

METABOLIC AND ENDOCRINE RESPONSES TO OVEREATING

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Kinesiology)
in The University of Michigan
2012

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Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.

Ithaka gave you a marvelous journey.
Without her you wouldn't have set out.
She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.
Wise as you will have become, so full of experience,
You'll have understood by then what these Ithakas mean.

BY C. P. CAVAFY 1863–1933

TRANSLATED BY EDMUND KEELEY AND PHILIP SHERRARD

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Acknowledgements

I would like to acknowledge the work of my advisor, Dr. Jeffrey Horowitz, for help writing this dissertation, support, and guidance. I would also like to thank my committee, Dr. Katarina Borer, Dr. Ariel Barkan, and Dr. Jessica Schwartz for their participation.

I would like to thank Dr. Alexander Hinko, Dr. Minghua Li, Lisa Locke Michael, Sean Newsom, Rachael Nelson, Sanghee Park, Chris Paran, Kristin Thomas, Simon Schenk, and Nick Knuth for their kind assistance in the Substrate Metabolism Laboratory.

A special thanks to Kathleen Symons for her contribution to the analysis of GH, IGF-1, and cortisol.

I would also like to thank Po-Ju Lin and Dr. Katarina Borer for their assistance in the Exercise Endocrinology Laboratory. I would also like to thank Katsuhiko Funai, George Schweitzer, Carlos Castorena, Ed Arias, and Dr. Greg Cartee for their assistance in the Muscle Biology Laboratory.

I would like to thank all the staff and nurses of the Michigan Clinical Research Unit at the Michigan Hospital, but especially Daniel Myers, Paula England, Laura Foess-Wood, Marti Hartdegen, Judy Flaherty, Therese Smallwood, and Cindy Boersma.

I would like to thank my parents, Marcia E. Cornford and Eain M. Cornford, for their love, guidance, and financial support. I would like to thank my brothers, Chris A. Cornford and Daniel M. Cornford for their love and believable empathy.

I would not be able to complete this work without the friendship of Lisa Locke Michael and her husband Lowell Evan Michael Ph.D. M.D., Sudha Krishnamurthy, Ph.D., Jennifer Kirk, the Ann Arbor Track Club: Mitch Garner, Randy Gravel, Ken Castle, Lauri Mendenhall, Dorit Hammerling, Nico Howson, Nico's cats Ada and Vera, Kt Tomey, Michael Linkevich, Marty Betts, who's yoga-flex class is awesome, Steve Hinzmann, Susanna Mettler, Ph.D., Heather Huggins, Nicole Erlacher, Knitting Groups: Merrill Hodnefield, Rosalind Keith Ph.D., Debbie Huntzinger, Kt Tomey, Jim's family: Mr. Jim & Joan Whitehead, Jeff & Christina Lower, Dan & Marianne Mutscher, and Brad Whitehead (& fiancé Jayma Hollenbeck), for making me part of your family. I must thank Sacha Fierstein for her kindness. Writing buddies Julie Lesnick, Jen Kirk, and Rachel Afi Quinn.

I would also like to thank Sweetwaters in Ann Arbor and Joe and Rosie in Dexter.

Last, but not least, I must thank James Anthony Whitehead, for love, support, and tolerating me through all this.

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Abstract

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Chair: Jeffrey F. Horowitz

Although metabolic abnormalities common in obesity are well-defined, the metabolic and endocrine responses that occur in the early stages of weight gain are not. The overall objective of my dissertation was to characterize the metabolic and endocrine responses during 2 weeks of overeating. We conducted a battery of metabolic tests in a total of 23 healthy non-obese volunteers before, during, and after a 2-week overeating intervention. Subjects remained in the hospital for the entire 2-week overeating period to ensure strict control over their diet (i.e., they consumed 70kcal/kg FFM/day [\sim 4000kcal/day]) and their physical activity (i.e., they were limited to 1500 steps per day). STUDY 1 of my dissertation examined some important metabolic adaptations to overeating, and we found that 2-weeks of overeating increased whole-body insulin resistance. However, the impaired systemic insulin sensitivity was not accompanied by changes in markers of impaired insulin action within skeletal muscle. Interestingly, muscle lipid accumulation did not increase despite consuming about twice the normal amount of dietary fat (\sim 150 grams/day). STUDY 2 of my dissertation focused on the endocrine responses to

overeating, and as anticipated we found that the large and frequent meals provided during the overeating period resulted in a chronic elevation in plasma insulin concentration ($p < 0.05$). In STUDY 2 we report a nearly 80% suppression in 24h growth hormone (GH) concentration. This reduction in GH was likely a consequence of the inhibitory effects of the chronic hyperinsulinemia during overeating. In STUDY 3 we examined the metabolic consequences of this marked suppression of GH concentration. In a separate group of subjects, we provided multiple daily injections of exogenous GH during the same 2-week overeating intervention. Preventing the fall in plasma GH concentration exacerbated systemic insulin resistance and fasting lipemia was significantly increased. Therefore, the normal fall in plasma GH with overeating may actually help mitigate the rise of cardiovascular and metabolic risk factors. Overall, the findings from the three studies of my dissertation help expand our understanding about the metabolic and endocrine responses that occur in the early stages of weight gain, which may shed important light on the development of obesity-related diseases.

CHAPTER 1

Statement of the Problem

Because weight gain occurs when energy intake exceeds energy expenditure (i.e., overeating), overeating underlies the alarming rise in obesity and obesity-related disorders. During periods of overeating the human body must adapt to accommodate the surplus of available energy. However, the metabolic and endocrine adaptations to overeating are poorly understood.

To further expand our understanding about metabolic adaptations to overeating, there is also great interest in the regulation of storage of the excess energy in different tissues. Clearly, the vast majority of the surplus energy is stored as triacylglycerides in adipose tissue. However, triacylglyceride depots in other tissues, such as liver and skeletal muscle, can also expand in response to overeating. Importantly, under conditions of an energy surplus, storing the excess energy as a neutral lipid (i.e., triacylglyceride) has far fewer negative health implications than an accumulation of other lipid intermediates (e.g., diacylglycerol, ceramide), especially in non-adipose tissues. For example, in skeletal muscle an accumulation of diacylglycerol and ceramide has been found to potently impair insulin sensitivity. Therefore, increasing intramyocellular triacylglyceride synthesis may be a favorable adaptation to overeating. However, the regulation of muscle triacylglyceride metabolism in response to overeating is not known. Furthermore, the accumulation of skeletal muscle lipid may be regulated systemically, through the availability of fatty acids in the plasma.

Endocrine responses to overeating help dictate the metabolic adaptations that occur in response to an energy surplus. However, the endocrine responses to overeating have not been well described. Because overeating profoundly impacts the storage and mobilization of endogenous fuels, it is of particular interest to understand how excessive exogenous energy availability affects hormones that regulate these processes. Specifically, we are

interested in characterizing changes in insulin, and growth hormone (GH) in response to overeating. In our preliminary studies, we found that even just a few days of overeating resulted in a profound suppression of GH. Because GH is known to help regulate many metabolic processes, including: lipolysis, protein synthesis, and insulin sensitivity, this suppression in GH may have important implications on the metabolic responses to overeating. The factors regulating this suppression in GH with overeating are not known, and the metabolic consequences of this lower GH exposure during overeating have not been explored.

My dissertation addressed:

1. The metabolic responses to overeating with regard to substrate availability and the regulation of skeletal muscle lipid metabolism (**STUDY 1**).
2. The endocrine responses to overeating (**STUDY 2**).
3. Factors regulating the suppression in GH secretion in response to overeating (**STUDY 2**).
4. The metabolic consequences of the suppression in GH secretion during overeating (**STUDY 3**).

In order to address these questions, we overfed non-obese subjects for two weeks while they were admitted to the hospital (in order to strictly control energy intake and physical activity level). We collected a muscle biopsy sample before the overeating period and again after two weeks of the diet to assess the accumulation of intramyocellular triacylglyceride and diacylglyceride, as well as to compare the abundance of key lipogenic enzymes. To assess changes in important regulatory and counter-regulatory hormones (i.e., insulin, cortisol, and GH), we collected serial blood samples (every 20 minutes for 24h) before the overeating period as well as on days 3-4 and days 13-14 of overeating. In addition, we measured potential metabolic/hormonal candidates that may be regulating the suppression in GH secretion we observed with overeating in our

preliminary studies. To assess the metabolic consequences of an overeating-induced suppression in GH we compared the metabolic responses to overeating in two separate groups of subjects who received either: 1) no treatment (our preliminary studies indicated that GH secretion is markedly suppressed with overeating), or 2) four GH bolus injections each day of the overeating period (to mimic natural GH secretion pattern under normal feeding conditions). We assessed the effects of the changes in GH concentration on muscle protein synthesis, whole-body proteolytic and lipolytic rates, insulin sensitivity, as well as some intracellular markers in skeletal muscle involved in regulating these processes.

CHAPTER 2

Review of Literature

Clearly when energy intake exceeds energy expenditure (i.e., overeating) weight gain occurs, and chronic overeating underlies the development of obesity and obesity-related diseases (e.g., type 2 diabetes, cardiovascular disease). Metabolic and endocrine profiles of obese adults have been well described (e.g., insulin resistance, hyperinsulinemia, elevated cortisol secretion, reduced growth hormone (GH) secretion, high concentration of intramyocellular triacylglyceride (IMTG)). However, whether these metabolic/endocrine profiles are a consequence of being obese, or whether some of these adaptations begin to develop in response to overeating is not clear. This review will address some important metabolic and endocrine adaptations that occur during overeating, as well as discuss the potential ramifications for these changes. More specifically, we will characterize the effect of overeating on metabolic responses, including skeletal muscle triacylglyceride metabolism, and the potential impact of changes in muscle triacylglyceride metabolism on insulin resistance. We will focus on the effect of overeating on the secretion of insulin, cortisol, and GH. Finally, we will then address how changes in GH secretion with overeating may impact the regulation of some key metabolic processes (e.g., energy expenditure, lipolytic rate, protein metabolism, and insulin resistance).

Metabolic responses to overeating

Several weeks of overeating and the accompanying weight gain can increase basal metabolic rate (i.e., measured after an overnight fast) (24, 32, 105, 124). In contrast, basal metabolic rate was found not to increase after relatively short overeating periods where weight gain was minimal (39, 65, 68). Because resting energy expenditure is tightly correlated with lean body mass (47), and an increase in lean body mass typically accompanies body weight gain, the increase in basal metabolic rate after prolonged overeating is largely due to an increase in lean (i.e., metabolically active) tissue (24, 94, 105). Although short-term overeating has not been found to increase basal energy expenditure, acute overeating has been found to increase 24h metabolic rate (53, 59, 71,

131). Much of this increase in energy expenditure has been attributed to an increase in diet-induced thermogenesis (84). However, the effect of overeating on energy costly processes (e.g., protein turnover, substrate cycling) has not been well described.

Overeating can provide an abundance of exogenous amino acids and high plasma insulin response, which can stimulate protein synthesis (28, 35). Indeed, it has been found that overeating 1,600kcal of carbohydrate per day above energy requirements for ten days resulted in a positive nitrogen balance (i.e. the rate of protein synthesis exceeded the rate of protein breakdown) (132). Interestingly, despite the increased protein accretion with overeating, these authors reported that overeating also increased whole-body proteolysis (132). The simultaneous increase in protein synthesis and proteolysis that was observed with overeating represents a substrate “futile cycle,” an energy costly process that may contribute to increased 24h energy expenditure during overeating. Although an elevated whole-body protein turnover was observed with overeating (132), it is not clear if overeating specifically affects protein synthesis in skeletal muscle.

Basal lipolytic rate (i.e., triacylglyceride hydrolysis after an overnight fast) is elevated in overweight and obese individuals (52). In contrast, lipolytic rate during periods of overeating may actually be suppressed. Adipose tissue lipolysis is exquisitely sensitive to the anti-lipolytic effects of insulin (129), thereby elevated insulin secretion with overeating would be expected to suppress adipose tissue lipolysis. To date, much of the anti-lipolytic effects of insulin have been attributed to effects on hormone sensitive lipase (HSL) activity (63, 97). However, it has recently been recognized that lipolysis is primarily initiated by another lipase (i.e., adipose triacylglyceride lipase (ATGL)). Available evidence suggests that insulin can reduce the expression of ATGL (62), but it is not clear if insulin suppresses the activity of ATGL. Importantly, the anti-lipolytic effect of insulin can persist even after insulin concentration has returned to basal levels (88). Therefore, basal lipolysis during overeating may still be suppressed even if fasting insulin concentration is no longer elevated after an overnight fast.

While it is well known that insulin resistance is directly linked with obesity (13, 41, 61) it is less clear whether insulin resistance occurs in response to overeating before a

measurable increase in bodyweight. A positive caloric balance before an appreciable weight gain did not increase fasting plasma glucose or insulin concentration (93), but fasting glucose and insulin concentrations provide only a very crude index of insulin action. In contrast, Brons, et al (12) recently reported that overeating impaired hepatic insulin sensitivity in subjects that had not yet gained weight as measured by hyperinsulinemic-euglycemic clamp, which is the “gold standard” for assessing insulin resistance *in vivo*. These findings support earlier work in which a positive energy balance was also found to evoke impaired insulin action (31, 93). Alternatively, a negative energy balance has been found to evoke a marked increase in insulin sensitivity (4). The work of Brons, et al. (12) is particularly interesting because they reported that overeating increased hepatic insulin resistance without affecting peripheral glucose uptake (i.e., muscle glucose uptake). This may indicate that muscle is better able to adapt to a chronic caloric challenge compared with the liver. As I will discuss later in this review, one of the beneficial adaptations that skeletal muscle may incur with overeating is the accumulation of intramyocellular triacylglyceride (IMTG).

Endocrine response to overeating

The hormonal response to overeating can dictate much of the acute metabolic consequences to excessive energy availability. The transient rise in plasma glucose concentration after large and/or frequent meals markedly affects the secretion of insulin, and thereby changes the individual’s daily exposure to this hormone in the circulation. Clearly, because this hormone has potent effects on important metabolic processes (e.g., hepatic glucose production, lipolysis, protein turnover) these changes in insulin may have a profound metabolic impact. Insulin concentrations as little as 13 μ U/ml can inhibit lipolysis (42), an insulin concentration of 24 μ U/ml can disrupt hepatic glucose production (96), and concentrations of insulin as low as 16 μ U/ml can suppress proteolysis (37). In addition, because our preliminary findings indicate that overeating profoundly suppressed GH secretion (see Appendix I), the metabolic effects of changes in GH on key metabolic processes will be thoroughly addressed later in this review.

Pancreatic and adrenal hormone secretion with overeating

Eating stimulates insulin secretion, which drives many postprandial metabolic responses, including increasing glucose uptake (23), energy expenditure (134), protein synthesis (28, 36), and reducing lipolysis (97). Insulin is secreted from the pancreas in response to rising blood glucose and amino acid concentrations. Therefore, with greater amounts of carbohydrate in the meal, there is a greater insulin response. Additionally, the plasma insulin concentration rises with eating and then returns to basal levels within a few hours, but the metabolic effects of insulin are far more persistent. For example, longer-term metabolic influence from insulin may be due in part to insulin-stimulated transcription factors such as SREBP-1c (26, 29), thereby activating genes involved with metabolic processes like glucose uptake and triacylglyceride synthesis. Therefore, due at least in part to insulin's effect on gene transcription, insulin can impact metabolic processes even after insulin concentration has declined back to basal levels.

Overeating may affect the concentration of the adrenal hormone, cortisol. Cortisol can influence metabolism by stimulating whole body proteolysis (112), lipolysis (109), and it can contribute to insulin resistance in skeletal muscle *ex vivo* (50). Although the cortisol response to overeating has not been reported, weight gain may affect plasma cortisol concentration. Some evidence suggests that cortisol concentration is elevated with obesity (77), however, follow up studies by this same group suggested that early morning cortisol concentrations were not dependent on body mass index (BMI) (9, 73). The concentration of cortisol has been found to be low overnight and rises after waking (109), which may have contributed to the disparity in cortisol concentration with obesity in these studies. Furthermore, another group, using stable isotope tracer techniques, found the rate of cortisol production normalized to fat free mass in obese men did not change after weight loss (102). Therefore, cortisol concentration may reflect lean body mass, and may increase in response to overeating-induced weight gain if lean body mass increases. In response to eating alone, cortisol concentrations rise (3), therefore, cortisol secretion would likely be increased with overeating during waking hours. In addition to the impact of cortisol directly on metabolic processes, a rise in cortisol levels with overeating may regulate the secretion of other pituitary hormones, especially GH.

Regulation of GH secretion with overeating

GH may increase lipolysis (108, 115), protein synthesis (74), insulin resistance (8) and metabolic rate (116). Additionally, GH secretion is low in obesity (76, 119, 127), but it is not known how the obesity-related reduction in GH may contribute to metabolic disorders. Furthermore, it has been found that parenteral nutrition can suppress the concentration of GH (86). Consistent with this study, in our preliminary studies we found that GH secretion was reduced approximately 70% with a few days of overeating, before any measurable weight gain (see study 2). In light of this novel finding, I will focus the next several paragraphs of this review on factors that regulate GH secretion, thereby focusing on the potential mechanisms that may underlie the observed suppression in GH secretion with overeating.

GH secretion is primarily regulated by two hypothalamic factors: GH releasing hormone (GHRH) and somatostatin. GHRH is produced by the hypothalamus and acts in a paracrine fashion on the pituitary to induce the secretion of GH into the peripheral circulation. Somatostatin reduces GH secretion by acting directly on the pituitary and inhibiting GHRH release. It is important to note that GH can inhibit its own production and secretion as well. Other metabolic and endocrine factors can also influence the secretion of GH, either by acting directly on the pituitary or modulating GHRH signaling.

Elevated fatty acid concentration has been found to reduce secretion of GH (14, 57, 100, 101). *In vitro* experiments in primate (75) and rat (100, 101) pituitary cells have demonstrated that fatty acids can reduce the spontaneous secretion of GH, which may be caused by a fatty acid-induced reduction in the activity of protein kinase A in the pituitary (100). In addition, fatty acids have been found to reduce the transcription of GH, GHRH-receptor and GH secretagogue (GHS) receptor in cultured primate pituitary cells (75). Furthermore, studies using an infusion of heparin and intralipid in humans to increase plasma fatty acid concentration have demonstrated that the GH response to GHRH is significantly reduced (14, 57). Conversely, treatment with acipimox, an inhibitor of lipolysis, can increase the GHRH-induced GH response when fatty acid concentrations were reduced (99). Interestingly, despite the marked elevation in plasma

fatty acid concentration during fasting, GH secretion actually increases (48, 108). This effect may be due, at least in part, to the fasting-induced reduction of IGF-1 (6) a known potent suppressor of GH secretion (75, 128, 139). Therefore, it is important to recognize the complex, multifactorial regulation of GH secretion. During overeating, fatty acids are unlikely to influence GH secretion due to the suppression of lipolysis by insulin.

Insulin may also suppress GH secretion. Incubation of primate and rat pituitary cell lines with insulin caused a drop in mRNA levels of GH (75, 137, 138), the GHRH receptor (75), and the GHS receptor (75). *In vivo*, GHRH stimulated GH secretion was blunted by insulin during a euglycemic-insulinemic clamp in humans (69). Importantly, GH secretion was significantly reduced only when insulin was at least ~35uU/ml, a concentration that could be seen after a meal, and not at fasting insulin concentrations (69). This suggests that after a meal, insulin can reduce the pituitary production and secretion of GH at the level of transcription. However, it is not known how persistent the effect of insulin would be on GH secretion after insulin concentration returns to basal levels.

The gastrointestinal peptide hormone, ghrelin, may also influence GH secretion during overeating. Ghrelin is released from the stomach with extended fasting, and ghrelin secretion is reduced with eating. The role of ghrelin in promoting GH secretion from rat pituitary cells was first studied using a synthetic peptide (64). Some evidence has suggested that this synthetic peptide ghrelin may induce the secretion of GH via stimulation of the GHS receptor (64, 121) and it has been reported to induce GH secretion in humans (121). Therefore, if overeating reduces plasma levels of ghrelin, it may reduce the impetus for GH secretion. However, some researchers reported that endogenous ghrelin does not influence spontaneous GH secretion (5). In addition, the nature of using a synthetic peptide calls into question the applicability of the above findings to the endogenous hormone function.

As mentioned above, insulin-like growth factor-1 (IGF-1), which is produced and secreted by the liver in response to GH-receptor stimulation, can reduce the secretion of GH (75, 128, 140). Mice lacking hepatic IGF-1, the source of ~80% of the circulating

IGF-1, have elevated GH concentrations (128). Pituitary cells incubated with IGF-1 demonstrated a reduced GH secretion as well as a reduction in the mRNA of GH, GHRH-receptor, and GHS-receptor (75). Therefore, circulating IGF-1 appears to act centrally to directly reduce pituitary GH secretion (75, 139). Additional regulation of IGF-1 occurs in plasma, where most of the circulating IGF-1 is complexed with binding proteins (e.g., IGF binding protein 1 (IGFBP1) or IGFBP3). Consequently, the abundance of IGFBP1 and IGFBP3 in the circulation can greatly influence the bioactivity of IGF-1, and as such, the binding and sequestering of IGF-1 to these proteins can affect the regulation of GH secretion. While a previous study reported that overeating can increase plasma concentration of IGF-1 (33), there is little data regarding the abundance of IGF-1 binding proteins in response to overeating.

Summary of endocrine responses to overeating

Eating induces an increase in the secretion of cortisol and insulin after a meal. Additionally, our preliminary findings indicate that GH secretion is markedly suppressed with overeating, yet the underlying cause for this phenomenon remains uncertain. Potential explanations for the reduction in GH could implicate ghrelin, insulin, IGF-1, and/or IGF-1 binding proteins, which are all factors that could lead to the suppression of GH secretion during overeating. To our knowledge, IGF-1 binding proteins have not been examined during overeating, and may potentially play a role in the suppression of GH secretion.

Metabolic effects of growth hormone

Growth hormone can influence several metabolic processes. For example, evidence suggests that GH can help regulate energy expenditure (98, 135), lipolytic rate (115), and protein synthesis (95). In addition, GH has been found to increase insulin resistance (60, 67, 115). Because our preliminary findings indicate overeating results in the suppression of GH secretion with overeating, we want to explore how this reduction in GH concentration could affect some important metabolic processes. Additionally, because our preliminary findings indicate that overeating reduces GH secretion, the following

section will elaborate further on the effect of GH on energy expenditure, lipolytic rate, protein metabolism, and insulin resistance.

Effect of GH on energy expenditure

The role of GH on the rate of energy expenditure is equivocal. Some studies have suggested that GH treatment can increase metabolic rate (98, 116, 120, 135). GH may increase energy expenditure by increasing the activity of thyroid hormones (116, 135). Another study has suggested that GH can increase the expression of uncoupling protein 3 (UCP3) in skeletal muscle (98), which may increase energy expenditure by reducing the efficiency of mitochondrial ATP synthesis. The effect of GH treatment on 24 hour energy expenditure in GH-deficient adults is not clear because some studies have reported an increase (116), and others found no change (22, 78). It is important to note that discrepancies in studies using a GH-deficient model to examine the role of GH on energy expenditure may be due to accompanying co-morbidities that may influence metabolic rate. In an obese model, administration of a GH secretagogue was reported to increase resting energy expenditure concomitant with an increase in GH and IGF-1 concentration (120). However, a recent meta-analysis of the effect of GH administration in obese humans concluded that GH treatment had no effect on bodyweight or resting energy expenditure (82). Therefore, the effect of GH on energy expenditure remains uncertain.

Effect of GH on lipolytic rate

Studies have reported GH increases whole-body lipolytic rate in humans (108, 115, 119). Recent findings from our lab indicate that GH may be the primary regulator of the augmented lipolytic rate during short-term fasting, but may not be important for regulating lipolysis in the postabsorptive state (108). Increased lipolysis in the presence of GH may be due to an increase in HSL activity, which has been demonstrated in response to GH in adipocytes (25, 144). Additionally, GH has been found to increase the mRNA of patatin-like phospholipase domain containing 3 (PNPLA3), a novel lipase, in the adipose tissue of GH-deficient men (146). To my knowledge, no studies have examined muscle HSL activity or PNPLA3 activity in response to GH in humans. While GH replacement therapy in GH-deficient adults did increase HSL mRNA in skeletal

muscle (125), it was not found to do so in adipose tissue (146). Furthermore, GH treatment with weight loss in obese individuals did not affect HSL transcription in muscle or adipose tissue (104). However, in this study (104) the effect of weight loss on lipolysis may have masked the independent effect of GH on HSL.

