of 3T3-L1 adipocytes reveals that inhibition of ATGL dramatically decreases total adipose acyl-hydrolase activity, indicating that ATGL may have a significant long-term effect on lipolysis (Kershaw et al., 2006; Zimmermann et al., 2004).

Lipolysis is down-regulated by several antilipolytic factors. A recent study has shown that chronic high insulin levels may inhibit the activation of PKA by disrupting the close apposition of β-adrenergic receptors in cell culture (Zhang, Hupfeld, Taylor, Olefsky, & Tsien, 2005). In addition, factors like adenosine, neuropeptide Y, and prostaglandin E bind to α-adrenergic receptors located on the cell surface, which negatively couple to adenylate cyclase by the inhibitory guanine-nucleotide regulator protein (Gi) and suppress lipolysis (Castan et al., 1994).

Expansion of Adipose Tissue

The enlargement or expansion of adipose tissue involves both hyperplasia, the increase of adipocyte numbers, and hypertrophy, the enlargement of individual adipocytes. Both hyperplasia and hypertrophy occur during periods of positive energy balance (Hausman, DiGiralamo, Bartness, Hausman, & Martin, 2001). In humans, hyperplasia occurs predominantly during the first year of life and puberty, but slows down at adolescence, the point in which hypertrophy becomes more critical (Van Harmelen et al., 2003). The first hallmark of hyperplasia is the conversion of resident fibroblasts into preadipocytes, the precursors of mature adipocytes (Gregoire, 2001). Once converted, preadipocytes are gradually differentiated to lipid-filled and functional mature adipocytes and, thus, the cell population is markedly increased (Hausman et al., 2001). Hypertrophy is induced primarily by lipid accumulation within mature adipocytes. When lipogenesis is enhanced and/or lipolysis is retarded, the intracellular
lipid droplet size is increased. Therefore, in obese subjects, enlargement of the preexisting adipocytes is one way to increase adipose tissue mass (Villena et al., 2004).

Regional Adipose Tissue Distribution

Due to gender, age and other factors, the metabolism, cell composition, and endocrine capabilities of adipose tissue from various anatomical locations are not identical (Bouchard et al., 1993). Because of this, the accumulation of fat as TAG varies from fat pad to fat pad (Van Harmelen, Rohrig, & Hauner, 2004; Van Harmelen et al., 2003; Villena et al., 2004). The differential accumulation of adipose tissue can be described in several ways. Gynoid and android adiposity is one manner in which researchers distinguish between differing patterns of adipose tissue deposition. Gynoid adiposity refers to adipose tissue mass accumulated particularly in the lower part of the body. Gynoid adiposity is observed more frequently in women than in men. The other pattern of adipose tissue distribution is named android distribution or central distribution, referring to the accumulation of adipose tissue either subcutaneously or viscerally in the abdominal area. This type of adipose tissue distribution is found more frequently in men and post-menopausal women (Bouchard, 1991a, 1991b).

Besides android and gynoid adipose tissue distribution, adipose tissue can also be distinguished as subcutaneous adipose tissue and intra-abdominal adipose tissue (Smith et al., 2001). Subcutaneous adipose tissue refers to the fat accumulated beneath the skin; intra-abdominal tissue includes the fat mass surrounding internal organs, such as epididymal, perirenal, retroperitoneal and omental fat pads (Cinti, 2001). Intra-abdominal adipose tissue is more important in terms of the health risks associated with excess adipose tissue because it is associated with a reduction in insulin sensitivity.
(Gastaldelli et al., 2002). Studies have also shown that intra-abdominal obesity is a closely related to metabolic syndrome (Merino-Ibarra et al., 2005; Mori, Hoshino, Yokota, Yokose, & Tajima, 2005).

Methods for Adipocyte Sizing

Adipose tissue mass can vary from 3% of the total body weight in humans, as might be found in elite athletes, to 70% of body weight, as in morbidly obese individuals (DiGirolamo, 2001). Moreover, individual adipocytes may increase their diameters 20 fold and their volumes thousands of fold by elevated accumulation of TAG (Fruhbeck, Gomes-Ambrosi, Muruzabal, & Burrell, 2001). Hence, measuring the size of adipocytes is an important method to study the metabolism of adipose tissue.

Several methods for measuring adipocyte size have been described, among which direct measurement of adipocyte diameter is a common method (DiGirolamo, Mendlinger, & Fertig, 1971; Farnier et al., 2003). In this method, shredded adipose tissue is digested with collagenase. Adipocytes are then isolated by gentle centrifugation. The isolated cells are stained and the diameter of each cell is measured under the light microscope (DiGirolamo et al., 1971; Farnier et al., 2003). This method provides a direct observation of adipocytes (DiGirolamo, 2001). However, to obtain the cell size of a large number of adipocytes with this method is very tedious and time-consuming (Chen & Farese, 2002). Furthermore, no permanent visual record can be saved for future reference by this method because the cell suspension can not be kept for a long time (Tchoukalova, Harteneck, Karwoski, Tarara, & Jensen, 2003). Finally, during the process of isolating the adipocyte, large adipocytes are likely to be broken as they are extremely fragile (Hirsch & Gallian, 1968), which may distort the value of mean cell size.
Recently, microscopy with digital imaging capabilities has altered the manner in which diameters of the isolated adipocytes can be measured. As with the method of measuring isolated adipocytes under the microscope, adipocytes are digested with collagenase and then isolated. Subsequently, the cell size is determined with digital images of adipocyte suspension. In the program, a threshold value is interactively determined to separate the cell from background. A connected algorithm is then applied to the image to determine the areas or diameters of the cells (Bjornheden et al., 2004; Tchoukalova et al., 2003). This computer-based measurement provides a permanent record (image of adipocyte suspension) that can be assessed at a convenient time. The diameters of large numbers of adipocytes can be obtained rapidly (Bjornheden et al., 2004; Tchoukalova et al., 2003). Nevertheless, since this method still uses isolated adipocytes for the measurement, the problem of large cell damage remains unresolved.