Effect of GH on plasma triacylglyceride concentration

In contrast to the stimulatory effect of GH on adipose tissue lipolytic rate, GH has been found to inhibit lipoprotein lipase (LPL) activity (104), which catalyzes plasma triacylglyceride lipolysis. Inhibition of LPL activity may suppress plasma triacylglyceride clearance, and indeed, GH treatment has been found to increase plasma triacylglyceride concentration (136). However, contrary to the reported effect of GH on plasma triacylglyceride lipolysis, another study found GH treatment did not alter the concentration, nor the clearance, of very low-density lipoprotein (VLDL) triacylglyceride (67). The VLDL kinetics reported by Krag et al. (67) were measured under non-steady state conditions during a three hour time period, which may not have been sufficient to discern differences between treatment groups. However, further study is necessary to determine how GH may regulate plasma triacylglyceride concentration.

Effect of GH on protein synthesis

Although some studies suggest GH increases muscle protein synthesis (10, 34, 38), several studies have cast doubt on this theory (45, 103, 141, 143). The study of Fryburg, et al. demonstrated a net amino acid uptake across skeletal muscle in response to GH infusion, however, uptake of amino acids may have reflected amino acid oxidation, not protein synthesis (34). Indeed, in healthy males the fractional synthetic rate of isotope labeled leucine incorporated into muscle after 12 weeks of GH treatment was not found to be different than placebo (133, 141-143), therefore muscle protein synthesis was no different with or without GH treatment. Some work has suggested that GH could increase muscle protein synthesis through its stimulation of IGF-1 (38, 74). However, the increase in protein synthesis in response to IGF-1 may only occur in response to the muscle-specific isoform of IGF-1, and not the endocrine IGF-1 secreted by the liver (80, 128, 141, 143). Indeed, in mice overexpressing the skeletal muscle-specific isoform of IGF-1

in skeletal muscle, hypertrophy, and the associated muscle protein synthesis, was observed (90). Furthermore, although Loughna, et al. (74) linked GH to IGF-1-induced muscle protein synthesis, GH does not affect the skeletal muscle-specific isoform that is associated with hypertrophy in humans (45). The increase in skeletal muscle protein synthesis that Loughna et al. (74) found may be attributable to interspecies differences, given that their study used rats as the experimental model. Additionally, studies have described GH as “protein sparing” due to its stimulation of lipid oxidation (87), which preserves protein and amino acids from oxidation by increasing the availability of fatty acids for energy. However, in studies by Yarasheski, et al. GH treatment in healthy men did not reduce leucine Ra (141, 142). Therefore the role of GH on protein metabolism remains questionable.

Effect of GH on insulin resistance

Several studies have reported that GH can induce insulin resistance. An infusion of GH decreased the glucose infusion rate during the euglycemic-insulinemic clamp in humans (60, 67, 115), which is indicative of impaired insulin action. This effect on insulin signaling may be related, at least in part, to the lipolytic action of GH which can increase fatty acid availability, thereby interfering with insulin signaling (27) as mentioned previously. In fact, when GH was infused continuously in conjunction with the euglycemic-insulinemic clamp, fatty acid concentrations were elevated, and insulin resistance was increased (60). However, the expected decrease in insulin signaling with GH infusion was not observed (60). This could be related to the timing of the muscle biopsies or the difficulty in measuring insulin signaling events. In contrast to the findings of Jessen et al. (60) GH has also been demonstrated to interfere with insulin signaling independently of its lipolytic effects. It has been reported that mice overexpressing human placental GH have an elevated concentration of the regulatory subunit of PI3-K (p85 α) in skeletal muscle (7, 8). Although the p85 subunit is required for the insulin-stimulated activation of PI3-K, the p85 α subunit has been linked to a reduction in IRS-1 associated PI3-K activity (7, 8, 126). The overabundance of p85 α could interfere with the activation of the heterodimer p85:p110 complex at IRS-1 through competitive binding at IRS-1 (7, 8). It has also been demonstrated that increased levels of p85 α may stimulate

the activity of JNK (122, 123), which is known to induce insulin resistance (1, 91). Additional work suggests that suppressors of cytokine signaling (SOCS-1) and SOCS-3, which can be induced by GH (92), can promote the degradation of IRS-1 and interfere with insulin signaling (107). It is not known if the reduction in GH concentration that we observed in response to overeating in our preliminary studies, may actually provide a protective effect against an accumulation of p85 α , SOCS proteins, as well as the activity of JNK in skeletal muscle, and thereby help protect against the development of insulin resistance.

Summary of metabolic effects of GH

Growth hormone is known to influence many metabolic processes. While the influence of GH on energy expenditure is not clear, GH can increase lipolytic rate through its influence on HSL and GH can impair insulin action. The suppression in GH during overeating may preserve insulin signaling by reducing the insulin resistance induced by lipolysis and GH signaling. However, it seems unlikely that protein synthesis would be affected, given the studies that indicate that GH may have a limited role in these processes.

Intramyocellular triacylglyceride metabolism

Intracellular lipid moieties can affect insulin sensitivity and pro-inflammatory responses

Elevated IMTG concentrations are prevalent in obesity (40), and although elevated IMTG levels are associated with insulin resistance, the triacylglycerides themselves are most likely benign. Excess fatty acids that are not stored as triacylglyceride can lead to the accumulation of by-products of fatty acid metabolism or lipid intermediates. Although many of these by-products are critical for cell structure and function, the accumulation of these lipid intermediates can promote inflammation and lead to insulin resistance (17, 54, 58, 70). For example, intramyocellular diacylglycerol (DAG) accumulation can induce transcription of pro-inflammatory cytokines that can disrupt insulin action over an extended period of time. Elevated DAG concentration has been associated with increased activation of the transcription factor NF κ B, (58, 145), which in turn has been found to

cause insulin resistance through the transcription of the pro-inflammatory cytokines TNF- α and IL-6 (2, 111). These cytokines, not only have the potential to exacerbate insulin resistance in an autocrine fashion, but through paracrine signaling as well. For example, TNF- α produced in response to DAG accumulation can bind to TNF- α receptors on neighboring cells, resulting in an increased activity of c-Jun N-terminal kinase (JNK) that further promotes inflammation and reduces insulin action (1, 91). In addition, elevations in intramyocellular DAG concentration have been found to increase the activity of protein kinase C (PKC) (11, 58, 145), which has several cellular effects, including interfering with the association of insulin receptor substrate-1 (IRS-1) and phosphoinositide-3-kinase (PI3K) by phosphorylating IRS-1 on specific serine residues (27, 145). The resultant reduction in IRS-1 associated PI3K activity markedly impairs insulin-stimulated glucose uptake.

Intramyocellular ceramide accumulation has also been found to contribute to the development of insulin resistance. Ceramide has been reported to impair insulin action by decreasing the insulin-stimulated phosphorylation of Akt (16, 18, 44, 49), which is downstream of PI3K in the insulin signaling pathway. Akt phosphorylation is an essential step in insulin-mediated glucose transport, and a reduction in Akt activity induces insulin resistance. Studies have demonstrated that inhibiting ceramide synthesis rescued the lipid-induced impairment in insulin-stimulated phosphorylation of Akt (16, 17). The mechanism(s) mediating this ceramide-induced reduction in insulin signaling have not been clearly defined. It has been postulated that ceramide's actions may be working through alterations in factors such as PTEN (117), PP2A (18, 118), and PKC-zeta (44). Together, the detrimental effects of ceramide and DAG suggest that under conditions of excessive fatty acid availability, if fatty acids did not accumulate as these lipid intermediates, development of insulin resistance may be abated. One way to sequester these fatty acids and reduce the accumulation of these lipid intermediates may be to increase the synthesis of triacylglycerides.

Regulation of intramyocellular triacylglyceride

Triacylglyceride synthesis occurs through the succession of four reactions. Importantly, the first committed step of triacylglyceride synthesis is regulated by the enzyme glycerol-3-phosphate acyl transferase (GPAT), which catalyzes the production of lysophosphatidic acid (LPA) from fatty acyl-CoA and glycerol-3-phosphate. In addition, the final key step in the triacylglyceride synthesis pathway is regulated by the enzyme diacylglycerol acyl transferase (DGAT), which catalyzes the binding of a third acyl-CoA to DAG to create a triacylglyceride molecule. The intermediate reactions in the triacylglyceride synthesis pathway are regulated by 2-acyl-glycerol-phosphate acyl transferase (AGPAT) and phosphatidic acid phosphatase (PAP), ultimately resulting in the formation of DAG. In addition to these enzymes, other enzymes such as stearoyl-CoA desaturase (SCD) and acyl-CoA synthase, that use fatty acids to produce the appropriate substrate for the aforementioned acylating enzymes, may influence the rate of triacylglyceride synthesis. Because the accumulation of intermediate metabolites in the triacylglyceride synthesis pathway (e.g., DAG) can impair insulin sensitivity, the regulation of these lipogenic enzymes may have an important impact on insulin action.

It has been found that the overexpression of GPAT (56), DGAT (15, 72), and AGPAT (106) increased incorporation of exogenous fatty acids into triacylglyceride. Importantly, some of these studies report that the resultant increase in triacylglyceride synthesis was associated with improved insulin-stimulated glucose uptake (15, 72, 106). In addition, data from our laboratory has demonstrated that an increase in GPAT and DGAT protein expression after acute exercise coincided with an increased intramyocellular triacylglyceride (IMTG) accumulation, reduced ceramide and DAG concentration, with a concomitant increase in insulin sensitivity (110). These data suggest that enhancing the abundance and/or activity of triacylglyceride synthesis enzymes increased fatty acid flux toward storage as triacylglyceride, which may in turn reduce the accumulation of intramyocellular lipid intermediates known to impair insulin signaling. Alternatively, suppressed activity of the key enzymes within the triacylglyceride pathway may lead to lipid intermediate accumulation and impaired insulin action. Indeed, mice lacking mitochondrial GPAT displayed greater acyl-CoA accumulation in the liver and were

more severely insulin resistant when on a high fat, high sucrose diet compared with wildtype littermates (46). Although mice lacking DGAT1 are insulin sensitive, they also have a complex phenotype, including increased energy expenditure and lower bodyweight compared with wildtype littermates (19). Therefore, the improvement in insulin sensitivity in these knockout mice may be due to these phenotypic changes, rather than due to any change in regulation of triacylglyceride synthesis.

It is important to acknowledge that IMTG lipolysis can also provide substrate for the formation of lipid intermediates. Lipolysis is initiated by the enzyme adipose triacylglyceride lipase (ATGL) (147) and the enzymes HSL and monoacylglycerol lipase are largely responsible for completing the hydrolysis of the triacylglyceride into its component glycerol and fatty acids (147). Although ATGL catalyzes the reaction to produce DAG from triacylglyceride, some evidence suggests that ATGL may facilitate the partitioning fatty acids from triacylglyceride toward oxidation (55). Indeed, the overexpression of ATGL in cell culture led to the accumulation of DAG, however, this did not occur when HSL was also overexpressed (147). The coordination of these enzymes to induce the complete hydrolysis of triacylglycerides may be important for reducing the accumulation of lipid intermediates.

Effects of overeating on IMTG metabolism

It is well documented that IMTG levels are elevated in obesity (40), but the mechanisms responsible for this phenomenon are not understood. Accumulation of IMTG in obesity could be a consequence of chronically elevated fatty acid availability (51), which could contribute as a substrate for IMTG synthesis. Some studies report that fat oxidation in muscle is low in morbidly obese people (113, 114), which may result in fatty acids being stored locally, rather than being oxidized. Additionally, IMTG may accrue during periods of overeating. However, it is not known how triacylglyceride synthesis enzymes are regulated during these periods of caloric excess.

While excessive caloric intake stimulates fat storage in many tissues, including skeletal muscle, it is not known if this effect is merely the result of an increase in the substrate

(i.e. fatty acids) for acylating enzymes, or if the excess nutrients regulate these enzymes in some manner. For example, carbohydrate overeating has been found to stimulate adipose tissue transcription of stearyl response element binding protein 1c, (SREBP-1c) (85), which is a transcription factor for several lipogenic genes, including GPAT (30). Therefore, excess nutrients may regulate triacylglyceride synthesis by increasing the transcription of triacylglyceride synthesis enzymes. Interestingly, overeating only fat calories did not increase transcription of the acylating enzymes involved in triacylglyceride synthesis (83) supporting the potential role of carbohydrate and the resultant insulin response in augmenting triacylglyceride synthesis, rather than simply an increase in energy availability, per se. Insulin has been found to directly increase the expression of SREBP-1c in skeletal muscle (26, 29). In addition, insulin can stimulate DGAT activity in adipocytes, leading to the accumulation of triacylglyceride (81). Insulin may also affect skeletal muscle in a similar manner, but to our knowledge, no study has examined this. Available evidence suggests that acylating enzymes can be regulated post-translationally (81, 89). Therefore, measurements of protein abundance together with assessment of the activity of these enzymes may provide a more comprehensive evaluation of the impact of nutrient excess on the regulation of triacylglyceride synthesis in skeletal muscle.

Interestingly, *in vitro* work in L6 myotubes has suggested that fatty acids may stimulate AMP-activated protein kinase (AMPK) (130), which can inhibit GPAT (89). This would be counter-intuitive, given that AMPK, which is normally active during times of energy shortage, is likely not active during overeating because of the high energy availability. Given that *in vitro* studies may not reflect what actually occurs in whole body physiology, it is difficult to tell if fatty acids would actually *inhibit* GPAT activity. However, it is not known how overeating would affect GPAT or AMPK in skeletal muscle.

It is important to acknowledge that factors other than changes in the activity of the acylating enzymes can impact the rate and magnitude of IMTG synthesis. For example, simply an increase in fatty acid availability may increase IMTG synthesis independently

of an increase in enzyme activity. Additionally, overeating carbohydrate can contribute to *de novo* lipogenesis (68, 85), which may increase the amount of fatty acids for muscle triacylglyceride synthesis. Furthermore, IMTG concentration has been found to increase in high-fat fed rats compared with chow-fed, even before there were differences in bodyweight (43). This suggests that increased abundance of lipid substrate augments IMTG synthesis and accumulation. Alternatively, overeating has been reported to increase gene expression of acyl-CoA synthase long chain family 5 (83), which has been hypothesized to direct fatty acids toward triacylglyceride synthesis (79). Additionally, in muscle, overeating can stimulate the transcription of SCD-1 (83), the enzyme that produces unsaturated fatty acids, which may enhance triacylglyceride synthesis (20, 21, 66, 70). Therefore, aside from the triacylglyceride synthesis enzymes, accessory enzymes (i.e. acyl-CoA synthase, SCD-1) may be nutritionally regulated and augment triacylglyceride synthesis.

Summary of effects of overeating on the regulation of triacylglyceride synthesis

Although overeating can increase triacylglyceride synthesis, little is known about how triacylglyceride synthesis in skeletal muscle is regulated in response to overeating. While the expected increase in IMTG could be due to an increased abundance of substrate, it is unknown if overeating may increase the abundance and activity of key lipogenic enzymes in muscle. For example, a chronic elevation in plasma insulin concentration during overeating may increase the expression and/or activity of GPAT and DGAT. Additionally, insulin contributes to the accrual of IMTG through the insulin-induced inhibition of lipolysis via HSL. By sequestering lipid intermediates, IMTG may reduce the activity of inflammatory markers and abate the development of insulin resistance. Therefore, the metabolism of IMTG during overeating could influence whole body physiology during a positive caloric balance.

Overall summary of the review of literature

Overeating induces several physiological and endocrine changes, affecting energy expenditure, protein synthesis, lipolysis, and insulin resistance. The endocrine response to overeating can influence several metabolic processes including energy storage, protein

synthesis, lipolysis, energy expenditure, and insulin resistance. In particular, insulin and cortisol can influence the availability of glucose and lipid for energy production and storage. In addition, the influence of overeating-induced changes in GH secretion may alter several key metabolic processes (e.g., energy expenditure, lipolysis, protein synthesis, and insulin action). The synthesis of IMTG in response to a positive energy balance may abate skeletal muscle insulin resistance, however, it is not known if or how triacylglyceride synthesis enzymes in skeletal muscle respond to overeating. Most importantly, the plasticity of skeletal muscle to caloric surplus has not been fully described and may elucidate how several obesity-related metabolic abnormalities may develop.

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CHAPTER 3

DISSERTATION STUDY #1

Metabolic adaptations to overeating

Abstract

Prolonged overeating and the resultant weight gain are clearly linked with the development of insulin resistance, but metabolic adaptations that occur after relatively short periods of overeating are not completely understood. The primary aim of this study was to characterize changes (both at the whole body-level and in skeletal muscle) that may accompany the development of insulin resistance after 2 wks of overeating. Healthy, non-obese men (n=7) and women (n=2) were admitted to the hospital for 2 wks, during which time they ate ~4000 kcals/day (70 kcals/kg fat free mass/day). Insulin sensitivity was estimated during a meal tolerance test, and a muscle biopsy was obtained after an overnight fast to assess muscle lipid accumulation, as well as protein markers associated with impaired insulin signaling, inflammation, and the regulation of lipid metabolism. Whole body insulin sensitivity declined markedly after 2 wks of overeating (Matsuda composite index: 8.3 ± 1.3 vs. 4.6 ± 0.7 , $p < 0.05$). In contrast, muscle markers linked with insulin resistance and inflammation (e.g., phosphorylation of IRS-1-ser312, Akt-ser473, c-Jun N-terminal kinase (JNK)) were not altered by overeating. The concentration of intramyocellular lipids tended to increase after 2 wks of overeating (triacylglyceride: 7.6 ± 1.6 vs. 10.0 ± 1.8 nmol/mg ww, $p = 0.11$; diacylglyceride: 104 ± 10 vs. 142 ± 23 pmol/mg ww; $p = 0.26$), but these changes did not reach statistical significance. Overeating induced a 2-fold increase in 24h insulin response (area under the curve [AUC]; $p < 0.05$), and a 6-fold increase in 24h plasma triacylglyceride AUC, while 24h non-esterified fatty acid AUC declined by ~35% ($p < 0.05$) in response to overeating. Together our findings indicate that alterations in fatty acid availability limited skeletal muscle lipid accumulation and skeletal muscle metabolism may not contribute meaningfully to the marked insulin resistance observed after 2 wks of overeating.

Introduction

Abundant high-caloric food and a sedentary lifestyle have contributed to the increased prevalence of obesity and obesity-related diseases, such as cardiovascular disease and diabetes. Insulin resistance, hyperlipidemia, high ectopic fatty acid accumulation in skeletal muscle and liver, are just a few of the metabolic abnormalities common in obesity. Although the metabolic abnormalities in obesity are reasonably well defined, the metabolic responses during the early stages of weight gain are far less clear. Improved understanding of the metabolic changes that occur in response to overeating may shed light on the development of obesity-related metabolic complications, even before meaningful weight gain.

In the first few days of overeating (i.e., ~5 days) fasting plasma insulin concentration has been found to increase significantly (1, 6) which provides a crude assessment of systemic insulin resistance. However, in these same studies, measures of peripheral insulin resistance did not change (1,6). Because nearly all insulin-mediated peripheral glucose uptake is directed to skeletal muscle, these findings suggest that the very early stages of overeating (~5 days) may not provide a great enough perturbation to impair skeletal muscle insulin action. In animal models of overfeeding, mice have been reported to retain skeletal muscle insulin sensitivity after 2 weeks of overeating, despite a profound reduction in hepatic insulin sensitivity and a considerable increase in fat mass (27). However, whether the same is true in the first two weeks of overeating and weight gain in human subjects is not clear.

Ectopic lipid accumulation in skeletal muscle and liver can contribute to insulin resistance through the accumulation of fatty acid intermediates such as diacylglycerides (DAG) (30, 62). Accumulation of fatty acid intermediates can result in the activation of inflammatory factors, such as c-Jun-N-terminal kinase (JNK), that reduce insulin signaling (24). Lipid can accumulate in the liver in response to overeating even before a significant increase in bodyweight (54), but it is not clear if skeletal muscle responds in a similar manner.

The primary aim of this study was to characterize systemic metabolic alterations that occur in response to 2 weeks of overeating in healthy non-obese adults. An additional objective of this study was to assess the effects of overeating on skeletal muscle lipid metabolism.

Methods

Subjects

Nine healthy, non-obese subjects (7 men and 2 women) participated in a 2-week overfeeding study (initial body mass index: $23.9 \pm 0.3 \text{ kg/m}^2$, age: 24 ± 1 yrs). All subjects were determined to be healthy by a comprehensive medical exam prior to participation in this study and were not taking any medications, or participating in any regular physical activity or exercise program (< 2 h of physical activity per week, based on self report). All subjects provided written, informed consent prior to participation in this study. All procedures of this study were approved by the Institutional Review Board of the University of Michigan.

General Study Design

We conducted a battery of metabolic tests before (“Baseline”) and after a 2-week overeating period. During the “Baseline” trial, subjects were admitted to the Michigan Clinical Research Unit of the University of Michigan Hospital for an overnight stay, beginning the morning after an overnight fast. One fasting blood sample was drawn, and then a meal tolerance test (MTT) was administered at 0900, with blood samples obtained every 20 min for 2 hours for assessment of plasma glucose and insulin concentrations. Subjects were instructed to consume the entire meal as quickly as tolerable. The meal consisted of 10 kcal/kg bodyweight, 50% carbohydrate, 15% protein, and 35% fat. In addition to the blood samples collected during the MTT, we also collected samples every two hours for 24h to assess the 24h profile for plasma insulin, non-esterified fatty acids (NEFA), and triacylglyceride concentrations. In addition to the meal ingested for the MTT, subjects were also provided 2 additional meals (at 1200h and 1800h) to maintain energy balance. The total caloric intake was $41 \pm 1.5 \text{ kcal/kg FFM}$ for the Baseline trial.

Subjects remained in the hospital room overnight and the next morning, after an overnight fast, resting metabolic rate (RMR) was measured via indirect calorimetry (Vmax Encore, Sormedics, San Diego, CA). A muscle biopsy sample was then obtained from the vastus lateralis at 0800. Muscle samples were cleaned in sterile saline and immediately frozen in liquid nitrogen and stored at -80°C until further analysis. Body composition was determined by Dual-Energy X-ray Absorptiometry (DEXA) scan and then the subjects were released from the hospital.

Approximately one week after the Baseline trial, subjects were re-admitted to the hospital for the entire duration of a 2-week overeating intervention (i.e., subjects did not leave the hospital during this 2-week period). During this 2-week overeating period, subjects consumed ~4000kcal/day (70 kcal/kg fat free mass/day; 50% carbohydrate, 35% fat, and 15% protein), and were limited to 1500 steps/day as monitored by a pedometer. The daily diet was provided as 3 meals (at 0800, 1200, 1900) and 4 snacks (at 1000, 1400, 1600, 2200).

On the morning of the 13th day of the overeating period, subjects performed an MTT after an overnight fast (the meal ingested during this MTT was identical, in both composition and quantity of calories, to the one administered during the Baseline trial). Also starting in the morning of the 13th day of overeating, blood samples drawn every two hours for 24h to reassess plasma profiles of insulin, NEFA, and triacylglyceride concentrations. The next morning, RMR was measured again via indirect calorimetry and a muscle biopsy sample was obtained after an overnight fast as described above during the Baseline trial. Body composition was again determined by DEXA scan. After completion of all of the procedures on the 14th day of their hospital stay, the subjects were discharged.

Analytical Procedures

Plasma substrate and insulin concentrations

Standard commercial kits were used to measure plasma concentrations of glucose (glucose oxidase method, Thermo Scientific, Middletown, VA), non-esterified fatty acids

(NEFA) (Wako Chemicals USA, Richmond, VA), and triacylglyceride (Sigma, St. Louis, MO). Plasma insulin concentration was measured by radioimmunoassay (human insulin-specific RIA: Millipore, Billerica, MA). Cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by chemiluminescent assays (Siemens, Frimley, Camberley, U.K.). C-reactive Protein measurement was performed using the Human C-Reactive Protein (CRP) Elisa Kit (Invitrogen, Fredrick, MD).

Intramycellular triacylglyceride (IMTG) and DAG

Frozen muscle samples were rapidly homogenized in ice-cold saline and lipids then extracted in a single-phase mixture of chloroform-methanol-saline (1:2:0.8) (4). Internal lipid markers for IMTG, DAG, monoacylglycerol, NEFA, phospholipids and cholesterol esters with fatty acid moieties of odd carbon number were added at the start of extraction, for subsequent purity and recovery determinations (NuChek, Elysian, MN; Avanti Polar Lipids, Alabaster, AL). After 4h at room temperature, additional chloroform and saline were added to yield a two-phase mixture (2:2:1.8). After brief centrifugation, the lower chloroform phase containing lipids was transferred to a clean tube and dried under vacuum. The residue was dissolved in a small volume of chloroform and applied to a hexane-equilibrated, aminopropyl solid phase extraction cartridge (Supelco, St. Louis, MO). Individual lipid species were eluted using specific solvent mixtures (5). Fatty acid methyl esters (FAMES) were generated from purified glycerolipids by alkaline methanolysis, a transesterification process (5), while NEFA were converted to methyl esters by a methyl iodide procedure (44). FAMES were measured by gas chromatography and electron-impact mass spectrometry (Agilent 6890A GC and 5973N MSD, Palo Alto, CA), and quantified using FAME standards (NuChek, Elysian, MN).