Another frequently used method is to calculate the average adipocyte size indirectly by measuring the lipid content of adipocytes (Cushman & Salans, 1978; Hausman et al., 2003; Hirsch & Gallian, 1968). In this method, adipose tissue fragments are washed and fixed in osmium tetroxide. The fixed adipocytes are separated from the adipose tissue fragment using 250-µm and 25-µm mesh nylon filters. The number of adipocytes is determined using a coulter electronic counter. Mean adipose size is calculated by dividing the lipid weight of the fixed tissue sample by the total number of cells in the sample. This method is relatively convenient. However, the washing step leads to an unavoidable loss of smaller cells, and, therefore, the cell size is overestimated (Flint & Gardner, 1993). In addition, the expense of the coulter counter purchase and toxicity of osmium tetroxide are the concerns with this method (Chen & Farese, 2002).
A fairly new method has been described by Chen and Farese (2002). In this method, whole adipose tissue, not isolated adipocytes, is fixed in paraformaldehyde, embedded in paraffin, cut into thin sections and stained with hematoxylin and eosin. Computer programs, Adobe Photoshop (Adobe® system, San Jose, CA) and Image Processing Tool Kit (Reindeer Games®, Gainesville, FL), are used to assess the cross-sectional areas of adipocytes. Although using fixed adipose tissue to assess the adipocyte size has been available for years (Ashwell, Priest, Bondous, Sowter, & Mapherson, 1976), advances in computer software and digital technology have made the technique more automated and accurate. Compared to most of the methods described above, this method allows the researcher to measure a large quantity of adipocytes quickly. Moreover, adipose tissue used in the study is fixed and embedded in paraffin. Instead of an image record, the actual adipose tissue specimen can be saved permanently and potentially used for other microscopic analyses. A limitation of this method is that adipocyte size can be distorted due to cell damage during preparation. However, the distortion can be minimized by measuring a large number of tissues or adipocytes (Chen & Farese, 2002). In addition, in this method, all cells are assumed to be cut through their equators, thus revealing their maximum cross sectional areas (Ashwell et al., 1976). Therefore the cell size obtained with this method might be smaller than the actual cell size. Nevertheless, if the goal is to compare different conditions or fat pads, then all samples analyzed with this method should have a similar decrease in cell size; therefore, any bias would be uniform throughout all samples.
Growth Hormone

The GH protein, which is also known as somatotropin, contains 191 amino acids and has a molecular weight of about 22 kDa (Niall, Hogan, Sauer, Rosenbulm, & Greenwood, 1971). The three-dimensional structure of GH shows that the hormone consists of two disulfide bridges, four alpha-helices arranged in an “up-up-down-down” topology, and three shorter connective helices (de Vos, Ultsch, & Kossiakoff, 1992). GH is produced and secreted in a pulsatile manner by cells called somatotrophs located in the anterior pituitary gland. The production of GH is mainly mediated by the interplay between growth hormone releasing hormone (GHRH), which stimulates GH production, and somatostatin, a hormone that inhibits GH secretion. Stress, exercise, and sleep are also factors affecting GH production. Moreover, proteins like gonadotropin releasing hormone (GnRH), IGF-1, ghrelin, leptin, pituitary adenylate activating polypeptide (PACAP) and thyrotrophic releasing hormone (TRH) are also involved in GH secretion in some species (Anderson et al., 2004).

GH Receptors/Binding Protein

GH exerts its effects by binding to the GHR on cell surfaces. Once bound, the GH/GHR binding complex activates several signaling cascades, including the GH-induced Janus-Kinase 2 (JAK)/signaling transducer and activators of transcription (STAT) signaling pathway (Carter-Su et al., 1996). GHR is a trans-membrane protein belonging to the class I super-family of cytokine receptors (Moutoussamy, Kelly, & Finidori, 1998). Each GHR contains an extracellular domain that is capable of binding GH and an intracellular domain interacting with proteins of the signaling pathway that control the cellular function of the GH in target tissues (Leung et al., 1987). GHRs are
present in various biological tissues and cell types, such as hepatocytes, adipocytes, fibroblasts, chondrocytes, osteoblasts, beta-islet cells, macrophages, lymphocytes and ventral prostatic epithelial cells (Waters et al., 1990). The highest levels of receptors have been found in the liver, adipose tissue and kidney (Moffat, Edens, & Talamantes, 1999).

Growth hormone binding protein (GHBP) and GHR are the products of the same gene (GHR/BP gene) produced either by alternative splicing or proteolysis. Unlike GHR, GHBP are located in the circulation (Wang et al., 2003a). Structurally, GHBP contains only the extracellular hormone-binding domain, which is identical to the extracellular portion of GHR (Edens, Southard, & Talamantes, 1994). The transmembrane and intracellular domains are absent. Thus, GHBP has the capacity to bind the hormone but does not appear to induce the signaling cascade.

The binding of GH to GHR on the surface of the cell is considered the initial step of the GH-induced JAK/STAT signaling pathway (Carter-Su et al, 1996). This initial step involves two GHRs (GHR1 and GHR2) and one GH molecule with two receptor binding sites. It was believed for years that the activation of the signaling cascade resulted from the GH-induced dimerization of GHR (Fuh et al., 1992). However, a new model of GHR activation was reported recently suggesting that GH binds to a preformed receptor dimer (Brown et al., 2005). In this new model, the first binding site of a GH molecule interacts with GHR1, allowing the second binding site on the GH molecule to bind with GHR2. However, the two binding sites on the GH molecule are located asymmetrically, causing GHR2 to rotate with respect to GHR1 in order to bind to the second binding site of the GH molecule. This rotation results in a conformational change
of the GHR2 intracellular domain, which ultimately promotes the GH-induced signaling pathwya, by which GH regulates the transcription of GH-responsive genes (Piwien-Pilipuk, Hou, & Schwartz, 2002).

**IGF-1**

One gene that is significantly up-regulated in response to the GH signaling cascade is IGF-1. IGF-1 has its own distinct yet widespread cellular effects. Thus, GH can either impact cellular metabolism directly via its own signaling pathways or indirectly via the production of IGF-1 (Berneis & Keller, 1996). IGF-1 is a small protein containing 70 amino acids, with 48% of the amino acids identical to proinsulin (Moses, Young, Morrow, O'Brien, & Clemmons, 1996). IGF-1 molecules exist in circulation complexed to members of a family of six binding proteins, IGFBPs (Hwa, Oh, & Rosenfeld, 1999). Among these IGFBPs, IGFBP-3 carries the vast majority (about 80%) of the total IGF-1 molecules (Lewitt, Saunders, Phuyal, & Baxter, 1994). Among other functions, IGF-1 inhibits both GH secretion and GH transcription at the pituitary gland through a long loop negative feedback (Namba, Morita, & Melmed, 1989). Moreover, by binding to IGF-1 receptors, IGF-1 plays an important role to promote cell growth and differentiation (Garnett et al., 2004). Recent studies in animal models of IGF-1 deficiency or excess also reveal its role in carbohydrate and lipid metabolism in cooperation with GH and insulin (Berneis & Keller, 1996; Yakar et al., 2005).

**Functions of GH**

GH has a wide range of biological activities involving multiple organs and tissues. As its name indicates, the primary role of GH is to promote growth by stimulating the postnatal growth of bones and muscles. Accordingly, hyosecretion of
GH in humans during childhood leads to dwarfism while hypersecretion of GH results in gigantism during youth and acromegaly in adulthood (Stewart, 2000).

In addition to growth stimulation, GH plays a critical role in nutrient metabolism, modulating protein, lipid and carbohydrate utilization. For example, GH stimulates nitrogen retention and protein synthesis in skeletal muscles; thus, GH promotes increases in lean body mass (Russell-Jones et al., 1993). GH also functions in the metabolism of lipids by promoting lipolysis in adipose tissue and the turnover of carbohydrates by its anti-insulin effects, enhancing hepatic gluconeogenesis and glycogenolysis (Le Roith et al., 2001). The relationship between GH and macronutrient metabolism is demonstrated by adults with GH deficiency (GHD). These adults have reduced skeletal muscles, increased fat mass, insulin resistance, dyslipidaemia, increased risk of cardiovascular disease (McCallum, Petrie, Dominiczak, & Connell, 2002) and osteoporosis (Rosen, Whilemsen, Landin-Whilemsen, Lappas, & Bengtsson, 1997).