Immunoblot analysis

Cytosolic and crude membrane fractions of muscle homogenates were used for electrophoresis analysis of protein contents in muscle. Thirty micrograms of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. These membranes were incubated with antibodies for proteins of interest (see below), followed by a 60-min

incubation with the appropriate secondary antibody. The blots were developed with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and the bands for the proteins of interest were quantified using AlphaEaseFC (AlphaInnotech, San Leandro, CA). Blots were probed with the following antibodies: α -CD36/FAT (cat. no. 9154; Santa Cruz Biotechnology, Santa Cruz, CA), α -HSL (cat. no. 4107; Cell Signaling Technology, Danvers, MA), α -p-HSL Ser563 (cat. No.4139; Cell Signaling Technology), α -p-HSL Ser565 (cat. no. 4137; Cell Signaling Technology), α -p-HSL Ser660 (cat. no. 4126; Cell Signaling Technology), α -ATGL (cat. no. 2138; Cell Signaling Technology), α -GPAT1 (a gift from Dr. Rosalind Coleman), α -DGAT1 (cat. no. NB110-41487; Novus Biologicals, Littleton, CO).

Calculations

HOMA-IR

Homeostatic model assessment of insulin resistance was calculated by the equation $HOMA-IR = (\text{fasting plasma insulin} \times \text{fasting plasma glucose}) / k$, where k represents a constant 405 (39).

Matsuda Composite Index of Insulin Sensitivity

Matsuda Composite Index of Insulin Sensitivity was calculated using the equation = $10,000 / ((\text{fasting plasma insulin concentration} \times \text{fasting plasma glucose concentration}) \times (\text{mean plasma insulin concentration} \times \text{mean plasma glucose concentration}))^{1/2}$, where mean plasma glucose and insulin concentrations are the average plasma glucose and insulin during the 2h meal tolerance test (38).

Resting Metabolic Rate

Resting metabolic rate was calculated using expired VCO_2 and VO_2 values (measured via indirect calorimetry after an overnight fast), and the equation of Weir (60).

Area under the curve

Area under the curve was calculated using the trapezoidal rule with zero as the Baseline.

Statistical Analysis

Paired Student's t-test was used to determine significant changes after overeating compared with Baseline. A two-way ANOVA with Tukey's post hoc test was used to assess differences in 24h plasma concentrations of insulin, NEFA, and triacylglyceride, before and after overeating. Alpha level was set to 0.05 to distinguish statistical significance. All statistical analysis was performed using SigmaPlot software for Windows version 11.0. All data are presented as mean \pm standard error (SE).

Results

Weight gain, body composition and resting metabolic rate (RMR) with overeating

By the end of the two-week overeating period, subjects gained an average of 2.4 ± 0.6 kg in body weight ($p < 0.05$). The majority of this weight gain was fat mass (1.6 ± 0.3 kg; $p < 0.05$), and there was no significant change in fat free mass. 2 weeks of overeating tended to increase RMR slightly, ($p = 0.09$). Summary data for body weight, body composition, and RMR for our subjects before and after the overeating are provided in Table 1.

Markers of insulin sensitivity

Although fasting plasma glucose concentration was well maintained after 2 weeks of overeating (Table 2), fasting plasma insulin concentration doubled ($p < 0.05$; Table 2). As a result, HOMA-IR was also significantly elevated after overeating ($p < 0.05$; Table 2), suggestive of an increase in insulin resistance after 2 weeks of overeating. Impaired insulin action after 2 weeks of overeating was also confirmed by calculating the Matsuda Composite Index during the meal tolerance test ($p < 0.05$; Figure 1).

Although we did not collect a muscle biopsy in the insulin-stimulated state, elevated basal (non insulin-stimulated) phosphorylation of insulin receptor substrate-1 (IRS-1) at serine residue 312 (p-IRS-1 Ser³¹²) (9) and markers of increased activation of intracellular inflammatory pathways, such as increased phosphorylation of JNK (p-JNK) (24, 37) are associated with insulin resistance. In contrast to our finding that overeating

induced insulin resistance at the whole-body level, neither p-IRS-1 Ser³¹² nor p-JNK in skeletal muscle was affected by overeating (Figure 2). Similarly, elevated basal phosphorylation of Akt (p-Akt Ser⁴⁷³) in skeletal muscle has also been linked with insulin resistance (35), and p-Akt Ser⁴⁷³ in skeletal muscle remained unchanged after overeating (Figure 2).

Intramyocellular lipids

Although we found a trend for an increase in IMTG concentration after 2 weeks of overeating (Figure 3A), this increase did not reach statistical significance ($p=0.11$). Similarly, accumulation of DAG in skeletal muscle also did not increase significantly (Figure 3B, $p = 0.26$). In concert with the IMTG and DAG data, the protein abundance of the lipid synthesizing enzymes glycerol-3-phosphate acyltransferase (GPAT) and diacylglycerides acyltransferase (DGAT) also did not change in skeletal muscle with overeating (Figure 4). Interestingly, we actually found a trend for a reduction in the protein abundance of muscle sterol response element binding protein-1a (SREBP1a) ($p = 0.06$), which is a transcription factor reportedly involved in the gene activation of fatty acid synthesis enzymes in primary myocytes (46). Protein abundance of lipolytic enzymes adipose triacylglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in skeletal muscle did not change with overeating and the phosphorylation state of HSL associated with augmented lipolytic activity (p-HSL Ser⁵⁶³ Ser⁶⁶⁰) and suppression of lipolytic activity (p-HSL Ser⁵⁶⁵) were also not different after the overeating period (Figure 5). The transport of fatty acids into the myocyte to serve as substrate for intramyocellular lipids is obviously also a key factor in the regulation of muscle lipid accumulation, and we found that neither membrane-bound nor total protein abundance of the key fatty acid transporter, FAT/CD36 were altered by overeating (Figure 4).

Plasma concentrations of insulin, NEFA and blood lipid profiles

Overeating resulted in a chronic elevation in plasma insulin concentration throughout the day (Figure 6A) and 24h plasma insulin concentration area under the curve (AUC) nearly doubled (311 ± 34 uU/ml*h vs. 601 ± 93 uU/ml*h, at Baseline and 2 weeks, respectively,

$p < 0.05$). In conjunction with this chronic hyperinsulinemia, overeating suppressed plasma NEFA concentration throughout much of the day, this was especially evident in the overnight period (Figure 6B), and 24h plasma NEFA AUC was suppressed by about 35% (487 ± 70 mM*min vs. 317 ± 47 mM*min at Baseline and 2 weeks, respectively, $p < 0.05$). Plasma NEFA concentration after an overnight fast was also reduced after overeating (Table 2). In contrast, overeating did not affect plasma triacylglyceride concentration after an overnight fast (Table 2), but plasma triacylglyceride concentrations were elevated for much of the day while overeating compared with Baseline (Figure 6C). Additionally, 2 weeks of overeating increased plasma total cholesterol concentration (152 ± 10 mg/dl vs. 177 ± 11 mg/dl, at Baseline and 2 weeks, respectively, $p < 0.05$). The increase in total cholesterol was due to both an increase in HDL-cholesterol (39 ± 2 mg/dl vs. 47 ± 4 mg/dl, $p < 0.05$) and LDL-cholesterol (97 ± 10 mg/dl vs. 117 ± 11 mg/dl) but this increase in LDL-cholesterol did not quite reach statistical significance ($p = 0.06$).

Hepatic and plasma inflammatory markers

Compared with Baseline levels, 2 weeks of overeating doubled the plasma concentrations of ALT (16.1 ± 2.2 IU/L vs. 35.1 ± 5.8 IU/L, $p < 0.05$), and significantly increased AST (19.3 ± 1.3 IU/L vs. 24.5 ± 1.7 IU/L, $p < 0.05$). Despite these elevations in markers of liver inflammation, both AST and ALT concentrations remained in the “normal” range after overfeeding for all subjects. Additionally, 2 weeks of overeating did not significantly affect plasma C-reactive protein concentration (0.5 ± 0.2 mg/L vs. 0.7 ± 0.2 mg/L, Baseline vs. 2 weeks, respectively $p = 0.52$).

Discussion

One of the major findings of this study was that despite the increase in whole-body insulin resistance after 2 weeks of overeating, we did not find evidence for changes in markers of insulin signaling in skeletal muscle. Therefore, our findings support the notion that in the early stages of caloric excess and weight gain, insulin responsive tissues other than skeletal muscle (e.g., liver) may develop insulin resistance in advance of metabolic

alterations in muscle that can lead to impaired insulin action. Additionally, the hyperinsulinemia observed throughout the day while overeating, due to the large and frequent meals, likely had important metabolic consequences in our subjects. For example, because insulin regulates the activity of intracellular (8, 42) as well as extracellular lipases (i.e. lipoprotein lipase) (47, 49), the chronic hyperinsulinemia we observe may have helped direct the meal-derived fatty acids for storage in adipose tissue and away from skeletal muscle. The chronic elevation in plasma insulin concentration may also play an indirect role in the increased resting energy expenditure we found with overeating.

Our finding that even relatively short-term exposure to overeating can induce systemic insulin resistance agrees with previous studies (6, 13, 57). Systemic insulin resistance can develop as a consequence of reduced insulin action in one or more of the key insulin-responsive tissues (e.g., skeletal muscle, liver, adipose tissue). In frank diabetes, it is common for all of these insulin-responsive tissues to be markedly resistant to the effects of insulin in regulating glucose uptake (skeletal muscle and adipose tissue) and glucose production (liver) (28). However, the progressive alterations in insulin resistance that occur in the early stages of weight gain are not clearly understood. Although we did not directly measure insulin sensitivity in skeletal muscle in our study, we did find that key markers associated with impaired muscle insulin action remained unaffected after 2 weeks of overeating. For example, we found no change in the phosphorylation in the basal state (non-insulin-stimulated) increased phosphorylation of IRS-1 serine 312 (2), Akt (35), and JNK (51), all of which have been previously found to be associated with impaired insulin action. We interpret these indirect findings to suggest that insulin signaling in skeletal muscle in the fasting state may not be affected in the early stages of weight gain. In contrast, the liver may be more susceptible to the development of insulin resistance in the earlier stages of weight gain (6). Kleeman, et al (27) assessed the progressive sequence of the development of insulin resistance in liver, white adipose tissue, and skeletal muscle in mice on a high fat diet, and found that liver was the first to develop insulin resistance, followed by adipose tissue. Interestingly, these investigators found skeletal muscle did not develop insulin resistance even after 12 weeks of the high

fat diet, which was a bit surprising in light of the > 2-fold increase in body fat mass they reported (27). Nonetheless, their findings that skeletal muscle appears to be less susceptible to developing insulin resistance in the early stages of weight gain compared with other insulin-responsive tissues in rodents is consistent with our interpretation of the present findings in human subjects.

Results from human studies also support the hypothesis that insulin resistance may first emerge in the liver. Brons, et al. found increased hepatic insulin resistance after five days of overeating using a hyperinsulinemic-euglycemic clamp (6). Additionally, other investigators have found overeating significantly increased fasting insulin (1) and HOMA-IR (6, 57), which are believed to largely reflect hepatic insulin sensitivity (41). After a meal, the liver helps control blood glucose by inhibiting hepatic glucose production, and in response to overeating the liver can become less responsive to insulin (6). In contrast to short-term exposure to overeating, as in the present study and in Brons, et al (6), peripheral insulin resistance (i.e., largely skeletal muscle insulin resistance) has been found to develop after a slightly more prolonged exposure to overeating (28 days) in conjunction with a more meaningful increase in body fat mass (57).

If overeating does induce the development of insulin resistance in liver and adipose tissue in advance of skeletal muscle, it is possible that metabolic alterations in liver and adipose tissue may contribute the eventual development of impaired insulin action within skeletal muscle that occurs with more prolonged overeating and greater weight gain. In obesity, the relationship between altered adipose tissue metabolism and the development of skeletal muscle insulin resistance is well established (14, 32). For example, the excessive fatty acid mobilization from adipose tissue found in obesity is known to be a key contributor to the development of impaired insulin sensitivity in muscle (50). Additionally, skeletal muscle insulin resistance in obesity is also linked with an increased systemic inflammatory status resulting from alterations in the secretion of cytokines from adipose tissue (55) and liver (55). Our findings that neither fatty acid availability nor our marker of increased systemic inflammation (i.e., CRP) were elevated after our 2 week overeating intervention suggests that perhaps a more prolonged energy surplus, and/or a

greater gain in body fat are required to induce an appreciable change in systemic factors known to impair insulin action in muscle. Indeed, a slightly longer period of overeating has been found to augment systemic inflammation; Tam et al. (57) found increased plasma concentration of CRP, as well as monocyte chemotactic protein (MCP-1) in response to one month of overeating, which was accompanied by peripheral (i.e., skeletal muscle) insulin resistance. Similarly, a more prolonged period of overeating that results in a greater accrual of fat mass and the suppression of the potent anti-lipolytic effects of insulin augment systemic fatty acid availability (13) can suppresses insulin action in skeletal muscle.

Because our participants ate ~150g of fat per day, we anticipated we would find a relatively large lipid accumulation within skeletal muscle, perhaps comparable to what we (33) and others (20) commonly report in obesity. However, in contrast to our expectations, we did not find a robust increase in the concentration of either muscle triacylglyceride or diacylglyceride. Previous work from our lab (33) and others (58) suggest that fatty acid supply for esterification within skeletal muscle, rather than increased lipogenic capacity, is largely responsible for the accumulation of muscle lipids. After meals, most of the dietary fat is delivered to tissues as triacylglycerides packaged within circulating lipoproteins (i.e., chylomicrons), and indeed we found pronounced hyperlipidemia throughout much of the day in our subjects. Lipoprotein lipase (LPL), which resides on the luminal surface of the capillaries within skeletal muscle and adipose tissue, is the enzyme responsible for catalyzing the hydrolysis of the triacylglycerides, liberating the meal-derived fatty acids that may be taken up by the local tissues. Muscle LPL activity has been reported to be suppressed by insulin (26, 34), which provides an attractive explanation for our finding that muscle lipid accumulation did not increase despite abundant postprandial plasma triacylglycerides (i.e., the chronic hyperinsulinemia in our subjects may have inhibited muscle LPL, thereby limiting the availability and uptake of meal-derived fatty acids within skeletal muscle). However, the regulation of muscle LPL activity and the distribution of meal-derived fat into skeletal muscle appear to be more complicated than this. Conflicting evidence suggests that insulin may have no effect on skeletal muscle LPL activity (61) or may actually increase it (47). Additionally,

although the uptake of meal-derived fat into skeletal muscle has been reported to be negligible by some investigators (25), others have reported measurable extraction and storage of meal fat into skeletal muscle (3, 31, 47). In contrast to these equivocal reports in skeletal muscle, it is generally accepted that insulin augments adipose tissue LPL activity (48, 49, 52). This increase in adipose tissue LPL combined with the insulin-mediated reduction in intracellular lipolysis (7) results in a large fatty acid gradient across the adipocyte membrane, favoring uptake of a substantial proportion of meal-derived fatty acids into adipose tissue (12, 25, 31). We surmise that although some of the meal-derived lipids were likely being extracted by skeletal muscle in our study, the vast majority of the meal-derived fat was likely extracted and stored in adipose tissue. Albumin-bound NEFA are also a very important substrate for the synthesis of muscle lipids (10), and plasma NEFA concentration was suppressed throughout the day and night with overeating in our study, likely a consequence of the potent antilipolytic effects from the persistent elevation in plasma insulin concentration with large and frequent meals. Together, this chronically low plasma NEFA availability in combination with the likelihood that the vast majority of meal-derived fatty acids were being directed into adipose tissue and perhaps liver (see below), rather than skeletal muscle, help explain why we did not find a robust increase in muscle lipid accumulation in our study. We cannot rule out the possibility that overeating did increase muscle lipid synthesis, but lipids did not accumulate in muscle because the increased synthesis rate was matched by a similar increase in the rate of lipolysis. Our measurements of proteins involved in the synthesis (i.e., DGAT and GPAT) and degradation (i.e., ATGL, HSL) of muscle lipids do not support the notion that overeating evoked cellular adaptations to alter muscle lipid turnover. However, our measurements were limited to assessments of protein abundance of the synthesizing enzymes and the abundance and phosphorylation/activation state of the lipolytic enzymes, so our findings are not conclusive.

A substantial amount of meal-derived fat is also taken up by the liver (31). Once taken up by liver, the major fates of these fatty acids include: to be oxidized for energy, esterified into triacylglycerides for intrahepatic storage, or the esterified fatty acids may be released into the systemic circulation within very low density lipoproteins (VLDL). Jensen et al.,

reported that nearly 50% of dietary fat is oxidized within 24h after ingestion (25), and because the subjects in their study were on bed rest after the meals, skeletal muscle energy expenditure would have been quite low, so a high proportion of this oxidation was likely occurring in the liver. In general, hepatic fatty acids that are not oxidized are esterified into triacylglycerides, and overfeeding has been reported to increase hepatic triacylglyceride storage in humans (54). However, this increase in hepatic lipid content is not solely attributed to the uptake and subsequent storage of dietary fat, *de novo* lipogenesis can also contribute, especially in response to excessive dietary carbohydrate intake (13, 40). Hepatic steatosis has been linked with rather severe hepatic insulin resistance (17, 28). We did not directly measure hepatic lipid content, but we did find plasma AST and ALT to increase significantly, which are commonly used clinically as crude markers of hepatic steatosis (45, 53). One week of overeating a diet similar to ours (i.e., high in both carbohydrate and fat) has been found to induce a 2-3 fold increase in hepatic lipid content, which was accompanied by a 70% increase in plasma ALT concentration (54). Although the significant elevations we observed in AST and ALT with overeating in our study did not increase into the “abnormal” range, the notable increase in these markers of steatosis and liver damage, suggest that our brief overeating intervention was likely compromising normal liver function.

Our observation that RMR (in the overnight fasted state) tended to increase after 2 weeks of overeating is consistent with findings from many other overeating studies (11, 16, 59). In many cases, the higher RMR after overeating and weight gain has been accompanied by an increase in FFM, and this increase in metabolically active tissue could account for the elevated resting energy expenditure. However, FFM did not increase significantly with overeating in our relatively short overfeeding intervention, and we found the elevation in RMR to persist even when we normalized it to FFM. Therefore, our findings suggest that overeating augmented energy-requiring metabolic processes independently of any change in the accrual more metabolically active tissue. The chronic hyperinsulinemia may have contributed to the increased in RMR in our participants. For example, insulin can increase Na^+/K^+ ATPase activity (56), and the energy required by ion pumps to maintain resting membrane potential is known to be a major contributor to

resting energy expenditure (21, 22, 36). Consistent with the potential role of this ion pump to contribute to the increased resting energy expenditure found in our study, overfeeding has been found to increase the activity of Na⁺/K⁺ ATPase in the liver and skeletal muscle of mice (15). Other factors that may contribute to the increase in RMR include the process of *de novo* lipogenesis, which is stimulated by elevated insulin concentration and dietary carbohydrates (23), changes in sympathetic nervous system activity (19, 43) and other the neuroendocrine responses to overeating (18).

An important aspect of our study design was that in addition to the very high daily energy intake, our subjects were also tremendously inactive (~1,500 steps per day). While this was not bed rest, it is certainly possible/likely that the relative inactivity contributed to some of the metabolic adaptations we observed. It is important to note that our subjects were all very sedentary before their participation in our study. Our eligibility criteria required they not participate in structured physical activity programs and they accumulate <2h of physical activity per week; most did not engage in any planned physical activity and did not have active lifestyles or physically demanding occupations. Therefore, while 1500 steps/day was likely less than our subjects' habitual daily physical activity levels, the magnitude of the reduction in physical activity below their norm was far less than if we had recruited highly active participants. Along these lines, a recent study that parallels our study rather well enrolled habitually active adults (i.e., >10,000 steps/day) to dramatically reduce their daily physical activity for 2 weeks (i.e., to ~1,500 steps/day) without overeating (29). Very interestingly, they found that the marked reduction in physical activity induced a reduction in peripheral (i.e., skeletal muscle) insulin action, and markers for impaired insulin signaling in skeletal muscle, without evoking hepatic insulin resistance (29), which was basically the opposite of the adaptations we report here. Therefore, it appears that even a relatively brief period of physical inactivity has particularly profound metabolic consequences within skeletal muscle of people who are habitually very active, but without overeating and without weight gain this relatively brief reduction in physical activity does not affect liver insulin action. Complementary findings from our study logically suggest that the effects of physical inactivity on skeletal muscle metabolism may not be as evident in people who live habitually sedentary lives,

and it was likely the energy surplus (i.e., overeating) in our study that was largely responsible for the whole-body insulin resistance we observed. Nonetheless, we acknowledge that we cannot definitively distinguish between the effect of overeating from the effects of low physical activity, and our findings must be interpreted as such.

Overall, the present findings expand our understanding about metabolic adaptations that occur in the early stages of overeating. We found that just a few days of overeating suppressed insulin sensitivity at the whole body-level, but this was not accompanied by changes in markers of impaired insulin action in skeletal muscle, supporting findings from animal studies that indicate insulin resistance may develop first in other tissues, such as the adipose tissue and liver. Even this relatively short exposure to overeating elevated markers of hepatic steatosis and abnormal liver function (i.e. liver enzymes AST and ALT). We were surprised to find that despite eating ~150 grams of dietary fat (~2-fold greater than most dietary recommendations) we did not find a robust increase in skeletal muscle lipid accumulation. From our indirect assessments, we surmised that a lipid accumulation may not have accumulated in muscle as a consequence of: (1) the majority of meal-derived triacylglycerides (packaged within chylomicrons) may have been preferentially channeled toward storage in adipose tissue, and (2) the chronically low NEFA concentration limited substrate availability for esterification and storage in muscle. Importantly, we believe the chronic (i.e., 24h) hyperinsulinemia that occurs in the early stages of overeating plays a very important role in regulating the changes in substrate metabolism and resting energy expenditure, even in advance of substantial weight gain.

Table 3-1. Subject characteristics before and after 2 weeks of overeating

	Baseline	2 weeks
Bodyweight (kg)	75.0±2.6	77.1±2.7*
Body mass index [BMI] (kg/m²)	23.9±0.3	25.2±0.3*
Body fat percentage (%)	26.4±2.9	27.5±2.5*
Fat mass (kg)	19.8±2.4	21.2±2.0*
Fat free mass (kg)	55.2±2.7	55.9±2.8
Resting metabolic rate (kcal/kg fat free mass/day)	28.2±0.5	29.4±0.9†

Values are Means±SE * Significantly different from Baseline, $p < 0.05$. † $p=0.09$ compared with Baseline

Table 3-2. Plasma substrate and insulin concentrations before and after 2 weeks of overeating

	Baseline	2 weeks
Fasting plasma glucose (mg/dl)	86±3	83±2
Fasting plasma insulin (μU/ml)	6.6±0.9	12.1±3.1*
HOMA-IR	1.4±0.2	2.6±0.7*
Fasting plasma non-esterified fatty acid (mM)	0.39±0.02	0.18±0.02*
Fasting plasma triacylglyceride (mM)	0.97±0.13	1.16±0.23

Values are Means±SE * Significantly different from Baseline $p < 0.05$

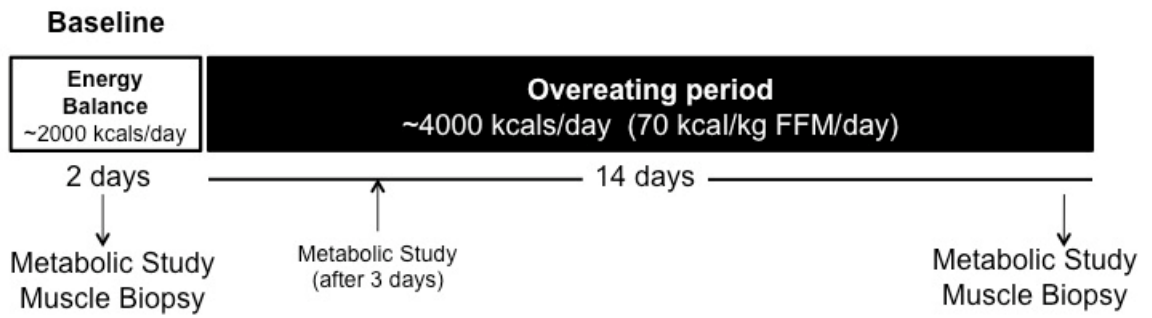


Figure 3-1. Diagram of the basic study design for the overeating period and the timing of the metabolic studies.