Effects of GH on Adipose Tissue

Adipose tissue is a well-documented target tissue for GH (Leung et al., 1987; Vikman, Carlsson, Billig, & Eden, 1991). A large body of evidence supports that GH has a profound effect on adipose tissue metabolism, development and distribution. In accordance with these effects, GH treatment markedly decreases adipose tissue in both human and animal models (Bengtsson et al., 1993; Frick, Leanard, & Goodman, 1990; Johannsson et al., 1997). The effects and mechanism of GH on adipose tissue are summarized below.

Metabolism. Two types of opposite metabolic effects of GH on adipose tissue metabolism have been described: acute effects and chronic effects. The acute effects of
GH are insulin-like, thereby stimulating glucose uptake, increasing blood glucose levels, and enhancing lipogenesis (Davidson, 1987). The insulin-like effect of GH is best observed soon after the adipose tissue is exposed to GH and in the adipose tissue obtained from hypophysectomized animals which are not exposed to GH beforehand. Acute effects of GH are transient and the physiological significance is elusive (Bengtsson, Brummer, Eden, Rosen, & Sjostrom, 1992).

On the contrary, chronic effects of GH on adipose tissue are the classic insulin antagonistic or diabetogenic effects (Davidson, 1987). Prolonged incubation of adipose tissue or isolated adipocytes with GH promotes lipolysis and suppresses lipogenesis, leading to a reduction in the lipid content of adipocytes (Dietz & Schwartz, 1991). The promoted lipolysis is, in part, due to an increase in HSL activity in adipocytes treated with GH (Vernon, Piperova, Watt, Finley, & Lindsay-Watt, 1993; Yip & Goodman, 1999). This GH-induced HSL activity enhancement involves two mechanisms (see Figure 3). On the one hand, GH increases response and sensitivity of the adipocyte to catecholamines, which, in part, is related to an increase in the number of β-adrenergic receptors on the cell surface (Kamel, Norgren, Elimam, Danielsson, & Marcus, 2000; Watt, Finley, Cork, Clegg, & Vernon, 1991). GH has also been found to increase lipolysis by accelerating post-receptor steps of lipolysis, promoting adenylate cyclase function and increasing cellular cAMP concentration, which lead to a higher rate of phosphorylation and translocation of HSL to the surface of the lipid droplet (Vernon et al., 1993; Yip & Goodman, 1999). On the other hand, GH decreases the response and sensitivity of the adipocyte to antilipolytic factors, including adenosine and prostaglandin E (Doris, Kilgour, Houslay, & Vernon, 1998). GH has been shown to reduce the
interaction between Gi and adenylate cyclase by either decreasing the abundance of Gi or impairing the ability of one or more of the iso-forms of Gi. As a result, the PKA-induced HSL phosphorylation and translocation are less inhibited, thereby lipolysis is elevated (Doris et al., 1998).

Figure 3. Growth hormone (GH) effects on adipocyte lipolysis. Gs = stimulating guanine-nucleotide regulator protein, Gi = inhibitory guanine-nucleotide regulator protein, ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, PKA = protein kinase A, HSL = hormone sensitive lipase, ATGL = adipose triglyceride lipase, TAG = triacylglycerol.

In addition to lipolysis, GH also has a long-term negative effect on lipogenesis (see Figure 4). The Richelsen group treated obese females with GH or placebo for 5 weeks and examined the LPL activity in adipose tissue biopsies taken from the
subcutaneous abdominal and gluteal regions (Richelsen et al., 1994). The results suggested that GH reduces LPL activity by approximately 50% in those adipose tissues. With similar results obtained in other studies (Barcellini-Couget, Peadines-Figueres, Rous, Dani, & Aihaud, 1993; Ottosson et al., 1995), we may draw a conclusion that GH plays an active role to inhibit lipogenesis by reducing LPL activity. Besides LPL, chronic treatment with GH also leads to suppression of adipocyte glucose uptake. \textit{In vitro} studies have shown that GLUT 4 transporter number present on adipocyte plasma membrane is significantly reduced after GH treatment (Kilgour, Baldwin, & Flint, 1995; Smith et al., 1997). As most of insulin’s actions are reduced by GH, GH may also affect some early steps in the insulin-stimulated pathway cascade (Yin, Clarke, Peters, & Etherton, 1998).

\textbf{Figure 4.} Growth hormone (GH) effects on adipocyte lipogenesis. TAG = triacylglycerol, LPL = lipoprotein lipase, GLUT4 = glucose transporter 4, Glucose-6-P = glucose-6-phosphate, CoA = coenzyme A, FFA = free fatty acid.
**Adipocyte proliferation and differentiation.** GH alters the total number of adipocytes by influencing proliferation and differentiation of adipocytes. However, the results vary as to how GH influences these processes according to the cell or tissue system used. In particular, the results obtained from the studies of GH effect on adipocyte proliferation remain controversial. For instance, GH has been demonstrated to inhibit proliferation in clonal mouse embryo cell lines, such as 3T3-F442A (Tang, Jeoung, & Sonenberg, 1995), but has been found to stimulate proliferation in cultures of primary adipocytes (Wabitsch et al., 1996). The ability of GH to regulate adipocyte differentiation is more consistent between cell lines versus primary adipocyte culture. In addition, GH effects on adipocyte proliferation and differentiation vary from species to species. In studies with primary cells, GH increases the proliferation of human and rat adipocytes (Wabitsch et al., 1996) whereas GH decreases preadipocyte proliferation in pig adipocytes (Gerfault, Louveau, & Mourot, 1999). The literature is lacking with respect to GH’s effects in mouse adipose tissue with only one study reporting that transgenic female mice expressing porcine GH have an inhibition of adipocyte differentiation (Chen et al., 2001).

GH also modulates adipocyte proliferation and differentiation indirectly via IGF-1. Similar to GH, the IGF-1 effect on these processes varies according to species. For example, while IGF-1 promotes proliferation in rabbit preadipocytes (Nougues et al., 1993), IGF-1 has been observed to either increase or decrease the proliferation in pig primary adipocyte cultures (Gerfault et al., 1999; Ramsay, White, & Wolverton, 1989; Wright & Hausman, 1995). Nevertheless, IGF-1 has been shown to enhance cell differentiation in both cell lines (Blake & Clarke, 1990) and primary culture (Hausman,
Studies with mouse adipose tissue are again limiting but one study with the mouse preadipocyte ob1771 cell line indicated that IGF-1 triggers adipocyte differentiation (Kamai, Mikawa, Endo, Sakai, & Komano, 1996). Overall, the effects of GH on adipocyte proliferation and differentiation are still debatable. Since GH and IGF-1 influence the production of each other, their roles in cell proliferation and differentiation are hard to differentiate (Louveau & Gondret, 2004).

*Site-specific effects.* GH has site-specific effects on adipose tissue distribution. For example, individuals with GHD are obese with increased body fat mass and decreased body lean mass. GHD individuals treated with GH exhibit increased muscle size and reduced intra-abdominal adipose tissue in preference to other depots (Bengtsson et al., 1992). This regional-specific impact of GH might be due to differing GHR expression levels. There is only one study available (LaFranchi, Hanna, Torresani, Schoenle, & Illig, 1985), in which rat adipocytes of epididymal, subcutaneous, and retroperitoneal fat pads were compared in their responsiveness to human growth hormone (hGH). The results showed that hGH binding in epididymal adipocytes is significantly higher than that in either subcutaneous or retroperitoneal adipocytes (LaFranchi et al., 1985). Such location-dependent GHR expression results in different sensitivities to GH between adipose tissue from various anatomic sites and explains why abdominal adipose tissue is specifically sensitive to GH administration in GHD patients. Another mechanism that might explain the regional difference of GH’s effect on adipose tissue is the depot-specific expression of proteins related to GH function. For example, LPL activity and expression varies from fat depot to fat depot (Rosenbaum et al., 1989), and GH has been shown to reduce more LPL activity in intra-abdominal adipocytes than in
subcutaneous adipocytes, retarding the adipocyte lipogenesis. These different LPL activities resulting from GH’s effects might also be responsible for the more pronounced reduction in the intra-abdominal fat depot than in subcutaneous adipose tissue after GH treatment (Rosenbaum et al., 1989).

Flint and Gardner (1993) conducted research on the GH site-specific effects on adipose tissue development. They found that long-term treatment with antiserum to rat GH (anti-rGH) on neonatal female rats caused a profound reduction, about 80%, in the number of differentiated adipocytes in parametrial and perirenal fat pads while the subcutaneous adipose tissue was reduced by only 20%. The results obtained from this study implicated that GH not only affects adipocyte metabolism, but also regulates adipose tissue development in a site-specific fashion (Flint & Gardner, 1993).

Transgenic Mouse Models with Altered Growth Hormone Signaling

There are numerous animal models available with varying levels of GH function. Three different mouse models are available for use in this study and were developed previously (see Figure 5). These three mouse models include mice expressing bovine growth hormone (bGH), expressing a growth hormone antagonist (GHA), and with a disruption of the GHR/BP gene (GHR-/-). These three mouse models have been documented to have different levels of GH signaling (Chen et al., 1991b; Zhou et al., 1997). The advantage of working with these three models is that they allow for direct comparison of excess, reduction and absence of GH function.
Figure 5. Mouse models with varying levels of growth hormone (GH) signaling. GH receptor/binding protein disrupted mice (GHR-/-), bovine GH antagonist (GHA) mice, bovine GH expressing (bGH) mice and wild type (WT) mice.

GHR -/- Mice

GHR -/- mice were generated by disruption of the mouse GHR/BP gene, resulting in the absence of GH-induced intracellular signaling (Zhou et al., 1997). The founder animal was made by homologous recombination resulting in the deletion of most of exon 4 and part of the intron 4 of the GHR/BP gene. Homozygous GHR -/- mice are produced by inbreeding of heterozygous GHR +/- mice. In GHR -/- mice, both alleles of the GHR/BP gene are disrupted. Therefore, the ability of target cells (e.g., liver or kidney) to bind the GH molecule is greatly decreased as compared to their GHR +/- littermates (Zhou et al., 1997).

The growth of GHR -/- mice is dramatically reduced (Zhou et al., 1997). At birth, the body size and weight of GHR -/- mice are similar to normal littermates (GHR +/-
mice). However, after three weeks, the GHR -/- mice are significantly smaller than their GHR +/+ littermates. At week eight, the body length of the GHR -/- mice is markedly shorter than GHR +/+ mice. Adult weight of the GHR -/- mice averages about 40% of the normal controls (Coschigano et al., 2000, 2003). The weights of major organs in GHR -/- mice were also examined. In GHR -/- mice, when normalized to body weight, the liver and kidney are disproportionally smaller whereas the brain is disproportionally larger than their normal littermates (Coschigano et al., 2003; Zhou et al., 1997).

IGF-1 levels are greatly decreased in the GHR -/- mouse line. The serum IGF-1 levels were reduced more than 80% in GHR -/- mice as compared to their littermate controls (Coschigano et al., 2003; Zhou et al., 1997). Because IGF-1 levels were decreased, it is not surprising that the major binding protein for IGF-1, IGFBP3, is also severely decreased in GHR -/- mice (Coschigano et al., 2003). Study has also demonstrated that the serum GH level is elevated in GHR -/- mice (Zhou et al., 1997). This elevation in GH despite an apparent lack of GH function is likely due to low IGF-1 levels, resulting in lack of negative feedback on GH secretion. Moreover, GHR -/- mice have been observed to have significantly decreased fasting serum insulin levels and glucose levels as compared to their littermate controls, suggesting that they are more sensitive to insulin (Coschigano et al., 2003).

GHA Transgenic Mice

GHA founder mice were generated by direct microinjection of foreign DNA into the male pronucleus of fertilized mouse eggs (Chen et al., 1991a). The bGH gene was mutated by a replacement of the codon for glycine at position 119 with a codon for a lysine residue in the third α-helix to produce the GHA gene. The expression of this GHA
gene is driven by the mouse metallothionein-I transcriptional regulatory element. The GHA protein fails to bind correctly to GHR and, therefore, can not activate the signaling cascade. In GHA transgenic mice, GHA competitively binds to GHR with an affinity similar to that of endogenous GH but cannot induce the signaling cascade, thus the GH-induced intracellular signaling is decreased.

Although less impacted as compared to GHR -/- mice, growth retardation has also been observed in GHA mice. The body weight of GHA mice is 61% of their respective controls at the age of four weeks (Coschigano et al., 2003). At 2 months of age, the mean growth ratio (body weight of transgenic mice per body weight of corresponding littermates) is about 0.7 (Chen et al., 1991a). In addition, the absolute weight of all organs except epididymal adipose tissue in GHA mice is significantly decreased as compared to the littermate controls. When the absolute organ weights are normalized to body weight, organs including liver, kidney, stomach and heart are disproportionately smaller than those of control mice (Coschigano et al., 2003).

The serum IGF-1 levels were also reduced 20-25% in GHA mice, associated with a decreased level of IGFBP3 (Coschigano et al., 2003). Due to this reduction in IGF-1 level, the pituitary GH concentration of GHA mice is significantly elevated (Chen et al., 1991a). In addition, research has found that, similar to their normal littermates, the GHA mice maintain normal fasting insulin and glucose levels and exhibit insulin resistance as they age (Coschigano et al., 2003).