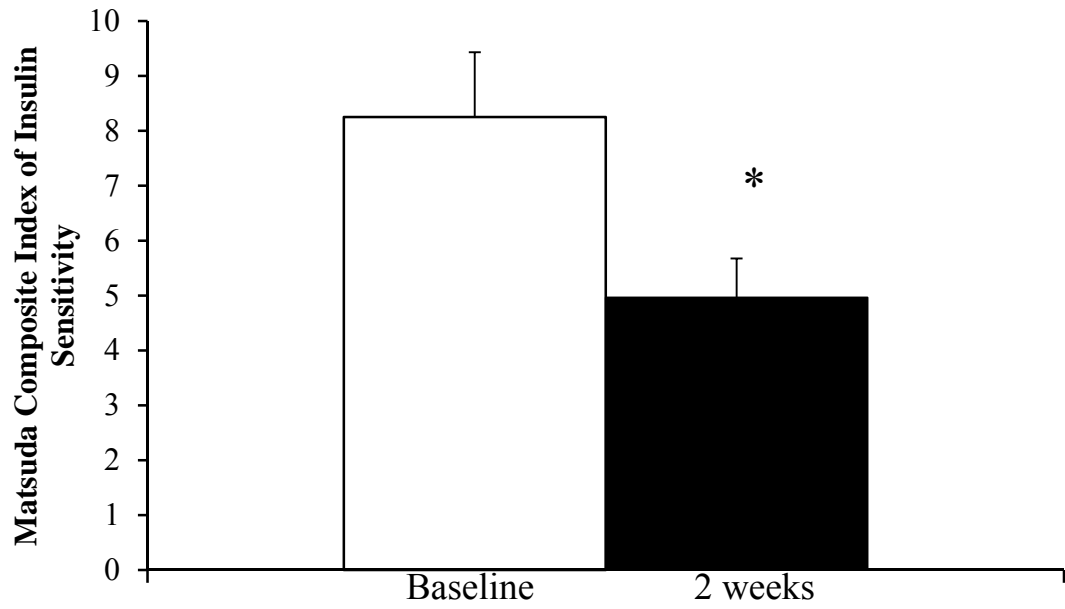
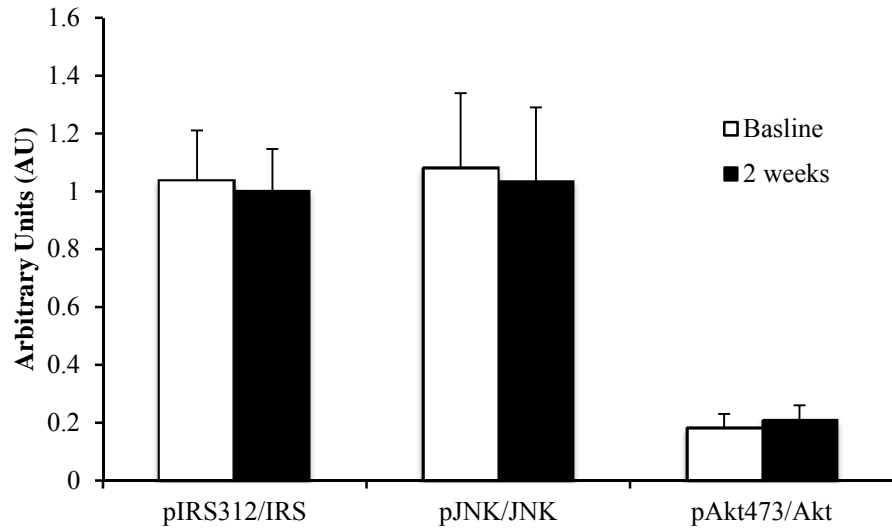


Figure 3-2. The Matsuda composite index of insulin sensitivity measured during a meal tolerance test before and after 2 weeks of overeating. * Significantly different from Baseline, $p < 0.05$.

A



B

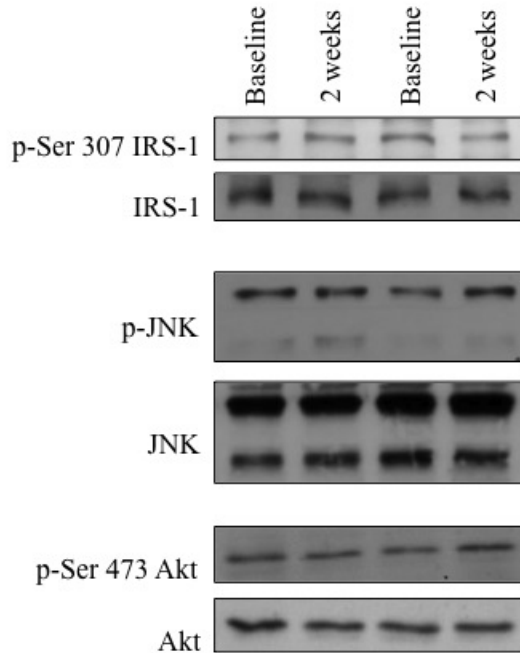
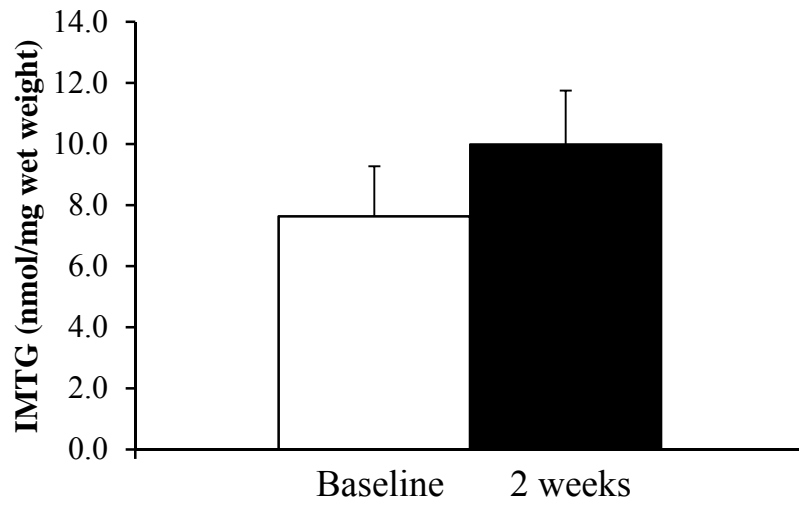


Figure 3-3. (A) Protein abundance of phosphorylated proteins in the insulin signaling pathway: insulin receptor substrate-1 (IRS-1) phosphorylated at Serine 312, c-Jun N-terminal kinase (JNK) phosphorylated at Tyrosine 185, and Akt phosphorylated at Serine 472 from skeletal muscle biopsy samples obtained after an overnight fast. (B) representative blots.

A



B

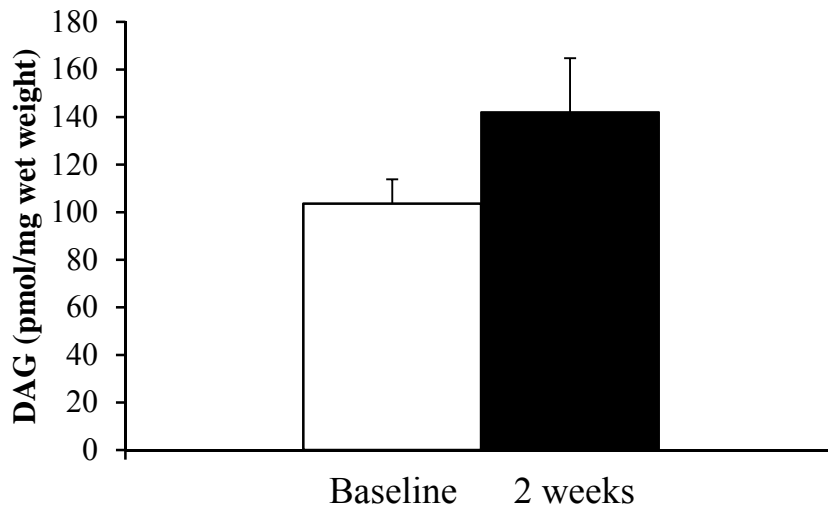


Figure 3-4. (A) Intramyocellular triacylglyceride (IMTG) and (B) Skeletal muscle diacylglyceride (DAG) concentration measured in skeletal muscle biopsy samples obtained after an overnight fast before and after 2 weeks of overeating.

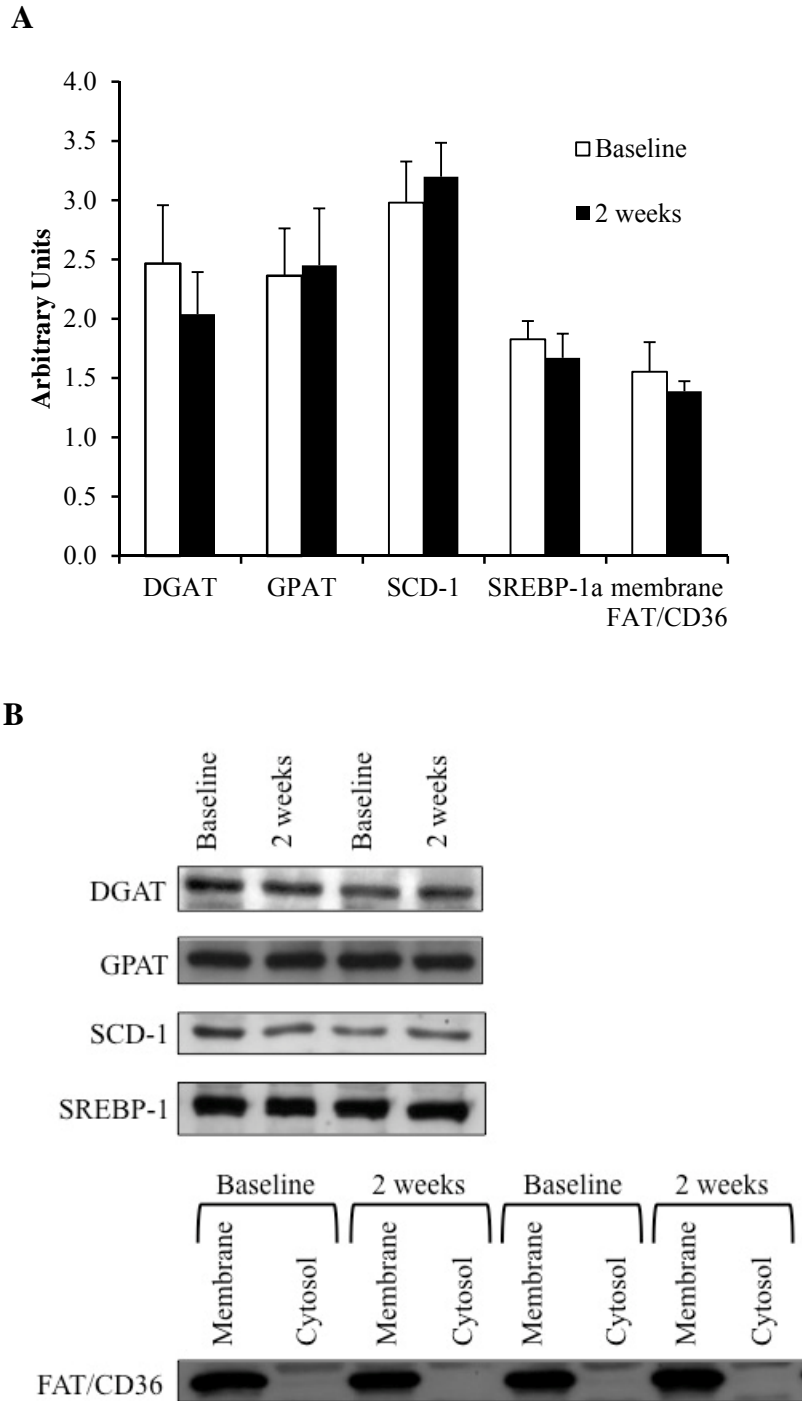


Figure 3-5. (A) Protein abundance of triacylglyceride synthesis enzymes glycerol-phosphate acyltransferase (GPAT), diacylglyceride acyltransferase (DGAT), and stearoyl-CoA desaturase-1 (SCD-1), transcription factor for lipid metabolism-related genes stearoyl response element binding protein-1a (SREBP-1a) and abundance of fatty acid

transporter CD-36 (FAT/CD36) on the crude membrane fraction (total abundance of FAT/CD-36 did not change) measured in skeletal muscle biopsy samples obtained after an overnight fast before and after 2 weeks of overeating. (B) representative blots before and after overeating.

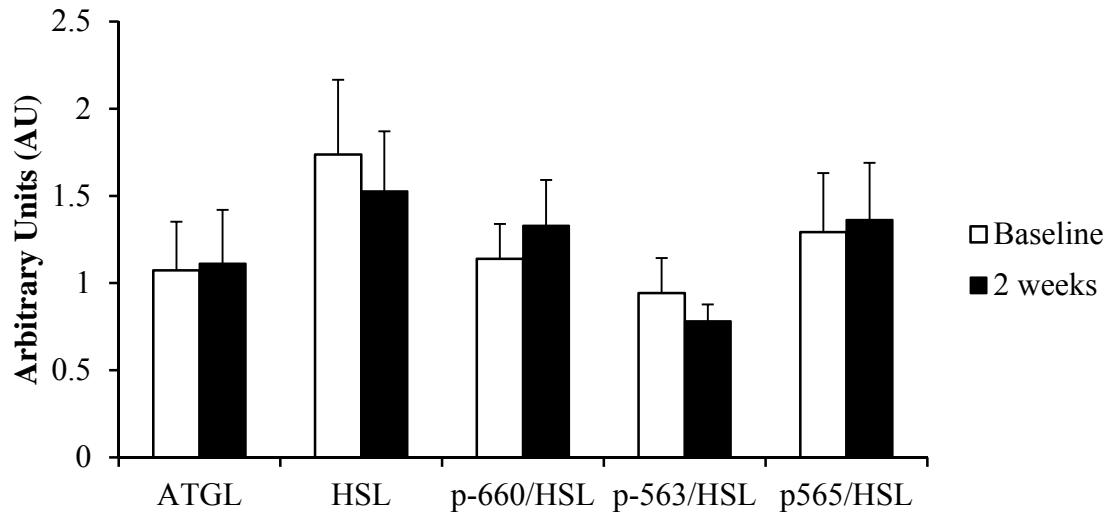
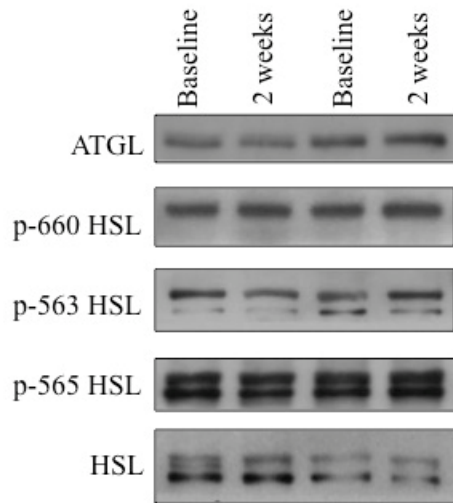
A**B**

Figure 3-6. (A) Protein abundance of lipolytic enzymes adipose triacylglyceride lipase (ATGL) and hormone sensitive lipase (HSL) measured in skeletal muscle biopsy samples obtained after an overnight fast before and after 2 weeks of overeating. Phosphorylation of HSL at Serine 565 (Ser⁵⁶⁵) is associated with an inhibition of lipolytic activity whereas phosphorylation at Serine residues 563 and 660 (Ser⁵⁶³ and Ser⁶⁶⁰) is associated with augmented lipolytic activity. (B) Representative blots.

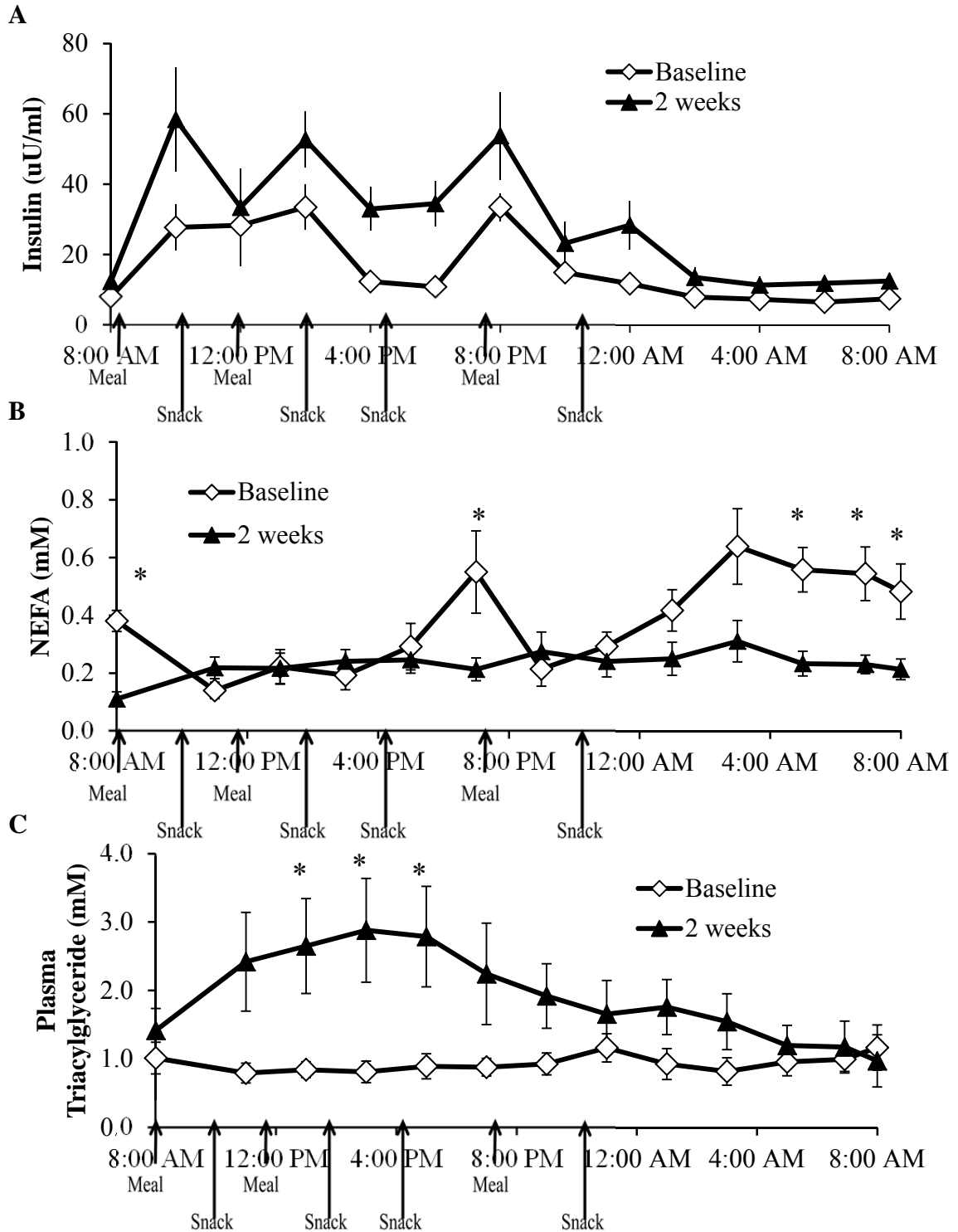


Figure 3-7. (A) Plasma insulin concentration, (B) plasma non-esterified fatty acid (NEFA) concentration, and (B) plasma triacylglyceride concentration over 24h before and after 2 weeks of overeating. * Significantly different from Baseline at that time, $p < 0.05$.

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CHAPTER 4

DISSERTATION STUDY #2

Changes in growth hormone and other endocrine responses to overeating

Abstract

Growth Hormone (GH) concentration is very low in obesity. This was traditionally attributed to the increased fat mass, however, the influence of overeating on GH secretion is not clear. The purpose of this study was to examine changes in GH secretion after 3 days and again after 2 weeks of overeating in healthy non-obese men (n=7; BMI: 24 ± 0.3 kg/m²; age: 24 ± 2 yrs). Subjects remained in the hospital throughout the entire 2 week overeating period and they ate standardized meals containing 70 kcals/kg fat free mass/day (~4000 kcals/day). 24h GH concentrations (Q20 min) were measured before overeating (Baseline), and on days 3 and at 2 weeks of overeating. By day 3, average 24h GH declined nearly 80% (1.3 ± 0.2 vs. 0.28 ± 0.1 ng/ml; $p=0.01$). This decline in GH concentration was due to a reduction in the GH pulse amplitude, and we observed no change in pulse frequency. Importantly, this marked suppression of GH secretion on day 3 occurred in absence of an increase in body weight (77.9 ± 2.3 vs. 77.3 ± 2.6 kg, Baseline and day 3, respectively). This was accompanied by a doubling in the average 24h insulin concentrations (15.9 ± 2.3 vs. 28.3 ± 5.4 μ U/ml, $p=0.03$). Two weeks of overeating significantly increased body weight (80.1 ± 2.1 kg; $p<0.001$ vs. both Baseline and day 3) and increased body fat by more than 10% ($p=0.002$). However, this did not translate to a further suppression of GH compared with day 3 (0.28 ± 0.1 vs. 0.32 ± 0.1 ng/ml, day 3 and day 13, respectively). In summary, only a few days of overeating markedly suppressed GH secretion prior to any measurable weight gain. This suppression in GH secretion was due exclusively to a reduction in GH pulse amplitude and was accompanied by chronic hyperinsulinemia.

Introduction

Obesity is characterized by abnormal metabolic and hormonal responses, including impaired glucose metabolism (14), high lipolytic rates with a resultant overabundance of circulating fatty acids (15), hyperinsulinemia, and low growth hormone (GH) secretion (25, 39, 49). The consequences of this reduced plasma concentration of GH in obesity has been the source of much speculation, with several studies suggesting that low GH contributes to the abnormal metabolic function in obesity (25, 39). The reduction in GH secretion in obesity has been attributed to total adiposity (49), abdominal adiposity (34), and intramyocellular and intrahepatic lipid content (3).

The underlying mechanisms responsible for this obesity-related suppression in GH secretion in obesity are not completely understood. Additionally, whether GH deficiency can precede (rather than follow) obesity, possibly facilitating its future progression, has not been investigated. Clearly, weight gain can only occur under conditions when energy intake exceeds energy expenditure (i.e., “overeating”). Therefore, the purpose of this study was to determine the effects of a short-term period of 2 weeks of overeating on the regulation of GH secretion.

Methods

Subjects

Seven men participated in a 2 week overeating protocol. All subjects were non-obese (body mass index: 24.0 ± 0.3 kg/m², age: 24 ± 2 yrs), weight stable, in good health, relatively sedentary (physical activity ≤ 2 hrs/week), and were not taking any medications. All procedures of this study were approved by the Institutional Review Board at the University of Michigan. Written, informed consent was obtained from all subjects before their participation in the study.

Experimental Design

Subjects were admitted to the Michigan Clinical Research Unit (MCRU) at the University of Michigan Hospital on two separate occasions. The first hospital visit lasted

two days (one overnight) during which we performed a battery of baseline metabolic tests (see details below) while the subjects consumed a weight maintaining diet (39 ± 1 kcal/kg FFM/day; 50% carbohydrate, 35% fat, and 15% protein). During the second hospital visit, subjects adhered to a supervised overeating intervention, and they remained in the hospital without leaving throughout this entire 2 week period. During this 2 week overeating period, each day subjects consumed standardized meals containing 70 kcal/kg FFM/day (~ 4000 kcals/day; 50% carbohydrate, 35% fat, and 15% protein), which represents about 75% more calories than a weight-maintaining diet. The daily diet was provided as 3 meals (at 0800, 1200, 1900) and 4 snacks (at 1000, 1400, 1600, 2200). During both the “Baseline” and overeating hospital visits, subjects were restricted to the MCRU ward, and their physical activity was limited to 1500 steps per day, as measured by a pedometer (Digi-walker SW200; Yamax, San Antonio). Daily energy intake and physical activity were strictly monitored by the research staff. Body weight was measured at 0730 each morning, and body composition was assessed using dual energy x-ray absorptiometry (DEXA; Lunar Prodigy Advance; GE Healthcare, Buckinghamshire, UK) before and after the 2 week overeating period.