**bGH Transgenic Mice**

To generate the bGH founder mouse for this study, a gene fusion between a mouse metallothionein transcriptional regulatory element and a bGH cDNA containing
the first intron was injected into the pronucleus of mice embryos (Berryman et al., 2004). As a result, GH signaling was found to be increased in this mouse model. Opposite to GHR -/- and GHA mice, the weight of bGH mice is dramatically increased, with a growth ratio of about 1.7 as compared to their littermate controls (Berryman et al., 2004; Chen et al., 1990). When normalized to body weight, organs including liver and heart are disproportionately larger whereas the brain is disproportionately smaller than those of their normal littermates (Berryman et al., 2004).

The serum level of IGF-1 in bGH transgenic mice is about twice that of control animals, thus resulting in a significant reduction in pituitary GH concentration (Chen et al., 1991a). Moreover, bGH transgenic mice are shown to have severe insulin resistance. One study demonstrated that, in bGH mice, the insulin-stimulated glucose uptake in skeletal muscles and WAT were reduced by 70% and 80%, respectively. This observation supports the role of GH in insulin resistance and development of type 2 diabetes in acromagalic patients (Higashimori et al., 2003).

The Size of Adipose Tissue Mass

The size of adipose tissue mass has been recently assessed in GHR -/-, GHA and bGH mice models (Berryman et al., 2004, 2006). The absolute fat body mass in these three mouse models are not significantly different from their corresponding controls, which is remarkable considering the severe differences in the absolute size of these mouse lines. However, when adipose tissue is normalized to body weight, GHR -/- and GHA mice had an increase in the percent adipose tissue mass compared to controls, whereas bGH mice had a decreased percent of fat mass compared to littermate controls.
Due to the site-specific effects of GH, adipose tissues from epididymal, retroperitoneal and subcutaneous fat regions are not equally affected by the level of GH signaling (Berryman et al., 2004). In GHR-/- mice, when normalized to body weight, subcutaneous and retroperitoneal adipose tissue are disproportionately enlarged (Berryman et al., 2004, 2006). In contrast, the size of epididymal fat pads of GHR-/- mice is decreased according to their body size (Berryman et al., 2004, 2006; Coschigano et al., 2003). In GHA mice, the subcutaneous adipose tissue increased dramatically, though the other two fat pads remained unchanged. In bGH mice, although no statistical difference has been observed in all three fat pads as compared to their littermate controls, the trend toward lower mass in epididymal and retroperitoneal fat pads was observed (Berryman et al., 2004, 2006).

Summary

WAT plays a critical role in storing and releasing TAGs to meet the body’s energy needs. GH has profound effects on adipose tissue, increasing lipolysis and decreasing lipogenesis. Mouse models with varying levels of GH signaling provide useful animal models to understand the GH effects and mechanism. These mouse models are also good systems to evaluate the effects of GH on adipocyte size.
CHAPTER 3

METHODOLOGY

Adipocyte cell size can be assessed using either whole adipose tissue or adipocytes that have been isolated from adipose tissue. Since both methods have advantages and disadvantages during tissue preparation and cell size measurement (Chen & Farese 2002; DiGirolamo et al., 1971; Farnier et al., 2003) and neither method had been implemented in our laboratory, both methods were initially tried to establish the best method for the laboratory to routinely assess adipocyte size.

Mice

Male GHR -/-, GHA, and bGH mice, as well as their corresponding littermate control mice, at the age of 6 months, were used in the study. GHR -/- mice were generated with a disruption of GHR/BP gene in a 129Ola/BalbC genetic background, as described previously (Zhou et al., 1997). For the adipocyte isolation, measurement, mice backcrossed eight generations with C57BL/6J mice were used. GHA transgenic mice were originally generated by pronuclear injection of a bovine GH minigene with a mutation in the third helix resulting in glycine 119 being replaced by lysine (Chen et al., 1991b). The GHA mice with a C57BL/6J-SJL genetic background were backcrossed greater than 20 generations into a pure C57BL/6J background. The bGH transgenic mice were generated using a metallothionein transcriptional regulatory element linked to the first exon of the bGH cDNA, including the first intron, and were
generated by pronuclear microinjection in a pure C57BL/6J background (Berryman et al., 2004). For measurement of adipocytes from whole tissue, adipose tissue samples were collected from the same mice described in a previous study (Berryman et al., 2004). Specifically, adipose tissues collected from the same fat depots in GHR -/- mice in the Ola/BalbC background (n = 3), GHA transgenic mice (n = 3), bGH mice (n = 3), and their corresponding littermates (WT1, GHR-/- littermates n = 3; WT2, GHA littermates n = 3; and WT3, bGH littermates n = 3) were used for this study. Separate littermate controls were necessary because of the varied genetic background of the mouse models.

For sizing adipocytes isolated from whole tissue, the adipose tissues were obtained from mice all in the C57BL/6J genetic background. However, because this method requires a large number of genetically modified mice and because it is difficult to generate these mice, we first explored how this method compares with the whole tissue method using isolated adipocytes pooled from five wild type mice. As described in the results section, it was determined to not use this method with the genetically modified mice and so no additional mice were used with this method of assessing adipocyte size.

Regardless of protocol, all mice used in these measurements were fed standard rodent chow after weaning at 28 days. The detailed animal generation, breeding, feeding and housing were described previously (Berryman et al., 2004). All protocols were approved by the Ohio University Institutional Animal Care and Use Committee and followed federal, state, and local guidelines.
Adipose Tissue Preparation

Whole Adipose Tissue

Adipose tissues were obtained from subcutaneous (inguinal), epididymal and retroperitoneal regions of each animal after sacrifice. Tissues were immediately placed in 10% formalin for fixation. Fixed samples were shipped to AMlabs for histology processing, embedding in paraffin, cutting 5 µm sections, mounting, and staining with hematoxylin and eosin. The stained slides were then shipped back for further analysis.

Isolated Adipocytes

To prepare the isolated adipocytes, fat pads from epididymal and inguinal subcutaneous areas were dissected from animals immediately after sacrifice and placed in high glucose DMEM held at 37°C. Since the retroperitoneal fat pad was very small, the adipose tissue from this depot was not harvested for this trial assay. To collect isolated cells, fat tissues were minced and digested in cell isolation media containing collagenase for 45 minutes at 37°C. Then, the liberated adipocytes were gently centrifuged and the cell suspension was washed with cell isolation media. The wash and centrifuge procedure were repeated three times. After the last wash, the adipocytes were stained with methylene blue at 37°C for 5 minutes. A complete protocol for adipocyte isolation is provided in Appendix A. Following the adipocyte staining, the cell suspension was placed in a well slide and individual adipocytes were sized using a microscope as described below. For each fat pad, three slides were used.

Measurement of Cell Size

For whole tissue, slides were examined with a Nikon Eclipse E600 microscope equipped with a SPOT RT (Real Time) digital camera at bright field and 20X
magnification. Digital images were taken for three non-overlapped microscope fields in each fat pad from the same animal. The measurement of cross-sectional area of adipocytes was accomplished with the combination of two software programs: Adobe Photoshop® 7.0, a program to manipulate image, and Reindeer ® Graphic Image Processing Tool Kit v.5, a plug-in software to Adobe Photoshop to measure the adipocyte area. With these two programs, the areas of a large amount of adipocytes can be measured (at least 200 cells for each fat pad) accurately and rapidly. This protocol is a slight modification of the method described by Chen and Farese (2002) and is described fully in Appendix B. The area results were exported to Microsoft® Excel Pro 2003. Cell size less than 10µm² were considered artifacts generated during the image processing and ignored.

For cell sizing of isolated adipocytes, well slides of adipocytes were viewed at 20x magnification under the same light microscope described above. One digital picture was taken for each well slide. The isolated adipocytes were identified by their spherical shape, a stained nucleus with one or two nucleoli, and stained cytoplasm. Measurements of cross-sectional areas of at least 100 adipocytes in each pad were done with Adobe Photoshop® 7.0 and Reindeer ® Graphic Image Processing Tool as described above.

Statistics

The size of the adipocytes from different animal models was expressed as mean ± SEM (Standard Error of Mean). To compare the cell size between different genotypes and fat pads, univariate 2x3 ANOVA was conducted first in SPSS 12.0 for Windows. When the results of 2x3 ANOVA permitted further statistic analysis, independent t-test were utilized to compare means of adipocyte size of GHR -/- mice, GHA mice, bGH...
mice and their corresponding littermate controls. One-way ANOVA were conducted to compare the cell size between different fat pads in each mouse model. In addition, the data obtained with whole adipose tissue were compared with data from isolated adipocytes by conducting an independent \( t \)-test. Significant differences were considered if \( p \leq 0.01 \).

To assess the depot-specific GH effects on adipose tissue, the percent change of adipocyte cross-sectional areas in epididymal, retroperitoneal and subcutaneous fat pads for GHR -/-, GHA and bGH mice were compared to their corresponding littermate controls. The formula used for the comparison was:

\[
\text{Percent change} = \left( \frac{\text{Mean cross-sectional area of GH genotype}}{\text{Mean cross-sectional area of littermate control}} \right) \times 100
\]
CHAPTER 4

RESULTS

Methods to assess adipocyte size using isolated adipocytes versus whole adipose tissue were compared. For the method using isolated adipocytes, only adipocyte isolated from subcutaneous and epididymal fat pads of five wild type mice were measured for their cell size as the retroperitoneal fat pad was too small to gather sufficient tissue for analysis. The results showed that the mean cross-sectional area of isolated adipocytes from epididymal fat pads was $889 \pm 19 \, \mu m^2$ ($n = 395$) and the mean cross-sectional area of adipocytes from subcutaneous depots was $406 \pm 13 \, \mu m^2$ ($n = 452$). To compare these two values to the results obtained from corresponding fat pads (WT2) using the whole adipose tissue method (see Table 1), the cross-sectional areas of adipocytes of isolated adipocytes are dramatically smaller in both epididymal ($889 \pm 19 \, \mu m^2$ vs. $1561 \pm 25 \, \mu m^2$, $p < 0.001$) and subcutaneous ($406 \pm 13 \, \mu m^2$ vs. $830 \pm 9 \, \mu m^2$, $p < 0.001$) fat pads. According to the distribution of the cell size (see Figure 6), the cross-sectional areas of isolated adipocytes were mostly under 2000 $\mu m^2$ in epididymal fat pads and less than 1500$\mu m^2$ in subcutaneous fat pads. In contrast, the cross-sectional areas obtained from whole adipose tissue were mainly between 1000 $\mu m^2$ - 5000 $\mu m^2$ in the epididymal depot and 1000 $\mu m^2$-3000 $\mu m^2$ in the subcutaneous area. Due to these huge differences in cross-sectional areas between the methods, it became clear that the results from these two methods could not be directly compared. Because of the larger number of mice needed
for the cell isolation procedure and because of the inability to assess all depots (retroperitoneal), only the method using whole adipose tissue was utilized to compare the adipocyte size between genotypes and fat pads.
Table 1

*Cross-Sectional Areas of Adipocytes Form Whole Adipose Tissue.*

<table>
<thead>
<tr>
<th></th>
<th>Epididymal</th>
<th>Retroperitoneal</th>
<th>Subcutaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean area ± SEM (µm²)</td>
<td>Median area (µm²)</td>
<td>n</td>
</tr>
<tr>
<td>KO</td>
<td>1875 ± 35</td>
<td>1573</td>
<td>1293</td>
</tr>
<tr>
<td>WT1</td>
<td>1990 ± 31</td>
<td>1789</td>
<td>1271</td>
</tr>
<tr>
<td>GHA</td>
<td>1732 ± 38*</td>
<td>1375</td>
<td>1116</td>
</tr>
<tr>
<td>WT2</td>
<td>1561 ± 25</td>
<td>1358</td>
<td>1308</td>
</tr>
<tr>
<td>bGH</td>
<td>1695 ± 33*</td>
<td>1426</td>
<td>1309</td>
</tr>
<tr>
<td>WT3</td>
<td>2946 ± 36</td>
<td>1856</td>
<td>1071</td>
</tr>
</tbody>
</table>

*Notes. n, number of cells measured.*

* p ≤ 0.01
Figure 6. Distribution of adipocyte size obtained from isolated adipocytes and whole adipose tissue. A: Isolated adipocytes vs. whole adipose tissue (WT2) in epididymal fat pads. B: isolated adipocytes vs. whole adipose tissue (WT2). Iso: isolated adipocytes. Whole: whole adipose tissue. Epi: epididymal fat pads. SubQ: subcutaneous fat pads.

The cross-sectional areas of adipocytes of GHR -/-, GHA, bGH, and their corresponding littermate controls were determined utilizing the whole adipose tissue method (see Figure 7 and Table 1). For GHR -/- mice (see Figure 8), the mean cross-sectional areas of adipocytes from retroperitoneal ($p < 0.0001$) and subcutaneous ($p < 0.001$) fat pads were significantly enlarged as compared to their littermate controls. However, the mean cell sizes of adipocytes from epididymal fat pads in GHR -/- mice and WT1 mice were not statistically different ($p = 0.013$). For GHA mice (see Figure 8), the cross-sectional areas from all fat pads, epididymal ($p < 0.001$), retroperitoneal ($p < 0.001$), and subcutaneous ($p < 0.001$), were significantly increased in GHA mice as compared to their littermate controls. In contrast to both GHR -/- and GHA mice (see Figure 8), the mean cross-sectional areas of adipocytes from epididymal ($p < 0.001$),
retroperitoneal ($p < 0.001$), and subcutaneous ($p < 0.001$) fat pads were significantly reduced in bGH mice as compared to those in their littermate controls.