Blood samples were obtained every 20 min for 24h during the Baseline visit, as well as on day 3, and day 13 of the overeating period. These samples were collected in tubes containing sodium heparin, and then centrifuged at 1,600 g for 20 minutes. Plasma was removed and stored at -80°C for later analysis. Plasma GH concentration was measured every 20 minutes during the 24-hour periods at Baseline, day 3 and 2 weeks. Plasma insulin and cortisol concentrations were assessed every 2 hours at Baseline, day 3 and 2 weeks. In addition, every morning of the 2 week overeating period (after an overnight fast), one blood sample was drawn and collected into tubes containing EDTA for assessment of fasting plasma concentrations of insulin, fatty acids, insulin-like growth factor-1 (IGF-1), and IGF-1 binding proteins (IGFBP).

Analytical Procedure

Plasma hormone concentrations

Plasma GH, free IGF-1, IGFBP3, and cortisol concentrations were determined using chemiluminometric assays from Siemens with an Immulite System (Los Angeles, CA). Total IGF-1 and IGFBP1 were determined by ELISA kits (Diagnostic Systems Laboratories (DSL); Brea, CA). Insulin concentration was determined by radioimmunoassay (Millipore; Billerica, MA).

Plasma substrate concentrations

Plasma concentrations of fatty acid (HR Series NEFA; Wako Chemicals USA, Richmond, VA), glucose (Glucose Oxidase; Thermo Fisher Scientific Inc., Waltham, MA), and triacylglyceride (Triglyceride Reagent; Sigma Aldrich Inc., St. Louis, MO) were measured by commercially available colorimetric assay kits.

Calculations

Analyses of GH pulse amplitude, pulse frequency, and interpulse level were conducted using the Pulse_XP hormone cluster analysis software through the generosity of Dr. Michael Johnson at the University of Virginia. Plasma GH concentrations used in this cluster analysis were measured every twenty minutes over a 24-hour period on three separate occasions (Baseline, Day 3, and 2 weeks). Measurement error, for the basis of pulse detection, was found through repeated sampling of a single specimen. Missing values were not included in the analysis and values below the point of detection were set at the lowest level of detection, 0.01ng/ml.

Statistical Analysis

Repeated measures one-way ANOVA in conjunction with a Tukey post hoc test was used to test for significant differences at Baseline, day 3, and 2 weeks. Analysis was conducted using Sigma Stat version 3.0.1a for Windows. Statistical significance was defined by a p-value less than 0.05. All data are presented as mean \pm standard error (SE).

Results

Changes in body composition

By day 3 of the overeating period subjects did not exhibit measurable changes in body mass (Table 1). However, after 2 weeks of overeating subjects gained 3.0 ± 0.5 kg body mass and 1.7 ± 0.3 kg of fat mass (both $p<0.01$ vs. Baseline). There was a tendency for fat free mass to increase after two weeks of overeating, however this did not reach statistical significance ($p=0.068$) (Table 1).

Changes in fasting plasma substrates

Overnight fasted plasma glucose concentration remained stable throughout the 2-week overeating period (Table 2). In contrast, fasting plasma fatty acid concentration was reduced by more than half in the first few days of the overeating period ($p<0.001$), and remained low at 2 weeks ($p<0.001$ vs. Baseline) (Table 2). Fasting plasma triacylglyceride concentration tended to increase by day 3 ($p=0.07$) and was significantly increased compared with Baseline at 2 weeks of overeating ($p=0.05$) (Table 2).

Plasma growth hormone concentration

24h average plasma GH concentration was 1.26 ± 0.2 ng/ml at Baseline (Figure 1). Only a few days of overeating suppressed 24h GH concentration by ~80% (0.28 ± 0.1 ng/ml by day 3 of overeating; $p<0.05$ vs Baseline; Figure 1). There was no further reduction in 24h average plasma GH concentration after 2 weeks of overeating (0.32 ± 0.1 ng/ml; $p<0.05$ vs Baseline). The reduction in the GH profile was due exclusively to a reduction in GH pulse amplitude (Table 3). There was no significant change in GH pulse frequency (Table 3) or the average GH concentration between pulses (i.e., “interpulse level,” Table 3).

Plasma insulin and cortisol concentration

Not surprisingly, mean 24h plasma insulin concentration was increased by about 2-fold, while overeating compared with Baseline (Figure 2A). In contrast, the increase in fasting plasma insulin concentration (8.1 ± 1.6 , 11.7 ± 2.9 , and 11.8 ± 4.5 μ U/ml the morning after an overnight fast at Baseline, day 3 and 2 weeks, respectively) did not quite reach

statistical significance ($p=0.12$). Overeating also did not affect 24h plasma cortisol concentrations (Figure 2B; all $p=0.56$).

Plasma IGF-1 concentration

Although GH is known to augment hepatic IGF-1 production, we found that the marked suppression in plasma GH concentration during overeating period did not translate into a change in total plasma IGF-1 (Figure 3A). However, plasma concentrations of IGF-1 binding proteins (IGFBP3 and IGFBP1) can impact the bioavailability of IGF-1. These binding proteins can have opposing signaling effects: IGFBP1 can inhibit the interaction of IGF-1 with its receptor (38), thereby reducing its bioactive potential, whereas IGFBP3 can augment growth-promoting IGF-1 signaling (9). Both binding proteins were affected by overeating (Figure 3B and 3C). Plasma IGFBP3 concentrations underwent a small but significant increase (3.5 ± 0.2 , 4.1 ± 0.1 , and 4.2 ± 0.2 ng/l at Baseline, day 3 and 2 weeks, respectively, $p=0.004$). In contrast, there was a significant main effect ($p=0.044$) for a reduction in IGFBP1 (36.2 ± 11.2 , 17.4 ± 4.7 , and 14.7 ± 3.1 ng/ml at Baseline, day 3 and 2 weeks). Furthermore, we also found a significant main effect ($p<0.05$) of overeating on plasma free IGF-1 concentration (1.3 ± 0.4 , 1.1 ± 0.2 , and 2.3 ± 0.4 ng/ml at Baseline, day 3 and 2 weeks, respectively; Figure 3D) with a significant increase in free IGF-1 concentration at 2 weeks ($p<0.05$ vs. day 3).

Discussion

Overeating induces complex endocrine responses that can alter metabolism. The major finding of this study demonstrated a marked reduction in daily GH secretion after only a few days of overeating. Although low GH concentration is a common feature associated with obesity, the reduction in GH concentration we observed in this study occurred before any change in body mass. Additionally, the reduction in GH did not coincide with changes in many factors known to regulate GH secretion (i.e., plasma concentrations of IGF-1, cortisol, fatty acids, or glucose during fasting). However GH suppression was accompanied by an increase in the average 24h insulin concentration, which has been demonstrated to reduce GH secretion *in vitro* (23, 51), and likely plays a key role in the suppression in the GH secretion we observed with overeating.

Excessive body fat has long been associated with suppressed GH concentration (3, 34, 49). Our findings suggest that overeating can markedly suppress GH secretion in advance of an increase in body fat. Although we did not assess body fat content after three days of the overeating period, body weight did not change, and previous studies have reported no measurable increase in fat mass or bodyweight after five days of overeating (4). Therefore, the suppression in GH after 3 days of overeating in our study was due to the more immediate metabolic or endocrine responses to overeating, *per se*, rather than increase in fat mass. Indeed, GH secretion is known to be regulated by several factors including IGF-1 (52), cortisol (45), fatty acids (5) and insulin (51).

The stimulatory effect of GH on IGF-1 production is well described (12, 19, 21), and we recently reported plasma IGF-1 production was determined by basal GH concentration rather than GH pulses (12). Therefore, given that the suppression in GH concentration we observed with overfeeding was a consequence of a reduction in GH pulse amplitude, with no change in basal GH concentration (i.e., plasma interpulse GH concentration), it was not surprising that total IGF-1 concentration was not affected by overeating in our study. Converse to the stimulatory effects of GH on IGF-1 production, IGF-1 provides a well-described negative feedback on GH secretion (30, 52-54). IGF-1 is known to inhibit GH secretion by decreasing GH mRNA and GH production in cultured primate pituitary cells (23). Although we found total plasma IGF-1 concentration to remain unchanged throughout the 2 week overeating period, total IGF-1 may not adequately reflect IGF-1 bioactivity (7). It has been reported that plasma free IGF-1, rather than total IGF-1, plays a key role in the inhibition of GH release (7). Only ~1% of all plasma IGF-1 is found in the free/unbound form (16). At least seven IGF-1 binding proteins (IGFBPs) can bind IGF-1 (7, 40), and in doing so, can modify or interfere with interactions between IGF-1 and its receptor (8). We measured the concentration of two key IGFBPs (IGFBP3 and IGFBP1) because approximately 90% of IGF-1 is typically bound to IGFBP3 (17), and IGFBP1 has been suggested to be pivotal in the inhibition of IGF-1 activity (22). Although most of IGF-1 is bound to IGFBP3 (17), the increase in plasma free IGF-1 concentration by the end of our overfeeding period corresponded with a slight, yet significant *increase* in IGFBP3. In contrast, the abundance of plasma IGFBP1 declined

during overeating, which is consistent with the known effect of chronic hyperinsulinemia on IGFBP1 (10, 29). Although we did find plasma free IGF-1 concentration to be elevated by the end of the 2 week overeating period, the large suppression in plasma GH concentration we observed occurred before any increase in free IGF-1. Therefore, free IGF-1 was unlikely to be contributing to the reduction in GH secretion after a few days of overeating, which agrees with work from previous studies in obesity (36, 37).

Glucocorticoids, such as cortisol, are known to augment pituitary GH secretion by increasing the pituitary expression of GHRH-receptor (27), and the GH-secretagogue receptor (45), as well as reducing the IGF-1-mediated feedback inhibition of GH release (46). However, because we found no change in the average plasma cortisol concentration over 24 hours during the overeating period, it is unlikely that plasma cortisol contributed to the suppression in GH secretion in our study. Additionally, fatty acids are known to impair GH secretion, by reducing the mRNA of GH, GHRH-receptor, and GH secretagogue receptor in cultured pituitary cells (23) and this may be due to a fatty acid-induced reduction in protein kinase A activity in the pituitary (33). Reducing plasma fatty acid concentration in obese adults using the antilipolytic agent, acipimox, was found to augment the GH response to GHRH (35). Therefore, the elevated fatty acid availability present in obesity (15) may be a major contributor to the obesity-related reduction in GH secretion. However, changes in plasma fatty acid concentration could not explain the suppression in GH concentration we found in our study because plasma fatty acid concentration remained very low during the 2 week overeating period, likely due in large part to the chronically elevated plasma insulin concentrations. Importantly, by itself the low fatty acid concentration in our study would be expected to enhance GH secretion. However, the potent suppressive effect of hyperinsulinemia has been found to override the sensitizing effects of low fatty acid concentration of GH secretion (20).

Previous studies demonstrated that insulin inhibits pituitary GH secretion by reducing the mRNA of GH, GH-releasing hormone (GHRH) receptor, and GH secretagogue receptor, as well as GH production in cultured pituitary cells (23, 26, 51). In humans, increasing plasma insulin concentration, via low-level insulin infusions, has been found to reduce

the GH response to GHRH in a dose-dependent manner (20). Although the GHRH-induced GH response was not significantly reduced when plasma insulin concentration was increased to only ~13mU/ml, increasing plasma insulin concentration to ~35mU/ml significantly reduced GH secretion (20). Because post-prandial plasma insulin concentrations often increase to levels well above 35 mU/ml, the findings from Lanzi, et al (20) indicate that even relatively small meals can suppress GH. This suppression in GH secretion after meals appears to be independent of the post-prandial rise in plasma glucose concentration, because hyperglycemia was found to not alter GH secretion when plasma insulin was kept constant (13). Furthermore, parenteral nutrition has been found to suppress GH concentrations (28). Therefore, the GH suppression induced by overeating in our study was likely due to the rapid and sustained elevation in plasma insulin concentration resulting from the frequent ingestion of high calorie meals and snacks during the overeating period.

The suppression in GH secretion with overeating may have important metabolic impact. Several studies have observed low plasma GH concentration in obese patients (32, 44, 48), leading some to suggest that reduced GH secretion may underlie some obesity-related metabolic abnormalities (18, 25). However, our data illustrate that overeating, *per se*, can suppress GH secretion prior to the actual weight gain. Importantly, overeating rapidly and consistently suppressed the GH pulse amplitude, which is known to selectively regulate several metabolic processes, including lipolytic rate (6, 44). As a result, GH suppression can influence metabolic substrate selection through its effect on lipolysis (31). Additionally, GH is also known to impair insulin sensitivity (31). The insulin desensitizing effects of GH have been attributed in part to fatty acid-induced insulin resistance that occurs in consequence to the GH-mediated increase in lipolysis (41). In addition, GH has been reported to induce insulin resistance through a down-regulation in the insulin signaling pathway via the p85alpha subunit of PI3K (1, 2). Therefore, the impairment in insulin sensitivity commonly found with overeating (4, 11) would likely be even more severe without this suppression in GH secretion. Additional experiments are needed to better understand the physiological impact of the rapid suppression of GH pulsatility with overeating.

It is important to note that during the 2 week protocol, our participants were not only overeating, but they were also restricted in physical activity level (1500 steps per day). Therefore, we cannot directly distinguish the effects of the overeating from the potential impact of very low physical activity levels. It is clear that exercise augments plasma GH concentration (50), and there appears to be a dose response because heavy resistance exercise (24) and sprinting (42, 43) can induce relatively high transient elevations in plasma GH concentration. However, plasma GH concentration returns to basal levels within a few to several hours after the exercise session. Moreover, available evidence suggests that plasma GH concentration is not associated with habitual physical activity or inactivity (47). Therefore, we do not believe that the reduction in physical activity in our sedentary subjects contributed much, if anything to the marked suppression in plasma GH concentration, but we cannot rule out this possibility.

In summary, overeating induced rapid and sustained suppression of GH pulse amplitude even before an increase in body weight. The accompanying hyperinsulinemia was a likely mediator of this rapid reduction in GH secretion.

Acknowledgements

This study was supported by the National Institutes of Health grant #R01DK71955, with additional support from the Michigan Clinical Research Unit (NIH-UL1RR024986), the Michigan Nutritional Obesity Research Center (P30-DK-089503) and the Michigan Metabolomic Obesity Center. We would like to thank Kathleen Symons for the analysis of GH, IGF-1, and IGF-BPs, as well as Lisa Michael, RD for her tireless efforts as research dietitian and study coordinator. We would also like to thank our participants, and the staff of the University of Michigan Clinical Research Unit.

Table 4-1. Subject Characteristics

	Baseline	Day 3	2 weeks
Body mass (kg)	77.9±2.3	77.3±2.6	80.1±2.1*
Body mass index (kg/m²)	24.0±0.3	24.0±0.4	25.4±0.2*
Percent Body Fat (%)	23.7±2.9	-	25.2±2.6*
Fat mass (kg)	18.7±2.7	-	20.4±2.5*
Fat free mass (kg)	58.8±1.6	-	59.7±1.5

Values are expressed as mean±SE. * Significantly different from Baseline, p<0.05

Table 4-2. Plasma substrate concentrations

	Baseline	Day 3	2 weeks
Plasma glucose (mM)	5.4±0.2	5.5±0.4	4.9±0.1
Plasma fatty acid (mM)	0.35±0.05	0.14±0.02*	0.14±0.01*
Plasma triacylglyceride (mM)	0.70±0.13	1.23±0.23	1.28±0.29*

Values are expressed as mean±SE. * Significantly different from Baseline p<0.05

Table 4-3. Average 24h plasma GH concentration and plasma GH profile

	Baseline	Day 3	2 weeks
24-hour Mean GH (ng/ml)	1.26±0.2	0.28±0.1*	0.32±0.1*
Pulse Frequency (per day)	3.6±0.4	2.7±0.8	3.1±0.5
Mean Pulse Amplitude (ng/ml)	6.38±1.11	2.46±1.03*	1.48±0.53*
Mean Interpulse Level (ng/ml)	0.32±0.07	0.20±0.11	0.24±0.11

Values are expressed as mean±SE. * Significantly different from Baseline p<0.05

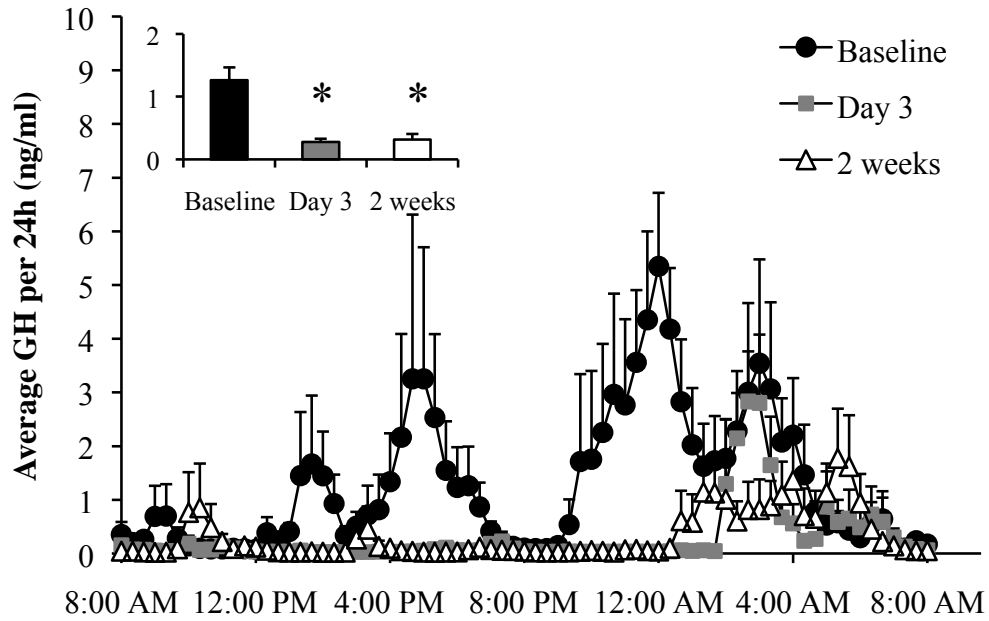


Figure 4-1. Mean plasma growth hormone (GH) concentration every 20 min for 24h before overeating (Baseline), on day 3 of overeating and at the end of the 2 week overeating period. Inset: the average plasma GH concentration at Baseline, day 3 and 2 weeks of overeating. * $p < 0.05$ vs. Baseline

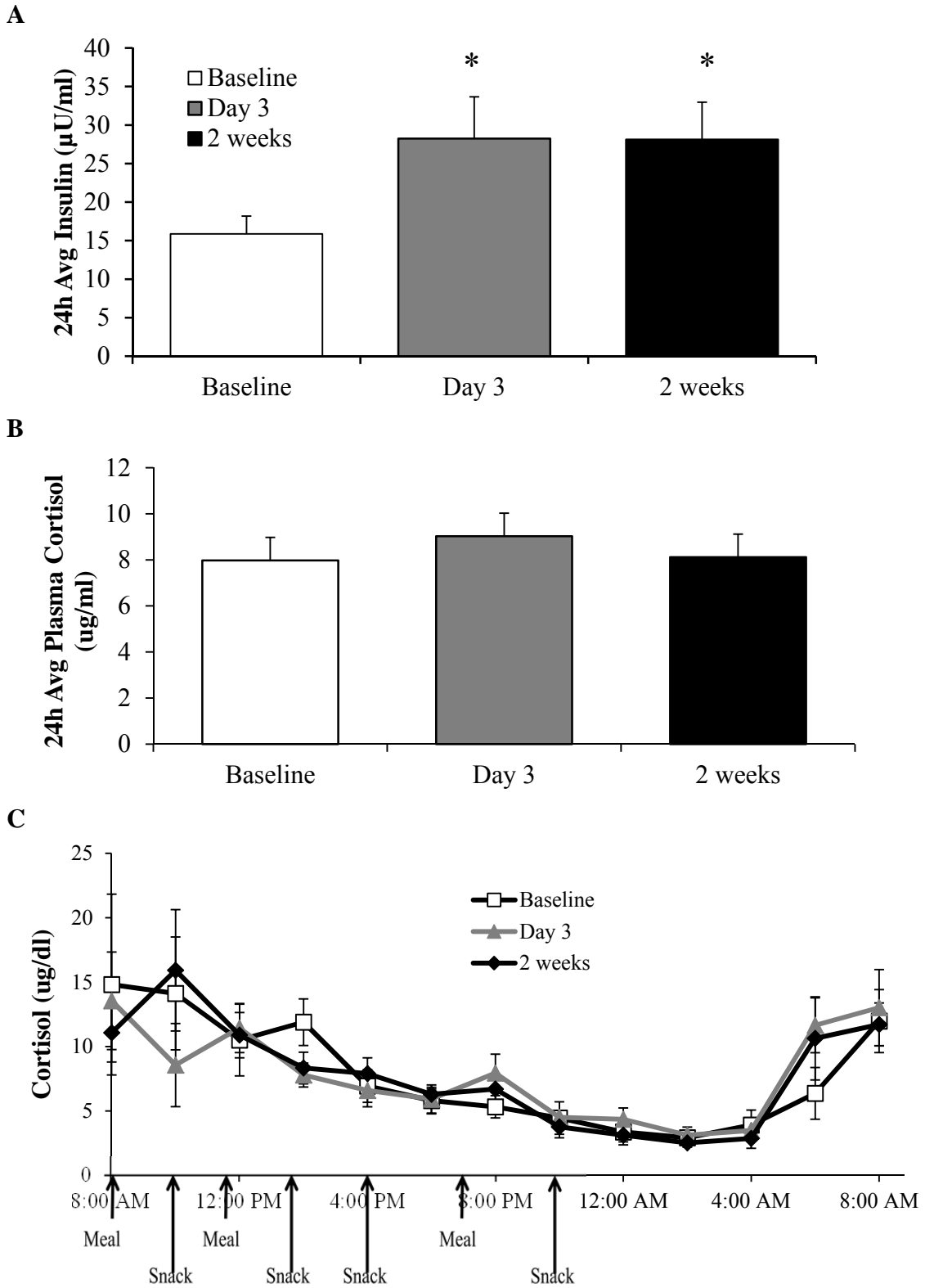


Figure 4-2. Mean 24h plasma concentrations of (A) insulin and (B) cortisol before overeating (Baseline), on day 3 of overeating, and at the end of the 2 week overeating period. (C) Plasma cortisol concentrations throughout the day * p<0.05 vs. Baseline

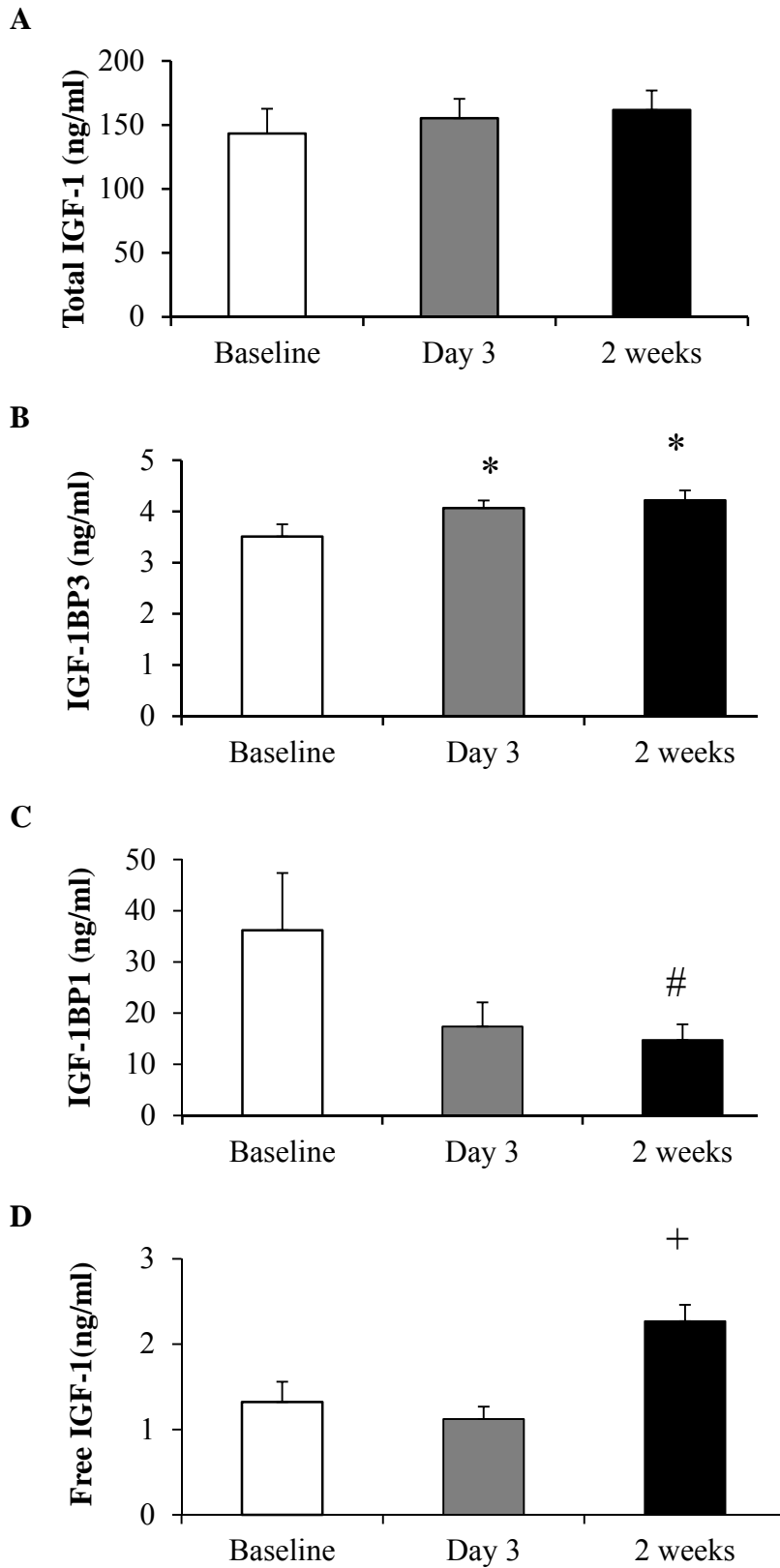


Figure 4-3. Mean plasma concentrations of (A) total IGF-1, (B) IGFBP-3, (C) IGFBP-1, and (D) free IGF-1 in the morning after an overnight fast before overeating (Baseline), on day 3 of overeating and at the end of the 2 week overeating period. * $p < 0.05$ vs. Baseline, + $p = 0.057$ vs. Baseline, # $p < 0.05$ vs. day 3