*Figure 7.* Histology of whole adipose tissue from mouse models with varying levels of GH signaling. Image captured at 10× magnification. A, WT1; B, GHR -/-; C, WT2; D, GHA; E, WT3; F, bGH. Only adipose tissues from the subcutaneous depot are shown. Scale bar represent 100 µm.
Figure 8. Mean cross-sectional areas of adipocytes obtained by the whole adipose tissue method. Adipocytes from Epididymal (Epi), retroperitoneal (Retro) and subcutaneous (SubQ) fat pads are shown. * $p \leq 0.01$. 
The increased and decreased adipocyte size was also reflected in median cross-sectional areas (see Table 1) and the distribution of cell size (see Figure 9). In mouse models with absent (GHR -/-) and decreased (GHA) GH signaling, there were more enlarged cells in all fat pads measured than their corresponding littermates, except for the epididymal fat pad in GHR -/- mice. In contrast, the adipocyte size distribution in bGH mice was shifted towards smaller cells.

Figure 9. Cells size distribution of GHR-/-, GHA, bGH mice, and their littermate controls. Epi, Epididymal; Retro, retroperitoneal; SubQ, subcutaneous.

In addition, the site-specific effect of adipose tissue was also assessed by calculating the percent of change in cross-sectional areas in all three mouse models with
varying levels of GH signaling. The results (see Table 2) indicated that adipocytes from subcutaneous depot in GHR-/- and GHA mice had the highest percent of change in size as compared to their littermate controls whereas adipocytes from epididymal fat pads had the least cell size change due to the absence or decreased GH signaling. Similar effects were also been observed in bGH mice with increased GH signaling, with adipocyte size being reduced more dramatically in retroperitoneal and subcutaneous fat pads rather than in the epididymal area.

Table 2

Percent Change in Adipocyte Cell Size for GHR -/-, GHA and bGH Mice Compared to Littermate Controls

<table>
<thead>
<tr>
<th>genotypes</th>
<th>Epididymal</th>
<th>Retroperitoneal</th>
<th>Subcutaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHR -/- vs. WT1</td>
<td>-5.8</td>
<td>10.6</td>
<td>33.3</td>
</tr>
<tr>
<td>GHA vs. WT2</td>
<td>11.0</td>
<td>27.7</td>
<td>74.4</td>
</tr>
<tr>
<td>bGH vs. WT3</td>
<td>-17.2</td>
<td>-41.5</td>
<td>-39.7</td>
</tr>
</tbody>
</table>
CHAPTER 5

DISCUSSION

Adipocyte Cell Size

GH is known to impact adipose tissue metabolism by increasing lipolysis and decreasing lipogenesis (Louveau & Gondret, 2004). A previous study (Berryman et al., 2004) using the same mice utilized in this study has shown that GHR -/- and GHA mice are obese with an increased percent of fat tissue whereas bGH mice are lean and have decreased percent body fat. Since changes in adipose tissue mass involve either a change in adipocyte number and adipocyte size or both (Hausman et al., 2001), it is important to determine adipocyte size and number in these mice with altered adiposity. In terms of cell size, one might expect the size of adipocytes to increase in mouse models with increased obesity, as with the dwarf GHR -/- and GHA mice. Likewise, adipocyte size should decrease in a mouse model with decreased obesity, such as the bGH increased GH function. The present data on adipocyte size agree nicely with these expectations in that GHR -/- and GHA mice have enlarged adipocyte size as compared to their littermate controls, whereas bGH have decreased adipocyte size as compared to their controls. In retroperitoneal and epididymal fat pads, the mean cross-sectional areas of GHA and GHR -/- mice are significantly larger than those in their corresponding littermate controls, but the median cross-sectional areas of these fat pads are similar in GH signaling altered mouse models and their controls; this reflects the existence of some extremely large cells.
in retroperitoneal and epididymal fat pads in GHA and GHR -/- mice. Although this study does not provide information about adipocyte numbers in these mouse models, the enlargement in adipocyte size appears to be at least partially responsible for the increased body fat mass in GHR -/- and GHA mice. In contrast, the decrease in percent fat mass of lean bGH mice is, in part, due to a reduction in size of adipocytes.

Existing data have shown that there are site-specific effects of GH on adipose tissue regarding adipocyte size, adipocyte number and adipose tissue mass in various species. For example, GH administrations in children are associated with specific significant reductions in both abdominal adipocyte size (Rosenbaum, Gertner, Gidfar, Hirsch, & Leibel, 1992) and the amount of adipose tissue mass (Roemmich, Huerta, Sundaesan, & Rogol, 2001). It has also been reported that long-term anti-rGH in rat results in an 80% reduction in the number of differentiated adipocytes in parametrical (an female intra-abdominal adipose tissue comparable to the epididymal fat depot in male mice) and perirenal adipose tissues, whereas the subcutaneous adipocyte numbers were only reduced 20% as compared to control mice (Flint & Gardner, 1993). Moreover, a recent in vitro study has shown that adipocytes from parametrical depots of GHR -/- mice fail to proliferate and differentiate normally, while the adipocytes from subcutaneous fat pads from the same mice are minimally affected and the preadipocytes development normally (Flint, Binart, Kopchick, & Kelly, 2003). All these data suggest that intra-abdominal adipose tissue is impacted more by the alteration of GH signaling than subcutaneous adipose tissue.

In contrast, current data show that the alterations of adipocyte size due to the GH signaling are most dramatic in subcutaneous fat pads and least significant in epididymal
fat pads in all three mouse models measured. Similar observations of GH site-specific effects on adipose tissue have also been reported (Berryman et al., 2004, 2006). In both of these studies, dwarf models (GHR -/- and GHA mice) have a preferential accumulation of fat mass in the subcutaneous region. Furthermore, in Flint and Gardner’s study in rats, short-term anti-rGH treatment led to a significant increase in subcutaneous adipose tissue weight, while the intra-abdominal adipose tissue depots were unaffected (Flint & Gardner, 1993), suggesting that the subcutaneous fat pad is more dramatically influenced by GH signaling than intra-abdominal pads. Taken together, although the specific depot impacted by GH signaling may vary according to the model and methods used, these data collectively indicate that the impact of GH on adipose tissue is not uniform and varies according to the location of the stores.

Methods for Cell Sizing

In this study, methods using whole adipose tissue and isolated adipocytes to determine adipocyte size were compared. Ultimately, only the method using whole adipose tissue was used to evaluate adipocyte size between different genotypes and adipose tissue depots. Although there are several advantages and disadvantages of these two methods, there are two reasons to utilize whole adipose tissue method instead of the method using isolated adipocytes.

First, the mean cross-sectional areas of isolated adipocytes from epididymal and subcutaneous fat pads are less than half of the size of adipocytes from corresponding fat pads using the whole adipose tissue method. The reduced cell size is likely because the extremely fragile large adipocytes tend to break easily during the cell suspension preparation (Hirsh & Gallian, 1968). Thus, reducing the wash in the isolated cell
preparation could increase the adipocyte size. The difference in cell size could also be
due to another limitation of the method using isolated adipocytes. In this study, the
isolated adipocyte size is assessed using digital image and computer programs.
Therefore, every cell in the digital image is assumed to be the largest cross-sectional area.
However, as the free-floating cells are at different levels within the microscope’s field of
view, it is impossible to assure all the cells in the field of view present their maximum
cross-sectional areas. Therefore, the mean cross-sectional areas of a population of the
isolated adipocytes are likely underestimated using this method.