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CHAPTER 5

DISSERTATION STUDY # 3

Metabolic consequences to the suppression in growth hormone secretion that occurs with overeating

Abstract

In STUDY 2 we found that overeating for only a few days markedly suppressed the secretion of growth hormone (GH). The primary aim of this study was to determine the role of this reduction in GH concentration on measures of insulin sensitivity, lipolytic rate, and protein metabolism throughout 2 weeks of overeating. 10 non-obese, healthy adults were admitted to the hospital for 2 wks, during which time they ate ~4000 kcals/day (70 kcals/kg fat free mass/day; 50% carbohydrate, 35% fat, 15% protein), and their plasma GH concentration was allowed to decline naturally (Control). An additional 8 subjects underwent the same overeating intervention, but they received exogenous GH treatment (GHT) administered in 4 daily injections to mimic physiologic GH secretion throughout the 2 wks of overeating. Plasma GH concentration was measured for 24h (Q20”) before overeating (Baseline), and again at 3-4 days and 2 weeks of overeating. We also measured plasma insulin and glucose concentrations, lipolytic rate (rate of appearance (Ra) of $^2\text{H}_5$ -glycerol in plasma), proteolytic rate (Ra of $^2\text{H}_3$ -leucine in plasma), and fractional synthetic rate (FSR) (incorporation of $^2\text{H}_3$ -leucine into skeletal muscle). 24h average GH concentration was reduced ~70% by 3-4 days of overeating in our Control subjects (1.3 ± 0.2 vs. 0.5 ± 0.2 ng/ml for Baseline and days 3-4; $p < 0.05$) and GH remained low at 2 weeks of overeating (0.4 ± 0.1 ng/ml, $p < 0.05$). GHT prevented the fall in plasma GH concentration, maintaining plasma GH concentration at Baseline levels (1.2 ± 0.2 ng/ml). Preventing the suppression in GH during GHT increased markers of insulin resistance, as noted by an elevated fasting plasma insulin concentration and the homeostatic model assessment of insulin resistance (HOMA-IR), and increased fasting lipemia (all $p < 0.05$ vs Control). In addition, preventing the suppression in GH with overeating also blunted the increase in systemic proteolysis ($p < 0.05$ GHT vs. Control).

In contrast, GHT did not alter lipolysis or FSR in response to overeating. In conclusion, the major findings from this study suggest that the suppression in GH secretion that naturally occurs during overeating may help attenuate the insulin resistance and hyperlipidemia that typically occurs with overeating.

Introduction

Weight gain can only occur when energy intake exceeds energy expenditure (i.e., positive energy balance), and even a relatively modest positive energy balance can result in an individual becoming overweight and obese over time. Although the metabolic complications of obesity have been well described (27, 31, 47), far less is known about the metabolic changes that occur in the early stages of weight gain. One hallmark of obesity is a low secretion of growth hormone (GH) (57), and several studies have suggested that the low plasma GH concentration may contribute to some of the metabolic abnormalities found in obesity (2, 48). Importantly, in STUDY 2 of my dissertation we reported that even just a few days of overeating markedly suppressed plasma growth hormone (GH) concentration in non-obese adults. However, the metabolic consequences of this acute suppression in plasma GH concentration with overeating are not known.

GH has been identified as an important regulator of several metabolic processes. For example, we (57) and others (8, 13) found that the normal pulsatile pattern of GH secretion augments lipolytic rate. Additionally, GH has been reported to impair insulin sensitivity through direct inhibition of insulin signaling (4), as well as indirectly by increasing lipolysis (29), which in turn can alter insulin action via increasing fatty acid infiltration into insulin-responsive tissues. GH may also contribute to hyperlipemia via alterations in the activity of lipoprotein lipase (46, 51, 60). GH has also garnered a great deal of attention for its role in regulating protein metabolism (14, 18, 71), but the direct effect of GH on muscle protein synthesis is controversial (49). Importantly, previous studies have found that overeating decreased insulin sensitivity (6, 61), exacerbated plasma triacylglyceride concentration (39), as well as altered protein metabolism (66), and the suppression of GH with overeating may be an important contributor to many of these responses. Therefore, the primary aim of this study was to examine how the

suppression of GH with overeating might influence insulin sensitivity, lipolysis, and protein metabolism with overeating. To address these goals, we measured metabolic responses to overeating while preventing the fall in plasma GH concentration via exogenous GH administration.

Methods

Subjects

A total of 23 young, healthy men (n=19) and women (n=4) participated in a 2-week overeating protocol (initial body mass index: $23.5 \pm 0.3 \text{ kg/m}^2$; age: $24 \pm 1 \text{ yr}$). Before the study, all subjects were weight stable, relatively sedentary (physical activity, $\leq 2 \text{ h/wk}$), and were not taking any medications. All procedures and protocols of this study were approved by the University of Michigan Institutional Review Board. Written, informed consent was obtained from all subjects before their participation in the study.

Study Design

GH treatment

In STUDY 2 we found that overeating markedly suppressed plasma GH concentration. In order to assess the metabolic consequences of this suppression in GH, in this study we prevented the reduction in plasma GH throughout 2 weeks of overeating by providing exogenous GH. Eight subjects (7 men and 1 woman) received exogenous GH ($0.3 \mu\text{mol Genotropin/m}^2/\text{day}$ – diluted in a 5% dextrose solution) in four intravenous injections each day. In attempt to mimic endogenous GH secretion, half of the daily exogenous GH dose was divided equally in 3 daytime bolus infusions, each lasting 20 min (at 0700h, 1100h, and 1600h), and the other half of the daily GH dose was injected over 60 min starting at 2300h. Metabolic responses to overeating in this cohort of subjects who received exogenous GH treatment (GHT) to prevent the suppression in plasma GH concentration was compared with the responses in 10 subjects (7 men and 2 women) in whom plasma GH concentration was allowed to decline naturally during overeating (Control). Control subjects were the same subjects from STUDY 1. Control subjects received daily infusions of 5% dextrose to match the vehicle infusion in GHT. In

addition, as a secondary aim of this study we also explored the effects of high GH concentrations during overeating in a separate, smaller cohort of 5 subjects (4 men and 1 woman) who received a relatively high daily dose of GH (“High GHT”; $1\mu\text{mol Genotropin/m}^2/\text{day}$), administered in the same manner as described above for GHT.

Overall experimental protocol

Before the overeating intervention (i.e., Baseline) subjects were admitted to the Michigan Clinical Research Unit at the University of Michigan Hospital for a two-day experiment, and they ate a weight-maintaining diet consisting of 39 kcal/kg FFM/day (~2250 kcal/day; 50% carbohydrate, 35% fat, and 15% protein) on the first day. We collected blood samples every 20 minutes for 24h to assess their 24h plasma growth hormone and insulin profiles. In the morning of the second day of the trial we performed a “metabolic study” (see details below) after an overnight fast. On a separate occasion (often about 1 week after the Baseline trial), subjects were admitted to the Clinical Research Unit for a 2-week overeating intervention, and they remained as in-patients throughout the entire 2-week period. During the 2-week study, subjects ate 70kcal/kg FFM/day (~4000kcal/day; 50% carbohydrate, 35% fat, and 15% protein). Each day, the subjects were provided 3 meals (0800h, 1200h, and 1900h) and 4 snacks (1000h, 1400h, 1600h, and 2200h). Every morning of the 2-week intervention we collected a blood sample after an overnight fast for measurements of plasma concentrations of insulin, glucose, non-esterified fatty acid (NEFA) and triacylglyceride, we also measure plasma insulin-like growth factor 1 (IGF-1) in fasted plasma samples collected at Baseline and at the end of the 2 weeks overeating period. We reassessed 24h plasma growth hormone and insulin profiles on days 3-4 and again at 2 weeks of the overeating period. At 2 weeks we also performed the same “metabolic study” as performed during the Baseline trial. Subjects were limited to 1500 steps per day (as monitored by a pedometer). Body composition was measured using Dual-Energy X-ray Absorptiometry (DEXA) during the Baseline trial and at the end of the 2-week overeating period.

Metabolic Studies

Before the overeating intervention (Baseline) and at 2 weeks of overeating we measured resting metabolic rate (RMR) at 0600h using indirect calorimetry (Vmax Encore, Sensormedics, San Diego, CA). After measuring RMR, we began a 6 hour, primed, constant rate infusion of $^2\text{H}_3$ -leucine, which was used to calculate whole-body proteolytic rate (Leucine rate of appearance in plasma (Ra)) and skeletal muscle protein synthesis (fractional synthetic rate (FSR)). At 0900h we started a primed, constant rate infusion of $^2\text{H}_5$ -glycerol for assessment of whole body lipolytic rate (glycerol Ra). Three arterialized blood samples were obtained at 1050, 1055h, and 1100h for the determination of glycerol Ra and leucine Ra [glycerol Ra was also measured on day 4 of the overeating period]. Muscle biopsies were obtained at 0800h and 1300h to measure the incorporation of the infused $^2\text{H}_3$ -leucine into skeletal muscle. Muscle biopsies were performed under local anesthesia, using a Bergstrom biopsy needle. Muscle samples were immediately cleaned with sterile saline, dried, frozen in liquid nitrogen, and stored at -80C until later analysis. Blood samples were also collected at the time of the muscle biopsies to determine the isotope enrichment of the precursor pool (α -ketoisocaproate (α -KIC)).

Analytical Procedures

Plasma concentrations of hormones and substrates.

Plasma GH concentration was determined by chemiluminescent assay on a Siemens Immulite System (Diagnostic Products, Los Angeles, CA), plasma IGF-1 concentration was determined via ELISA kit (Diagnostic Systems Laboratories, Brea CA), and plasma insulin concentration was determined by radio-immuno-assay (Millipore, Billerica, MA). Plasma concentrations of glucose (glucose oxidase; Thermo Fisher Scientific Inc., Waltham MA), NEFA (HR Series NEFA; Wako Chemicals USA, Richmond, VA), and triacylglyceride (triglyceride reagent; Sigma Aldrich Inc., St. Louis, MO) were all determined by colorometric assay.

Tracer:tracee ratios (TTR) of $^2\text{H}_5$ -glycerol, $^2\text{H}_5$ -leucine, and $^2\text{H}_3$ - α KIC in plasma

Plasma samples (250 μl) were deproteinized in ice-cold acetone. For TTR measurements of $^2\text{H}_5$ -glycerol, the deproteinized samples were derivatized in pyridine and acetic anhydride as previously reported (57). Glycerol TTR was determined using gas chromatography/mass spectrometry (GC/MS) with selectively monitored mass-to-charge ratios (m/z) of 145 and 148. For $^2\text{H}_3$ -leucine TTR, the deproteinized plasma samples were derivatized in 1:1 acetonitrile:*N*-methyl-*N*-tert(butyldimethylsilyl)tri-fluoroacetamide as previously described (25). Leucine TTR was determined using GC/MS with m/z of 202 and 203. For the plasma enrichment of the precursor pool, α -KIC, 250 μl of plasma was deproteinized in ice-cold acetonitrile and derivatized with methoxyamine HCl after samples were titrated to pH 10 with KOH and incubated at 60 C for 1 hour. Samples were dried and the methoxyime derivative of α -KIC was extracted using acetonitrile. Samples were again derivatized in 1:1 acetonitrile:*N*-methyl-*N*-tert(butyldimethylsilyl)tri-fluoroacetamide. TTR of α -KIC was determined using GC/MS with m/z of 216 and 219.

Skeletal muscle leucine TTR

Leucine TTR in skeletal muscle was determined as previously described (25). Briefly, ~20mg of muscle tissue was homogenized in 1 mL 3% trichloroacetic acid and centrifuged at 2800 rfc for 20 min. The resulting pellet was washed in 1 N saline four times before incubation in 6 N HCl at 100 C for 24h. Hydrolyzate was then dried under vacuum and derivatized in 1:1 acetonitrile:*N*-methyl-*N*-tert(butyldimethylsilyl)tri-fluoroacetamide. Muscle leucine TTR was determined using GC/MS with m/z of 202 and 203.

Skeletal Muscle mTOR signaling

We measured the protein content of the S6 ribosomal protein and phosphorylated S6 (Ser235/236), a downstream target of the mTOR, TORC1 complex, using immunoblot analysis as previously described (34). Briefly, 30 micrograms of protein were separated on SDS-PAGE and transferred to nitrocellulose membrane. These membranes were incubated with anti-S6 ribosomal protein (no. 2212, Cell Signaling Technology, Inc.) and

anti-phospho S6 ribosomal protein (no. 2211, Cell Signaling Technology, Inc.). The blots were washed and developed via chemiluminescence (GE Healthcare) and quantified using AlphaEaseFC (AlphaInnotech, San Leandro, CA).

Calculations

HOMA-IR

Homeostatic model assessment of insulin resistance was calculated by the equation $HOMA-IR = (\text{fasting plasma insulin} \times \text{fasting plasma glucose}) / k$, where k represents a constant 405 (38).

Resting Metabolic Rate

Resting metabolic rate was calculated using expired VCO_2 and VO_2 values and the equation of Weir (65).

Glycerol Ra and Leucine Ra

Glycerol Ra and Leucine Ra were calculated by the steady-state Steele equation (54). Rate of appearance is equal to the rate of infusion of tracer divided by the change in tracer to tracee ratio.

Skeletal muscle protein FSR

FSR was calculated as the rate of [2H_3]-leucine tracer incorporated into skeletal muscle protein using the plasma α -KIC enrichment as the precursor pool (68) and the following equation: $FSR (\% h^{-1}) = [(E_2 - E_1) \times 100] / [E_p \times (t_1 - t_0)]$, where E_2 and E_1 represent muscle [2H_3]-leucine enrichments after 6h and 1h, respectively, E_p represents the average plasma [2H_3]- α KIC enrichment, and $(t_1 - t_0)$ represents the time between muscle biopsies (5h).

Statistical Analysis

Two-way repeated measures ANOVA with one factor repeated was used to determine significant differences with respect to day and treatment group. Tukey's post hoc analysis was then used to determine significant differences with alpha level set to 0.05. All statistical analyses were performed using SigmaPlot software for Windows version 11.0. All data are presented as mean \pm standard error (SE).

Results

Plasma hormone and substrate concentrations

As reported in STUDY 2 overeating suppressed 24h plasma GH concentration by about 70% ($p < 0.05$). As designed, GHT prevented the reduction in systemic GH concentration while overeating (Figure 1A). Also as designed, the separate group of subjects who received the higher daily doses of GH throughout the overeating intervention ("High GHT") had markedly elevated 24h plasma GH concentrations (0.4 ± 0.1 ng/ml, 1.2 ± 0.1 ng/ml, and 4.1 ± 0.3 ng/ml at 2 weeks for Control, GHT, and High GHT, respectively; all $p < 0.05$).

As expected, overeating significantly elevated 24h average insulin concentration compared with Baseline ($p < 0.05$; Figure 1B), reflecting the greater total amount of insulin secreted in response to the larger and more frequent meals during overeating. Preventing the fall in GH during overeating resulted in an even greater elevation in 24h plasma insulin at 2 weeks in GHT compared with Control ($p < 0.05$; Figure 1B). In addition, fasting plasma insulin concentration was elevated above baseline levels after only four days of overeating ($p < 0.05$; Figure 2A), and preventing the fall in GH nearly doubled fasting plasma insulin concentrations in GHT compared with Control ($p < 0.05$; Figure 2A). Despite the marked elevation in fasting plasma insulin concentration in GHT vs. Control, fasting plasma glucose was identical between these two trials throughout the overeating period (Figure 2B), suggesting overeating induced a greater insulin resistance in GHT compared with Control. This is supported by the calculation of HOMA-IR,

which was more than 2-fold greater in GHT compared with Control (5.40 ± 0.70 vs. 2.59 ± 0.74 , respectively; $p < 0.05$).

Unlike fasting plasma glucose concentration, which was reasonably well maintained throughout the overeating period (Figure 2B), overeating suppressed fasting plasma NEFA concentration to low levels throughout the intervention in both groups (Figure 2C). Overeating did not significantly elevate fasting plasma triacylglyceride concentrations in Control, but preventing the suppression of GH with overeating augmented fasting lipemia (Figure 2D). Fasting plasma triacylglyceride concentrations during GHT were significantly greater than baseline levels by the fifth day of overeating, as well as greater than Control throughout much of the second week of overeating ($p < 0.05$; Figure 2D). Plasma IGF-1 concentration remained relatively stable throughout the 2-week overeating period in Control (151.6 ± 15.8 vs. 169.4 ± 15.4 , Baseline vs. 2 weeks, respectively; $p = 0.54$), but GHT markedly increased plasma IGF-1 concentration (139.1 ± 12.6 vs. 324.5 ± 28.2 , Baseline vs. 2 weeks, respectively $p < 0.05$). In subjects receiving High GHT, changes in plasma insulin, glucose, NEFA, triacylglyceride and IGF-1 in response to overeating were generally similar to GHT, with no significant differences between these groups (data not shown).

Body composition

Our 2-week overeating intervention increased body weight by approximately 5% and fat mass increased nearly 10% in both groups (both $p < 0.05$; Table 1). We observed a trend ($p = 0.1$) for a greater weight gain when we prevented the reduction in GH with overeating (in our GHT group) compared with a relatively low GH concentration in Control (Table 1). There was no difference in the amount of fat gained between the groups (Table 1), so the trend for a difference in weight gain between Control and GHT appeared to be due to a subtle, yet significant ($p < 0.05$) difference in gain of fat free mass (Table 1). Subjects receiving the High GHT during the 2-week overeating period increased their body weight by 3.1 ± 0.7 kg and increased fat mass by 1.1 ± 0.3 kg (both $p < 0.05$ vs. Baseline), which were similar to the gains in body weight and fat mass in Control and GHT. Also similar

to GHT, the High GHT group demonstrated a significant increase in fat free mass after 2 weeks (2.3 ± 0.6 kg, $p < 0.05$ vs. Baseline).

Resting metabolic rate (RMR)

RMR increased slightly, yet significantly after the 2 week overeating period in both Control and GHT (Figure 3). RMR was not different between Control and GHT. RMR also increased with overeating in our subjects receiving the High GHT, but after 2 weeks of overeating RMR was similar in Control, GHT, and High GHT.

Lipolytic rate (Glycerol Ra)

Fasting lipolysis was suppressed by the morning of the fourth day of overeating in both Control and GHT (Figure 4A; $p < 0.05$). However, by the end of the 2-week overeating period, the absolute rate of lipolysis ($\mu\text{mol}/\text{min}$) had returned to baseline levels in both groups (Figure 4A). Importantly, when expressed relative to fat mass, lipolytic rate was still suppressed by the end of the 2 weeks (Figure 4B). Therefore, the apparent “rebound” in the absolute lipolytic rate observed by the end of the 2-week intervention in Figure 4A was likely not a consequence of an intrinsic increase in the rate of lipolysis per unit fat mass, but rather due to a greater absolute lipolytic rate stemming simply from the greater fat mass accrued from overeating. There were no differences in glycerol Ra between Control and GHT. Similarly, lipolytic rate in High GHT did not differ from Control or GHT.

Protein metabolism

Whole body proteolytic rate (leucine Ra) in the overnight fasted state increased significantly with overeating in Control, (Figure 5; $p < 0.05$). Interestingly, preventing the reduction in systemic GH during overfeeding with GHT effectively prevented the increase in leucine Ra (Figure 5, $p < 0.05$ vs. Control at 2 weeks). We also found a trend for 2 weeks of overeating to increase the rate of muscle protein synthesis after an overnight fast in both Control and GHT (Figure 6A), but this increase did not quite reach statistical significance ($p = 0.09$). There was no difference in the rate muscle protein

synthesis between Control and GHT. We also found that overeating did not alter the phosphorylation of the ribosomal protein S6 in skeletal muscle obtained from either group (Figure 6B), which is a downstream target of mTOR complex 1, and a key component in the regulation of protein synthesis. Similar to our GHT group, High GHT also prevented the increase in proteolysis during overeating that we found in our Control subjects and did not significantly impact muscle protein FSR (data not shown).

Discussion

Our findings from STUDY 2 provided the first evidence demonstrating the profound reduction in GH secretion with overeating. Our objective here was to examine the metabolic consequences of this marked suppression in GH during overeating by preventing the overeating-induced reduction in plasma GH by administering exogenous GH throughout the overeating period in a manner to mimic normal endogenous GH secretion (in terms of both magnitude and pulsatility). Major findings from our study indicate that preventing the fall in plasma GH during overeating elevated fasting plasma insulin concentration, without altering plasma glucose concentration. Additionally, preventing the suppression in plasma GH concentration during overeating increased fasting plasma triacylglyceride concentration. Therefore, it appears that the suppression in GH secretion that normally occurs during overeating may help attenuate the insulin resistance and hyperlipidemia that typically occurs with overeating (40). We also found that overeating increased whole-body proteolytic rate in the overnight fasted state. Importantly, the suppression in plasma GH with overeating may underlie this increase in protein hydrolysis because preventing the reduction in plasma GH concentration during overeating largely blunted this increase in systemic proteolysis. In contrast, muscle protein synthesis and whole body lipolytic rate appear to be unaffected by the suppression in plasma GH during overeating. Together, the natural suppression in GH that occurs with overeating may help mitigate the rise of cardiovascular and metabolic risk factors.

Our finding that fasting plasma glucose concentration was identical in Control and GHT despite nearly a 2-fold greater fasting plasma insulin concentration in GHT, suggests

insulin resistance was greater in GHT compared with Control, which was supported by our calculation of HOMA-IR. Indeed, GH administration has been clearly linked with the development of insulin resistance (4, 29, 43), but our novel findings indicate that the natural fall in GH secretion that occurs with overeating helps protect against insulin resistance. The link between GH and systemic insulin resistance has been largely attributed to effects of GH on the insulin signaling pathway in liver and skeletal muscle. For example, GH-induced insulin resistance has been attributed to increased expression of suppressor of cytokine signaling-3 (SOCS-3) an protein that may lead to the degradation of insulin receptor substrate-1 (IRS-1) (43), which is a key component of the insulin signaling pathway. GH may also increase the abundance of the regulatory subunit of phosphoinositol-3 kinase (PI3K), p85 α , which can impede insulin signaling when available in excess (4, 5). Additionally it has been reported that p85 α may indirectly inhibit insulin signaling by potentiating the phosphorylation and subsequent activation of c-Jun N-terminal kinase (JNK), a key inflammatory factor that can suppress insulin signaling (62). Therefore, the natural suppression in GH during conditions of caloric excess may help protect against further development of insulin resistance by attenuating GH-induced signaling events in liver and skeletal muscle that normally disrupt insulin action.