Second, in order to obtain a comparable number of cells for sizing, the procedure
of cell isolation should be repeated three times and would require at a minimum two mice
per time. To accomplish the study using isolated adipocytes, at least six mice for each
genotype were needed. Unfortunately, mice with altered levels of GH signaling are not
easy to generate, making this method more challenging. For example, GHR -/- mice
have a smaller litter size but higher perinatal or immediate postnatal mortality rate of
newborns (Zhou et al., 1997). Therefore, using a lot of animals to measure adipocyte cell
size with isolated adipocytes is more costly and unnecessary especially when another
method is available that uses fewer animals.

When using whole adipose tissue to assess and compare the size of adipocytes,
adipocytes are not spherical. Because adipocytes are embedded in the whole tissue, the
adipocyte shape is dependent on surrounding cells making the adipocytes not circular and
uniform in shape as would be found in a cell suspension. Thus, the cross-sectional area
of each adipocyte from the same section might be either smaller or larger than the cross-
sectional areas through the equators, and the values obtained for adipocyte size would be
affected. Nevertheless, this study is to compare the cell size among different mouse models. If the whole adipose tissue method is consistently used in all fat pads and genotypes and a large number of cells are measured, the data will be effective to compare the size of adipocytes between fat pads or mouse models with varying levels of GH signaling.

Although there are advantages to using whole adipose tissue to compare cell size, the method still has some limitations. For example, the adipose tissue sample must be well prepared. Since adipocytes are very fragile because of their large lipid droplet, they are easily broken or distorted during the histological process of sample preparation (Chen & Farese, 2002). Thus, the accuracy of the cross-sectional areas of adipocytes might be affected if the sample sections are not well prepared. In addition, whole adipose tissue does not contain adipocytes exclusively as would be the case with isolated adipocyte suspension. Other cells within adipose tissue may push and distort the adipocytes; therefore, the cross-sectional areas of adipocytes might be affected.

Perspectives

This study indicates that GH influences adipocyte size according to the location of the store. Based on these findings, a number of future studies in these mice are warranted. For example, it may be useful to compare the GHR expression levels among adipose tissue depots. Previous reports have indicated that the expression of GHR is different between different adipose tissue depots in rat, with hGH binding to epididymal adipocytes being higher than those in retroperitoneal and subcutaneous fat pads (LaFranchi et al., 1985). Therefore, one would expect that the differences in adipocyte size among different adipose tissue in GHR-/-, GHA and bGH mice may be a result of
different levels of GHR expression levels in various adipose tissue depots. These results may elucidate the mechanism of how GH impacts adipose tissue mass, and adipocyte size in a site-specific manner will be better understood.

The same mouse models could be used to assess the impact of GH on adipocyte number. Since the alteration of adipose tissue mass can be due to increase or decrease of both cell size and cell number, studying the changes of adipocyte differentiation rate between adipose tissue depots in mice with varying levels of GH signaling is another important aspect to evaluate. A previous study in female GHR -/- mice revealed that the parametrical adipose tissue development is impaired due to a decreased number of adipocytes and reduced proliferation and differentiation of preadipocytes, whereas the subcutaneous adipose tissue development and the preadipocyte proliferation and differentiation were least affected (Flint et al., 2003). Nevertheless, no study has been done to assess the depot-specific rates of adipocyte differentiation using male GHR -/-, GHA and bGH mice. Since GH secretion and GH effects are related to genders (Cohen et al., 2002; Eckstein et al., 2002; Koutkia, Eaton, You, Breu, & Grinspoon, 2006), data about depot-specific rates of adipocyte differentiation in male mice, along with the results obtained from this study, will provide insight into the mechanism of how GH signaling alters adipose tissue mass.

In summary, this is the first study to evaluate the effects of GH on adipocyte size in GHR -/-, GHA and bGH mouse models. Adipocyte size is increased in mouse models with absent and decreased GH signaling and is reduced in mouse models with elevated GH signaling. In addition, this study demonstrates that GH influences the adipose tissue in a site-specific manner. Given the prevalence of obesity and the need of a better
treatment of obesity (Scacchi et al., 1999; Shadid & Jensen, 2003), this study provides more understanding of the relationship between GH and adipose tissue.
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Appendix A

Isolated Adipocyte Protocol
Isolated Adipocyte Protocol

1. Dissect adipose tissue from epididymal, and subcutaneous (scapular and inguinal) regions and get the weight of adipose tissue from each fat pad separately.
2. Soak adipose tissue immediately in high glucose DMEM held at 37°C.
3. Thoroughly mince adipose tissue in the DMEM with sharp scissors to dice the fat tissue into very fine pieces for 5-7 min.
4. Pour DMEM/diced adipose tissue into filter/mesh system.
5. Rinse with PBS 3 times.
6. Disassemble filter/mesh system and push the minced adipose tissue onto a piece of weighing paper.
7. Weight the adipose tissue for both subcutaneous and epididymal and record weight of each.
8. Place adipose tissue into Erlenmeyer flask. Add appropriate amount of cell isolation media (ISO) containing collagenase. For each gram of adipose tissue, use 1 ml of ISO and 1 mg collagenase (No. X58H100).
9. Digest the tissue for 45 minutes at 37°C and 80 cpm shaking.
10. When digestion is complete, pour the solution with digested adipose tissue into the 50 centrifuge tube through a mesh. Rinse the mesh and the flask several times with ISO.
11. Spin catheter placed in the centrifuge tube to carefully mix the cells and medium
12. Centrifuge the tube for 2 minutes at the speed of 1000 rpm at room temperature.
13. Remove the medium under the fat layer using catheter and a clean syringe with 18 gauge needle.
14. Add about 40 ml of ISO to the tube.

15. Repeat step 11-14.


17. Record the approximate volume of fat cell layer. Make a 20% cell suspension with ISO.

18. For each 1 ml of cell suspension, add 1 ml water and 1 drop of methylene blue solution (20 mg in 1 ml water). Gently stir to mix.

19. Incubate for 5 min at 37°C.

20. Gently stir cell suspension and out 3-4 drops on glass slide and covered with spacer. Make 3 slides for each fat pad.

21. Examine adipocytes under microscope with 20X objective.
Appendix B

Adipocyte Size Measurement Protocol
Adipocyte Size Measurement Protocol

1. Open image by Photoshop.

2. Go to Image ➔ adjustment ➔ Auto Contrast, then, go to Image ➔ Grey Scale.

3. Circle each cell by Magnetic Lasso Toll: fill the space selected with black; then border the space with white (10px).

4. Make clear image: go to select ➔ color range ➔ sample color (black) ➔ copy.

5. Create a new image with the same size as the original image and white background ➔ paste black area ➔ flatten image ➔ save new image.

6. Import calibration: Filter ➔ Measure global ➔ Calibrate Magnification ➔ import calibration

7. Measure: Filter ➔ Measure features ➔ Measure all features