Overeating, *per se*, did not significantly elevate fasting plasma triacylglyceride concentration in our Control group. Previous studies have reported elevated fasting lipemia with overeating, but typically these overfeeding protocols contained a very high proportion of dietary carbohydrate ($\geq 70\%$ carbohydrate) (28, 39, 41), which is known to accelerate hepatic *de novo* lipogenesis, and increase fasting plasma triacylglyceride concentrations (15, 39). The proportion of dietary carbohydrate to total energy intake in our study was 50%, which is relatively low compared with the high proportion of carbohydrate provided in these other studies (28, 39, 41). In contrast to our Control group, fasting plasma triacylglyceride concentration increased 2-fold above Baseline levels with overeating when we prevented the fall in GH (with GHT). Although GH administration has been shown to increase plasma triacylglyceride concentration (46, 70), our study expands on these findings by demonstrating the reduction in GH that normally

occurs with overeating attenuates hyperlipidemia, at least in the short-term. The accumulation of plasma triacylglycerides in the overnight fasted state is largely a balance between triacylglyceride production and the hydrolysis of the triacylglyceride within the circulation by lipoprotein lipase (LPL). In the overnight fasted state, triacylglycerides are almost exclusively packaged within hepatically-derived very low-density lipoproteins (VLDL) (37), and available evidence suggests that GH may either increase apo-B synthesis (11), or have a limited affect (32) on VLDL-triacylglyceride production. Conversely, GH has been reported to inhibit both adipose tissue and skeletal muscle LPL activity (46, 51, 60). Therefore, the effect of GH on plasma triacylglyceride accumulation may be primarily through its effect on inhibiting plasma triacylglyceride hydrolysis, with perhaps some contribution from an increase in VLDL production, thereby increasing fasting plasma triacylglyceride concentration. Because hyperlipidemia is an important risk factor for the development of cardiovascular disease, the normal reduction in plasma GH that occurs with overeating may act to help reduce disease risk.

Although prolonged overeating is often found to induce at least a modest increase in FFM together with the more robust increase in fat mass (12, 17, 63), the relatively small increase in FFM in our Control group did not reach statistical significance, likely because the short duration of our intervention. In contrast, preventing the fall in GH throughout our brief, 2-week overeating intervention did indeed induce a measurable increase in FFM. This finding is largely consistent with previous work reporting that exogenous GH administration for several weeks increased FFM in obese women (50), as well as in patients with growth hormone deficiency (56). However, the daily GH doses in these previous studies (Richelsen et al., 1994; Stenlof et al., 1995) were four to five-fold greater than our GHT, and they were provided as a single GH bolus in the evening (rather than as more physiologic pulses throughout the day as in our study). Therefore, the effect of GH on FFM may be largely independent of the magnitude of the GH dose. This notion is further supported by our finding that the increase in FFM was identical in our GHT and High GHT groups, despite a three to four-fold greater exposure to GH in the latter. We must acknowledge that GH treatment can increase water retention (42), which could contribute to the measured increase in FFM. However, GH is known to increase

FFM beyond its effects on hydration (14), yet the specific mechanisms underlying the effect of GH on FFM are not completely understood.

The accrual of FFM depends on the balance of proteolysis and protein synthesis. Our finding that GHT attenuated the proteolytic rate during overeating suggests that a GH-mediated reduction in the rate of protein breakdown may have contributed to the increase in FFM in this group. Putative mechanisms for anti-proteolytic effect of GH are not well described. GH may suppress proteolysis indirectly, through the actions of the elevated plasma insulin and IGF-1 concentrations (9, 21, 53) that we observed in the GHT group. It has been reported that elevated insulin exposure reduced the abundance of ubiquitinated protein and the rate of protein breakdown in rat skeletal muscle (9). In addition, IGF-1 has been found to suppress the rate of proteolysis and prevent the induction of the proteolytic factors Muscle Ring Finger 1 (MuRF1) and Muscle Atrophy F-box (MAFbx) in C2C12 myotubes (53).

Importantly, the increased FFM in our GHT groups was not a consequence of increased muscle protein synthesis. Despite the common misconception that GH administration induces a marked increase in muscle mass, many well-controlled studies have confirmed that GH does not augment skeletal muscle protein synthesis in humans (49, 67, 71). Although there is some evidence suggesting that plasma IGF-1, which is secreted by the liver in response to GH, may increase muscle protein synthesis in animal studies (35), we found no increase in skeletal muscle protein synthesis despite a 3-fold increase in plasma IGF-1 concentration in our subjects, which supports previous findings in human subjects (71). Therefore, in our study, a GH-mediated reduction in systemic proteolytic rate, rather than increased skeletal muscle protein synthesis, likely led to the observed increase in FFM when we prevented the suppression in GH with overeating. Alternatively, our data also implies that the normal suppression in GH with overeating may augment the rate of protein degradation, at least in the short-term.

Although GH has been characterized as a lipolytic hormone (13, 57), our findings suggest that the suppression in GH with overeating did not alter whole-body lipolytic rate. Because our overfed subjects were hyperinsulinemic, it is likely that the potent

antilipolytic action of insulin countered any lipolytic effects of GH (33). It is well established that lipolysis is greatly diminished after a meal, due largely to the very potent antilipolytic effects of insulin (7, 44). Along these lines, the suppression in lipolytic rate we found by the morning of the fourth day of overeating was accompanied by a 2-fold increase in fasting plasma insulin concentration. Interestingly, despite a persistent elevation in fasting insulin concentration by 2 weeks of overeating, lipolysis returned to basal levels. This “rebound” in lipolytic rate by 2 weeks of overeating may be due to the competing influences of hyperinsulinemia that suppresses lipolysis and the increased fat mass that augments whole-body lipolytic rate (27). In support of this hypothesis, when we expressed lipolytic rate relative to fat mass to effectively remove the influence of fat mass on lipolysis, lipolytic rate remained suppressed at 2 weeks of overeating, which may largely reflect the antilipolytic effect of the hyperinsulinemia. Other factors that may have contributed to the “rebound” in lipolytic rate we found during the second half of our overeating period include increased sympathetic nervous system activity and plasma leptin concentration, both of which have been found to increase with overeating (20, 45), and can increase lipolysis (64).

Although long-term GH treatment has been found to augment RMR (10, 58, 69), this may be due largely to the accompanying increase in FFM with prolonged GH treatment (1, 10, 69). In contrast, the effects of short term and/or acute GH treatment on RMR have been equivocal; some studies have reported an increase in RMR (3, 30), while others (55), including the present study did not find an effect of GH on RMR. It is possible that issues related to the accuracy and precision of indirect calorimetry to measure RMR (52) may limit the ability to detect relatively subtle changes in RMR. Although preventing the suppression in GH with overeating did not affect RMR compared with overeating without GHT in our study, overeating did indeed result in the expected (12, 17) increase RMR, with and without GHT. Similar to the effects of long-term GH treatment, the increased FFM found with overeating may be an important contributor to the elevated RMR. However, while FFM may have contributed to the increased RMR after overeating, the elevation in RMR persisted even when we accounted for the increase in FFM. Therefore, our findings suggest that overeating augmented energy-requiring metabolic processes

independently of the gain in metabolically active tissue, even in the basal state (i.e., overnight fasted). It is possible that the persistent basal hyperinsulinemia may have contributed to the increase in RMR in our participants. For example, insulin can increase Na^+/K^+ ATPase activity (59), and in fact, overfeeding has been found to increase the activity of this enzyme in the liver and skeletal muscle of mice (16). Energy expenditure associated with the active transport of ions across cell membranes is known to be a major contributor to RMR (22, 23, 36). Additionally, the process of *de novo* lipogenesis, which is stimulated by elevated insulin concentration and dietary carbohydrates (24), may result in increased energy expenditure with the synthesis of lipids during the overfeeding period, although this contribution to energy expenditure is likely small (26). It is also possible that changes in sympathetic nervous system activity (20, 45) and other neuroendocrine responses to overeating (19) may also contribute to the increase in RMR that is independent of the increased FFM. Although perhaps not a major contributor to whole body energy expenditure, the trend for an increase in protein synthesis we found with overeating may also slightly increase RMR.

In summary, the suppression of plasma GH concentration that naturally occurs with overeating may influence insulin action, plasma triacylglyceride concentration, and whole-body proteolytic rate. The fall in GH concentration that occurs with overeating may act to mitigate the development of insulin resistance and hyperlipidemia that can arise with overeating. Alternatively, the suppression in GH may suppress the accrual of fat free mass that can occur with overeating, by accelerating proteolytic rate. Additionally, the GH fall during overeating did not influence lipolysis, reflecting the limited role of GH in mediating lipolytic rate during overeating, and suggesting that the suppression of lipolysis may be tied to the elevated fasting insulin concentration. The increase in RMR found with overeating was not dependent on plasma GH concentration, and may at least in part reflect physiological effects of chronic hyperinsulinemia. Overall, the key findings from our study indicate the suppression of GH secretion that normally occurs with overeating, may help protect against the development of metabolic and cardiovascular disease risk factors.

Acknowledgements

We would like to acknowledge the expert technical assistance from Dr. Alexander Hinko and Kathy Symons. We would also like to acknowledge the efforts of our research dietician and study coordinator, Lisa Michael. This work was supported by National Institutes of Health (NIH) Grant R01DK71955, with additional support from the Michigan Clinical Research Unit (NIH-UL1RR024986), the Michigan Nutritional Obesity Research Center (P30-DK- 089503), and the Michigan Metabolomic Obesity Center.

Table 5-1. Changes in bodyweight, fat mass, and fat free mass during the 2 week overeating period in Control and GHT.

	Control			GHT		
	Baseline	2 weeks	Δ	Baseline	2 weeks	Δ
Body weight (kg)	75.0±2.6	77.1±2.7*	2.4±0.6	75.3±3.3	79.1±3.8*	3.6±0.6‡
Fat Mass (kg)	19.8±2.4	21.2±2.0*	1.6±0.3	16.8±2.4	19.2±2.6*	1.7±0.2
Fat Free Mass (kg)	55.2±2.7	55.9±2.8	0.7±0.3	58.5±3.0	59.9±3.5*	2.2±0.3†

* Significantly different from Baseline, $p < 0.05$. † Significantly different from Control, $p < 0.05$. ‡ Trend for a greater increase versus Control, $p = 0.10$.

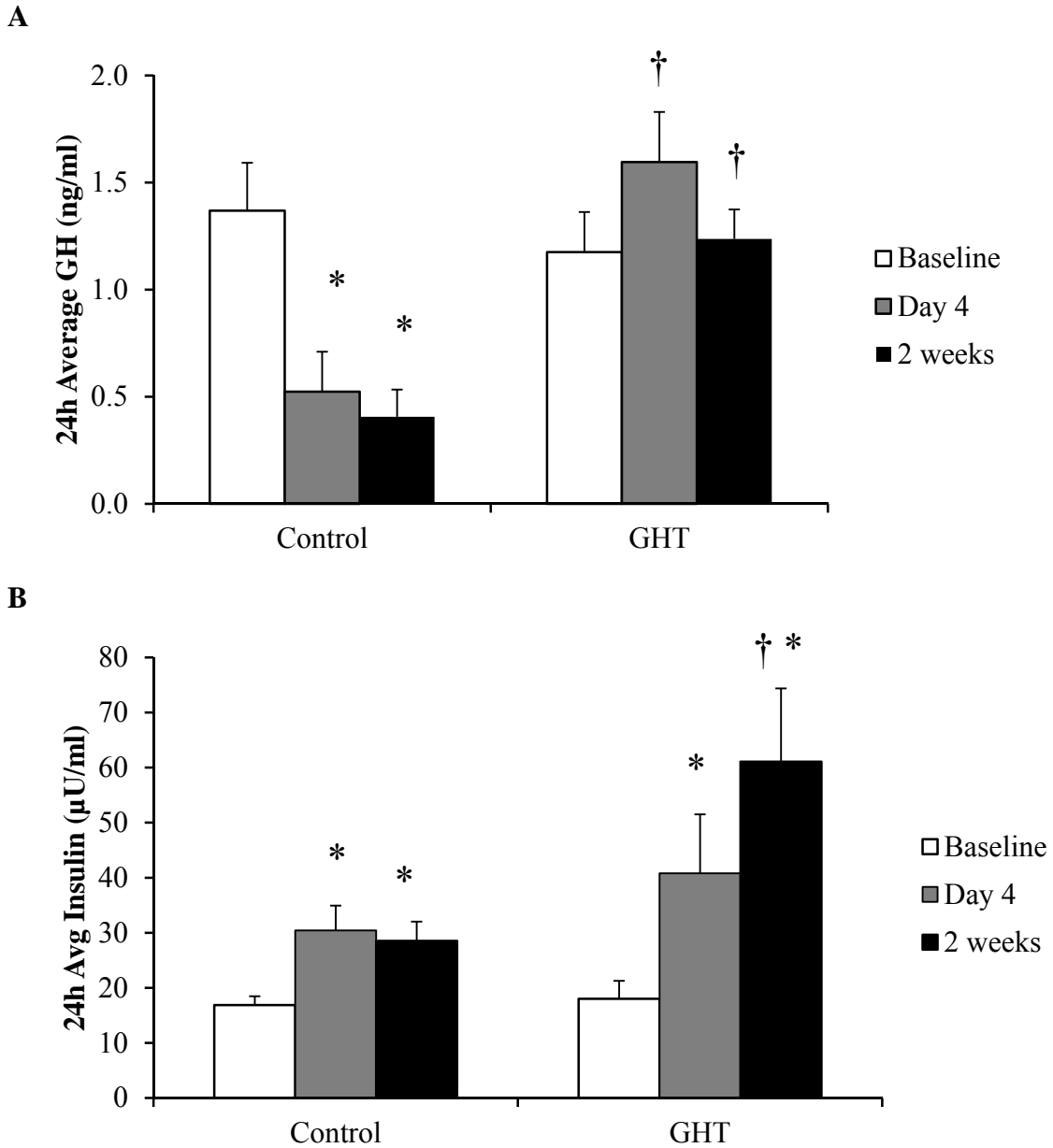
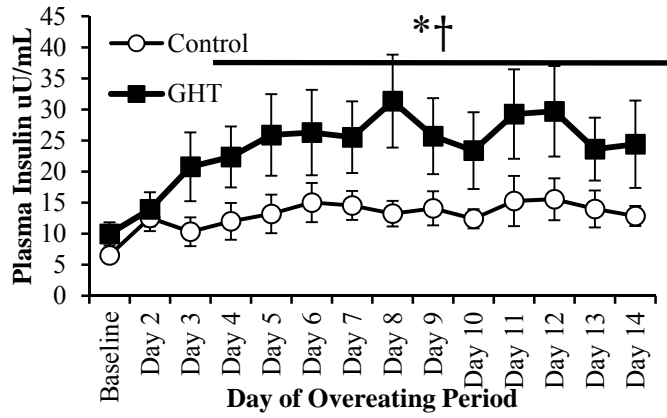
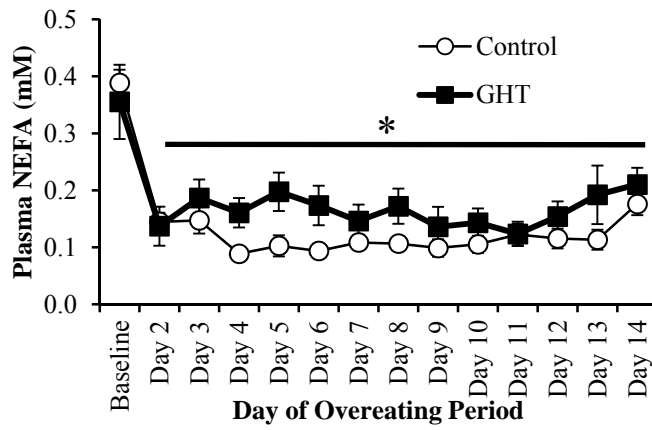


Figure 5-1. (A) 24h average plasma growth hormone concentration measured every 20 min for 24h and (B) 24h average plasma insulin concentration measured every 2h for 24h before overeating, by day 4 of overeating and by the end of the two-week overeating period in subjects who did not receive any growth hormone treatment (Control) and subjects who received low doses of growth hormone daily (GHT). *Significantly different from Baseline within Treatment, $p < 0.05$. † Significantly different from Control within Day, $p < 0.05$.

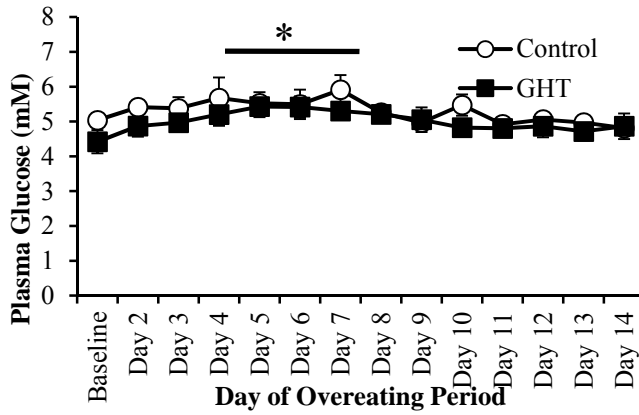
A



B



C



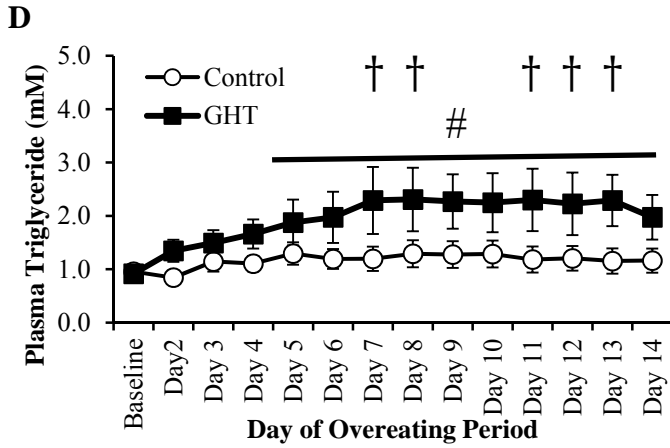


Figure 5-2. Fasting plasma concentrations of: (A) insulin, (B) glucose, (C) non-esterified fatty acids (NEFA), and (D) triacylglyceride, measured each morning after an overnight fast throughout the 2 week overeating intervention. *Significantly different from Baseline, $p < 0.05$. † GHT significantly different from Control within Day, $p < 0.05$. #Significantly different from Baseline within the GHT group, $p < 0.05$.

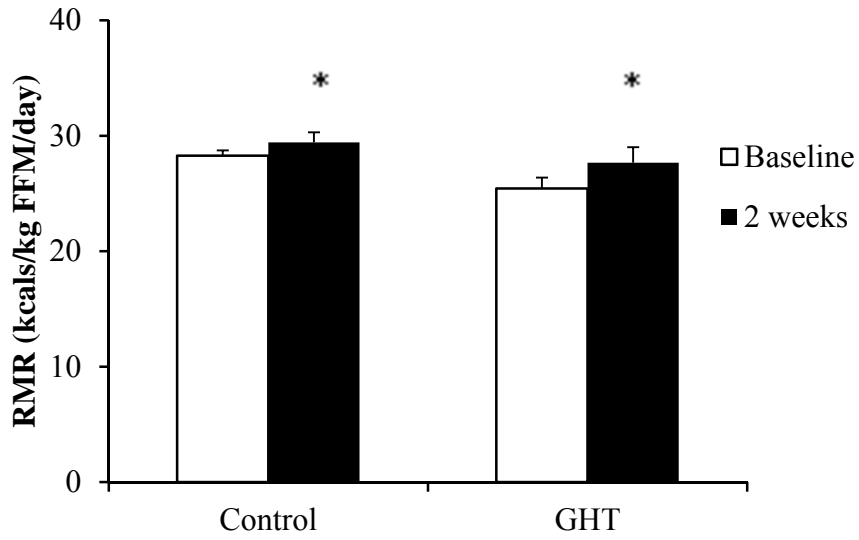
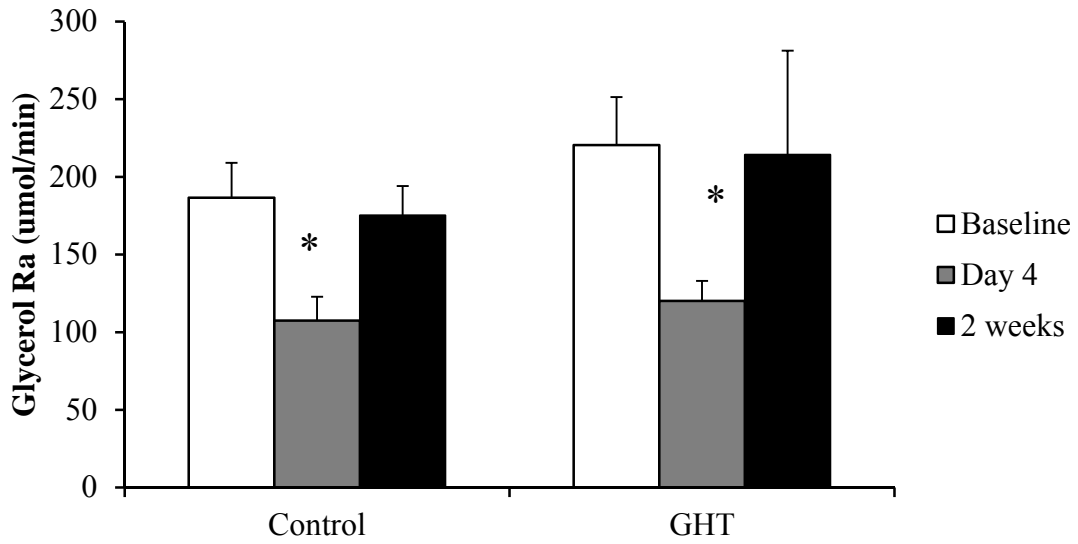


Figure 5-3. Resting metabolic rate (RMR) normalized to fat free mass (FFM) at Baseline and after 2 weeks of overeating in Control and GHT. *Significantly different from Baseline, $p < 0.05$.

A



B

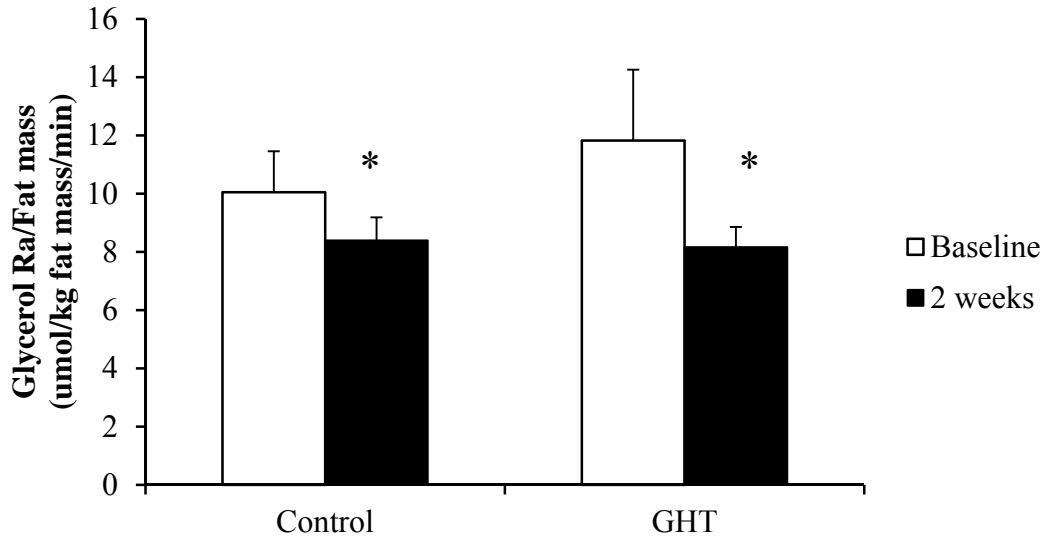


Figure 5-4. Lipolytic Rate (glycerol Ra) measured after an overnight fast before and after the 2 week overeating intervention (A) expressed in absolute terms ($\mu\text{mol}/\text{min}$) and (B) expressed relative to fat mass ($\mu\text{mol}/\text{kg}$ fat mass/ min) in both Control and GHT. *Significantly different from Baseline, $p < 0.05$. † Significantly different from Control at 2 weeks, $p < 0.05$. ‡ Trend for a difference between Control and GHT at 2 weeks, $p = 0.07$.

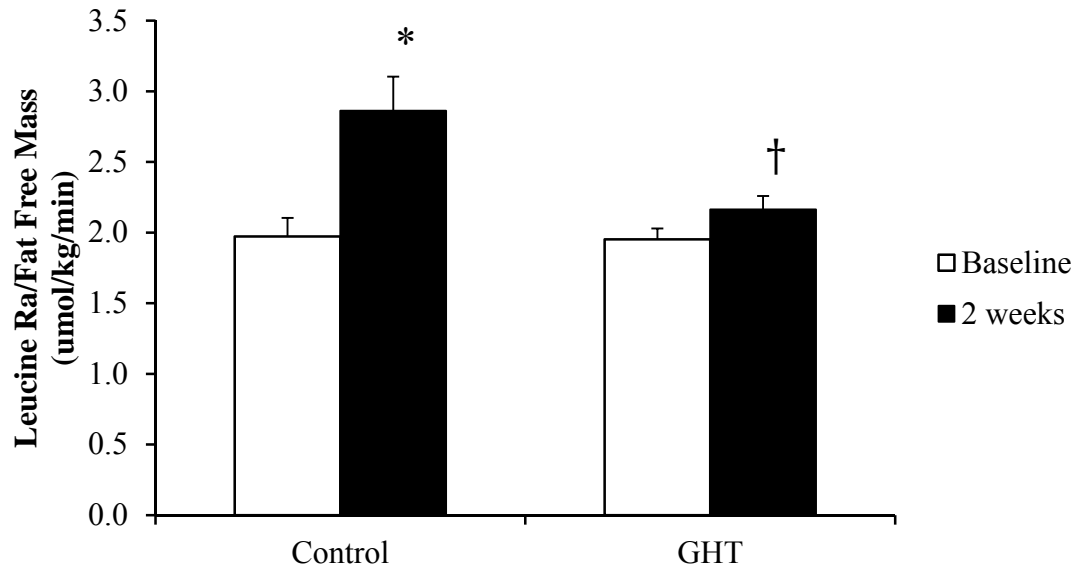
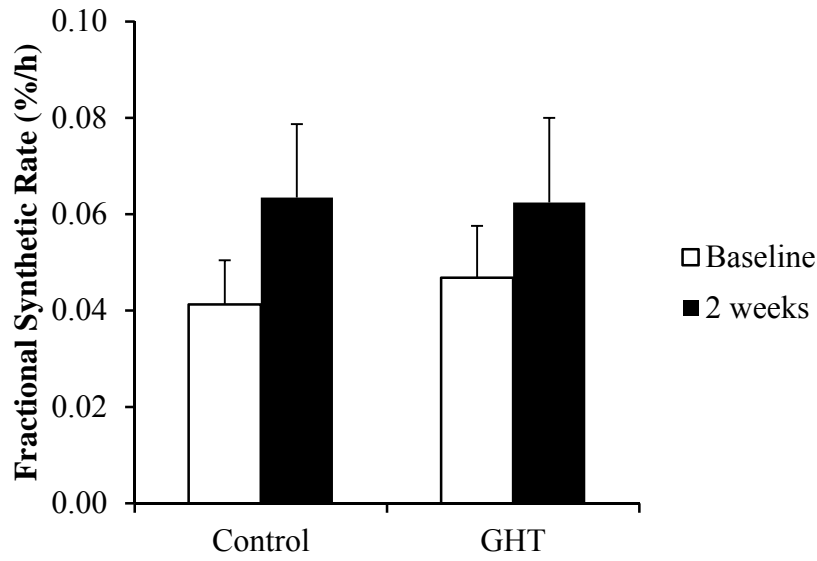


Figure 5-5. Proteolytic rate (leucine Ra) measured after an overnight fast before and after the 2 week overeating intervention in both Control and GHT. *Significantly different from Baseline within Treatment. † Significantly different from Control at 2 weeks, $p < 0.05$.

A



B

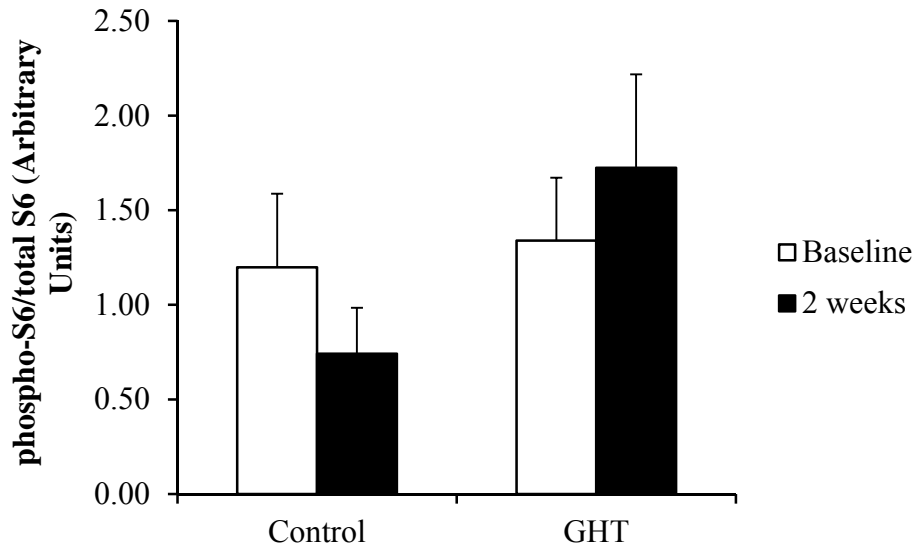


Figure 5-6. (A) Muscle protein fractional synthetic rate (FSR) of skeletal muscle protein and (B) abundance of phosphorylated S6 protein (expressed relative to total S6 protein content) in vastus lateralis muscle obtained after an overnight fast before and after the 2 week overeating intervention in both Control and GHT.

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CHAPTER 6

OVERALL DISCUSSION

In general, many of the metabolic health complications found in obesity are well defined, however, the metabolic responses during the early stages of weight gain are far less certain. Clearly, weight gain requires prolonged and/or recurring periods of “overeating” (defined as a greater energy intake relative to energy expenditure) and therefore, over time, overeating can lead to obesity and the disease risks that accompany it. By improving our understanding of the metabolic changes that occur in response to overeating, we can better understand the development of obesity-related metabolic complications, even before meaningful weight gain. The overall objective of my dissertation was to characterize the metabolic and endocrine responses during 2 weeks of overeating. In this overall discussion of my dissertation, I will attempt to expand on my findings, providing an integrative discussion of my different studies and explore interesting secondary findings. To this end, I have provided a schematic diagram that attempts to integrate the major findings from my projects regarding the metabolic and endocrine responses to overeating that I found (Fig 6-1).

Brief summary

As summarized in Figure 6-1, some of the major metabolic responses we found in response to 2 weeks of overeating included: the development of whole-body insulin resistance [STUDY 1], augmented 24h plasma triglyceride concentration [STUDY 1], accelerated proteolytic rate [STUDY 3], and a suppression in plasma fatty acid concentration [STUDY 1]. Although overeating increased whole body insulin resistance, this was not accompanied by changes in markers of impaired insulin action in skeletal muscle [STUDY 1], suggesting that tissues other than skeletal muscle (e.g., liver) may become insulin resistant in response to overeating in advance of skeletal muscle. Furthermore, skeletal muscle lipid accumulation did not increase despite consuming

twice the normal amount of dietary fat [STUDY 1]. This may be explained by a combination of a preferential partitioning of the fatty acids within plasma triglycerides to be stored within adipose tissue (rather than muscle), together with the chronic suppression of plasma fatty acid concentration, which greatly limited potential substrate for muscle lipid synthesis [STUDY 1]. As expected, overeating led to a chronic elevation in plasma insulin concentration (Figure 6-1), and the potent anti-lipolytic effect of insulin was likely responsible for the marked suppression in endogenous lipolysis [STUDY 3], which caused the chronically low plasma fatty acid concentration (Figure 6-1). One of the key findings of this project was the profound suppression in plasma growth hormone secretion after only a few days of overeating (Figure 6-1), which occurred well before any measureable weight gain [STUDY 2]. My findings suggest some key putative regulators of GH secretion (e.g., fatty acids, insulin-like growth factor-1 [IGF-1], and cortisol) were likely not involved in overeating-induced GH suppression [STUDY 2]. Instead, findings from STUDY 2 of my dissertation suggest that the chronic hyperinsulinemia resulting from overeating may have been the key factor in the rapid onset of GH suppression with overeating (depicted by the bold arrow in Figure 6-1). In turn, findings from STUDY 3 of my dissertation suggest the suppression in GH with overeating had some important metabolic consequences. For example, the suppression in GH with overeating appeared to underlie the overeating-induced elevation in proteolytic rate because the elevation in proteolysis was largely absent when we prevented the fall in GH during overeating in STUDY 3. Perhaps most importantly, the suppression in GH with overeating may mitigate risk factors for metabolic and cardiovascular diseases by attenuating the development of insulin resistance and preventing fasting hyperlipidemia [STUDY 3] (Figure 6-1).

We anticipated overeating would chronically elevate 24h plasma insulin concentration (in light of the large and frequent meals provided), which was a major contributor to many of the metabolic responses we observed. As noted above, the hyperinsulinemia likely caused the marked reduction in endogenous lipolysis and the chronic suppression of plasma fatty acid concentration. The reduced fatty acid availability limited the accumulation of muscle lipid, which may have prevented the induction of fatty acid-induced insulin resistance

and inflammatory factors within skeletal muscle [STUDY 1]. Therefore, the insulin-induced affect on fatty acid availability may have helped preserve skeletal muscle insulin sensitivity during overeating.

While overeating suppressed the mobilization of endogenous lipids, it augmented the mobilization of amino acids [STUDY 3]. Despite elevated insulin concentrations that are known to inhibit proteolysis, overeating actually increased the proteolytic rate [STUDY 3]. Previous work suggests that elevated fatty acid mobilization can suppress proteolysis (3), therefore, it may be plausible that the suppression of lipolysis and the reduction in fatty acid mobilization that we found during overeating stimulated proteolysis. This has been described as the “protein-sparing” effect of fat oxidation on proteolysis. Although we were unable to measure fat oxidation via indirect calorimetry in the present study due to the effect of *de novo* lipogenesis on carbon dioxide production, we indeed found a reduction in lipolytic rate per unit fat mass that coincided with an increase in proteolysis [STUDY 3]. Additionally, in cases where GH secretion is blocked and lipolysis is suppressed through the administration of somatostatin, protein degradation increased by ~40% (5). However, it remains uncertain if this mechanism is at work in the present study. When we prevented the fall in GH, the overeating-induced increase in proteolysis was attenuated, however, there was no effect on lipolysis. Preventing the fall in GH did increase insulin concentrations and fasting IGF-1 concentration, which could mediate the suppression of proteolysis in our GHT group.

The suppression of GH after only a few days of overeating was a unique finding of this study. We found that the suppression in GH mitigated the rise in insulin resistance and also curtailed the increase in hyperlipidemia with overeating (STUDY 3). Overall, the key findings from our study indicate the suppression of GH secretion that normally occurs with overeating may help protect against the development of metabolic and cardiovascular disease risk factors. Recent studies have reported that GH-deficient individuals suffer increased risk of cardiovascular disease (1) and mortality (2) due to GH treatment. These studies, along with the work from this dissertation, counter the prevailing theories regarding the GH deficiency in obesity as a problem. Treatment of

obesity with GH would likely worsen the metabolic profile and contribute to cardiovascular disease risk. Therefore, the use of GH as a treatment for metabolic diseases and obesity is counter-indicated, as reduced GH may assist in the preservation of metabolic health and reduce the rise in risk factors for cardio-metabolic diseases.

Predicting the magnitude of weight gain

One of the original hypotheses for this overall project was that individuals with high endogenous GH secretion may be somewhat protected against weight gain due to an elevated 24h energy expenditure [perhaps resulting from GH-mediated activation of some energy-requiring processes (e.g., triglyceride recycling, whole body protein turnover)]. This hypothesis was developed from preliminary findings in a small cohort of non-obese adults with high-normal 24h GH concentration (i.e., ~4ng/ml) who lost weight when placed on a diet calculated to maintain their body weight based on their fat free mass. However, enthusiasm for our hypothesis that high GH concentrations may help protect against weight gain was tempered when we discovered in STUDY 2 that GH concentration fell so profoundly after only a few days of overeating. Nonetheless, using data collected during STUDY 3 of my dissertation where we prevented the fall in plasma GH concentration during overeating (GHT groups) we were able to explore our original hypothesis regarding GH and energy metabolism during overeating a bit further (*Please note: data and discussion regarding our original hypothesis was not included in any of the three studies of my dissertation [Chapters 3-5] – and only described for the first time here in the Overall Discussion*). Interestingly, we did find a significant correlation of the change in GH with overeating to the change in RMR (normalized to FFM) with overeating (Figure 6-1, $p < 0.05$). Notably, this correlation was driven mainly by the individuals in the High GHT group ($n = 5$), who had the greatest increase in GH concentration. If all GHT subjects were removed and the regression analysis repeated, there was a trend for an association between the change in GH and the change in RMR in the Control group alone (Figure 6-2, $p = 0.12$), and when all subjects were included both before and after overeating we did find that 24h GH concentration was positively correlated with RMR (Figure 6-3, $p < 0.05$). In terms of possible underlying mechanisms to help explain the relationship between GH and RMR, we did not find significant

correlations between GH and any of the energy-requiring metabolic processes measured in my studies (i.e., lipolysis, protein synthesis, and proteolysis). Furthermore, our findings regarding the association between GH and RMR also did not appear to translate to protection against weight gain in our study, because we found that our GHT groups gained the same amount of body fat as Control, and even tended to gain slightly more bodyweight in comparison to Control. It is important to note that the habitual 24h GH concentrations of the subjects in our study (i.e., before overeating) were considerably lower (<2ng/ml) than the few subjects from our preliminary study (~4ng/ml) who seemed to demonstrate high daily energy expenditure relative to their fat free mass. Therefore, we believe that the relationship between GH and energy metabolism, in the context of weight management is still worthy of further investigation.

The regulation of energy expenditure and the resistance to weight gain during overeating provided an interesting perspective to our study. Using the very crude estimation that approximately 7000 kcals of surplus energy will result in a 1 kg increase in body fat mass, we calculated that the expected weight gain in our participants would be approximately 3 kg. This was surprisingly close to the actual weight gain in our participants, which was about 2.5 kg on average. It is of note that our calculation of weight gain for most of our participants overestimated their actual weight gain. This could certainly be a consequence of the inaccuracies associated with the estimate that there are 7000kcal/kg fat mass, but this might also be explained by the trend for an increase in RMR that we found with overeating (28.2 ± 0.5 kcals/kg FFM/day vs. 29.4 ± 0.9 kcals/kg FFM/day, $p = 0.09$). Minor changes in energy expenditure may have a meaningful effect on bodyweight and fat mass over a long period of time, but we acknowledge that we did not find a robust increase in RMR to explain all of the difference between the expected weight gain and the actual weight gain. Although non-exercise activity thermogenesis (NEAT) can prevent weight gain in some people during overeating (4), we attempted to control NEAT by limiting subjects to 1500 steps per day, however, this would not prohibit fidgeting, standing, or other energy-requiring non-stepping tasks. We did not observe the variability in weight gain that has been reported in previous overfeeding studies, which may be related to the manner in which physical

activity was controlled throughout our study. Furthermore, weight gain can also be influenced by changes in water retention and salt balance, which could also explain the some of discrepancy in expected weight gain and actual weight gain.

Future Directions

Findings from this study suggest that the insulin-induced regulation of lipolysis may remain sensitive to insulin even after the emergence of insulin resistance in other tissues, yet we know relatively little about how normal adipose tissue adapts to overeating. In obese adults, certain fat depots are more “lipolytically active,” meaning that they contribute more to the availability of fatty acids in the circulation, leading us to speculate as to how to channel the storage of fatty acids into certain depots in a manner that would limit fatty acid availability and be more beneficial to insulin sensitivity. It is not well known how the deposition of fatty acids is regulated among the different fat depots of the body. The study of fatty acid storage may be conducted in a manner similar to studying protein synthesis, where the incorporation of an isotopically-labeled fatty acid could be used to study the rates of triglyceride synthesis in different adipose tissue depots. Additionally, single biopsies of several different fat depots before and after overeating may reveal different responses to overeating in different depots. These experiments would be observational, but could reveal mechanisms that define the characteristics of different adipose tissue depots.

One limitation of our study was that our subjects were relatively inactive throughout the overeating period, which may confound some of the effects that we observed in this study, however, our subjects were recruited from a pool of relatively sedentary people, therefore it is unlikely that continued inactivity contributed substantially to the changes that we found here. Exercise is known to be beneficial to metabolic health, mitigating the risk of diabetes, heart disease, and cardiovascular disease. A single session of exercise can increase insulin sensitivity due to the depletion of glycogen during exercise, which stimulates glucose uptake and glycogen re-synthesis. Usually, this increased insulin sensitivity due to glycogen depletion is attenuated after glycogen is replenished, therefore exercise during overeating would likely limit the exercise-induced insulin sensitivity that

is driven by glycogen depletion in skeletal muscle. Exercise can increase insulin sensitivity even in obese individuals, which has been attributed, at least in part, to the effect of exercise to partition fatty acids toward triglyceride synthesis, thereby mitigating fatty acid-induced insulin resistance. We found in STUDY 1 that skeletal muscle lipid metabolism and the abundance of fatty acid intermediates is unchanged with short-term overeating, therefore the exercise-induced mechanism of insulin sensitivity under these conditions is unclear. Exercise also improves insulin sensitivity in the liver, which may better abate the decrease in hepatic insulin sensitivity with overeating. It is therefore possible that a single session of exercise after 2 weeks of overeating would improve whole-body insulin sensitivity, not through an increase in skeletal muscle insulin sensitivity, but through an increase in hepatic insulin sensitivity. Future studies of overeating and the effect of exercise may address this hypothesis.

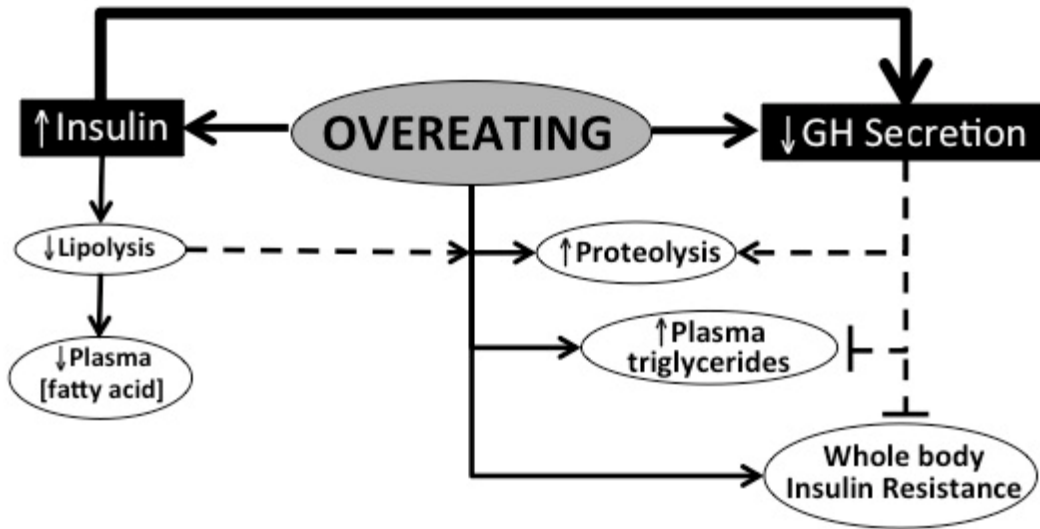


Figure 6-1. Schematic diagram of the endocrine and metabolic interactions that occur with overeating.

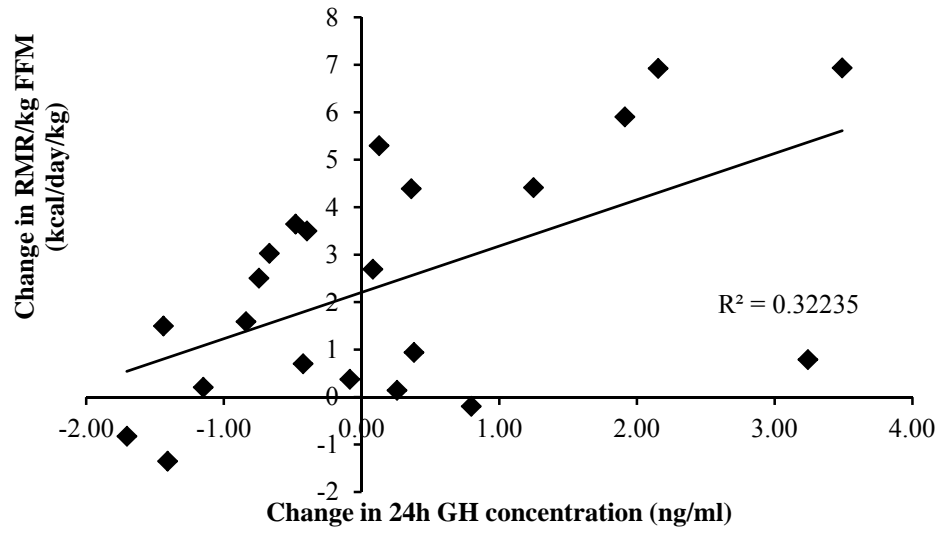


Figure 6-2. Regression analysis of the change in 24h GH concentration vs. the change in RMR, (n=23, p<0.05).

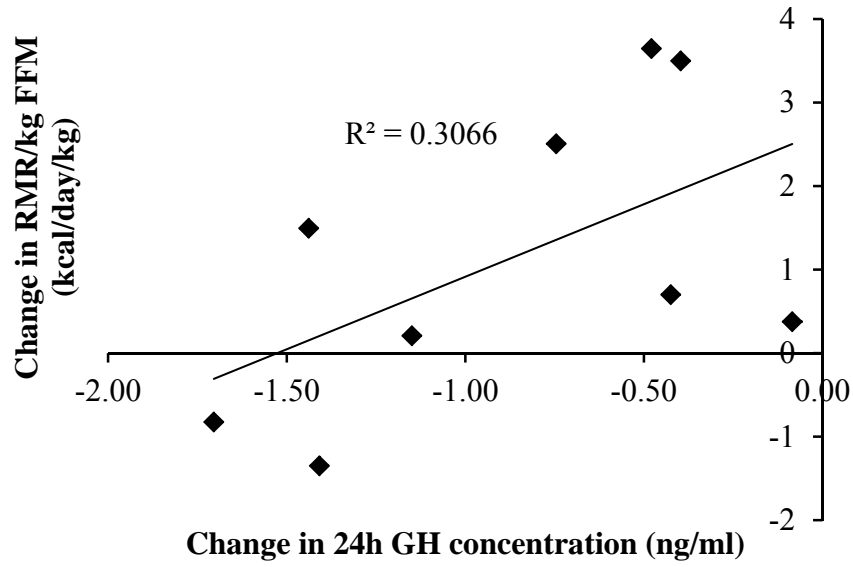


Figure 6-3. Regression analysis of the change in 24h GH concentration vs. the change in RMR, Control subjects only (n=9, p=0.21).

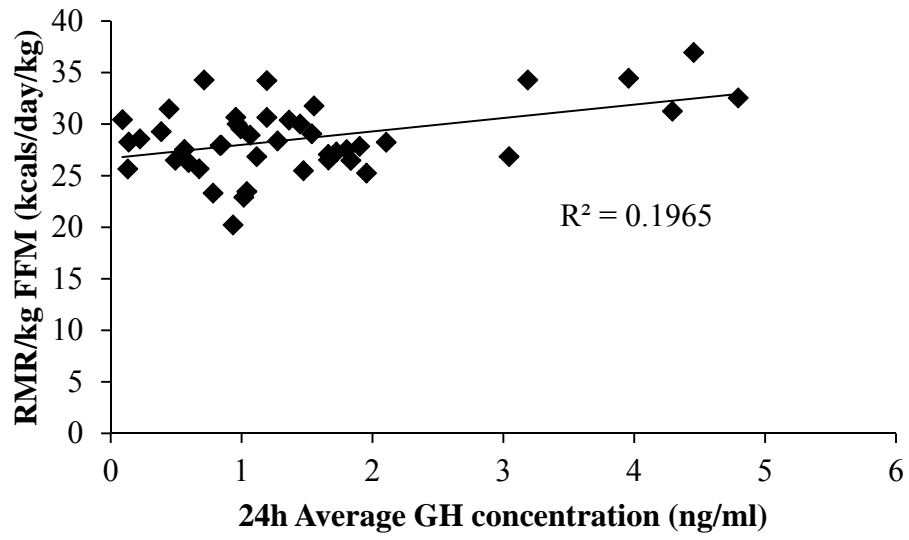


Figure 6-4. Correlation between 24h Average GH concentration and RMR normalized to FFM. (n=23, before and after treatment, $p < 0.05$).

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