The Effect of Ghrelin Isoforms on Skeletal Muscle Fatty Acid Metabolism

by

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Ghrelin is classically known as a central appetite-stimulating hormone, but has recently been recognized to have a significant role in peripheral tissue energy metabolism. Skeletal muscle is a major site for glucose and lipid disposal. However, the direct effects of ghrelin on this tissue remain understudied. We found that the two major ghrelin isoforms, acylated and unacylated ghrelin, were able to significantly increase skeletal muscle fatty acid oxidation while incorporation of fatty acids into major lipid pools remained unchanged. The increase in fatty acid oxidation was not accompanied by increases in AMP-activated protein kinase or acetyl-CoA carboxylase phosphorylation. Ghrelin isoforms significantly blunted epinephrine-stimulated lipolysis, but had no effect on lipolysis alone. This blunting effect did not appear to be due to decreased HSL phosphorylation. Taken together, these findings suggest that ghrelin isoforms have a direct, acute effect on fatty acid oxidation and lipolysis.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$^{14}$C palmitate</td>
<td>Radiolabelled palmitic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acetyl-CoA synthetase</td>
</tr>
<tr>
<td>AG</td>
<td>Acylated ghrelin</td>
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<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ATGL</td>
<td>Adipose triglyceride lipase</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>$\beta_1,\beta_2,\beta_3$</td>
<td>$\beta$1-3 adrenergic receptor</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>Epi</td>
<td>Epinephrine</td>
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<td>FA</td>
<td>Fatty acids</td>
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<td>GH</td>
<td>Growth hormone</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>GHRH</td>
<td>Growth hormone receptor hormone</td>
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<td>GHRP</td>
<td>Growth hormone releasing peptide</td>
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<td>GHS</td>
<td>Growth hormone secretagogue</td>
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<tr>
<td>GHSR-1</td>
<td>Growth hormone secretagogue</td>
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<tr>
<td>GLUT-1, GLUT-4</td>
<td>Glucose transport proteins</td>
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<tr>
<td>GOAT</td>
<td>Ghrelin-O-acyl transferase</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>ICV</td>
<td>Intracerebroventricular</td>
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<tr>
<td>IMTG</td>
<td>Intramuscular triacylglycerol</td>
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<tr>
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<td>Long chain fatty acids</td>
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<tr>
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<tr>
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<td>Neuropeptide Y</td>
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<tr>
<td>PKA, PKB</td>
<td>Protein kinases A and B</td>
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<td>Triacylglycerol</td>
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<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
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<tr>
<td>UnAG</td>
<td>Unacylated ghrelin</td>
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Chapter 1: Literature Review
Ghrelin effects on substrate regulation; focus on peripheral metabolism

1.0 Fuel homeostasis

Evolutionarily, starvation has been a critical threat to many species. In humans, there are various systems in place that regulate caloric supply in times of energy demand. In recent years, there has been an increase in nutrient availability and decrease in routine physical activity. The increase in caloric intake and decrease in energy expenditure has created an energy imbalance, lending towards the development of obesity. This has caused many researchers to shift their focus towards factors that may influence energy intake and substrate utilization.

Ghrelin is a peptide hormone commonly recognized for its orexigenic effects[1] and ability to stimulate growth hormone (GH) release[2]. It has also been suggested to play a role in the regulation of substrate (fat, carbohydrate) utilization[3]. Circulating ghrelin levels have been shown to increase in individuals with Prader-Willi syndrome[4] and anorexia nervosa[5], and decrease with obesity[6], suggesting a potential role for ghrelin in various nutritional states. Ghrelin has been shown to alter peripheral glucose and fatty acid (FA) metabolism[7], though the precise mechanism through which these alterations occur remains undefined.

2.0 Ghrelin background

2.1 Ghrelin discovery

Ghrelin is a 28-amino acid peptide hormone, differing in only 2 amino acids from humans to rodents[8]. Ghrelin was originally discovered around 1980 when Bowers, Momany, and colleagues generated a group of synthetic opioid peptide derivatives which they found could promote the release of growth hormone (GH) from the anterior pituitary[9]. Growth hormone,
also known as somatotropin, is a hormone responsible for many functions, including but not limited to, the stimulation of growth[10] in many tissues of the body. The molecules that stimulated the release of GH were later termed growth hormone releasing peptides (GHRPs). These GHRPs acted independently of growth hormone receptor hormone (GHRH), a class G protein coupled receptor found predominantly on pituitary somatotrophs[11], and somatostatin, a classical endocrine hormone[12]. In the following years, the growth hormone secretagogue receptor (GHSR-1) gene was identified in humans, and soon after came the discovery of the endogenous ligand agonist, ghrelin (“ghre” from the Proto-Indo-European root word meaning to grow)[13].

Ghrelin producing cells are distinct endocrine cell types found in the submucosal layer of the stomach, known as X/A like cells[14]. These X/A like cells contain round compact electron dense granules filled with ghrelin[15]. Ghrelin immunoreactive cells are also found in the small and large intestines, and in a limited amount in the region of hypothalamic arcuate nucleus[15][16]. The gastric X/A like cells are the major source of circulating ghrelin, both acylated ghrelin (AG) and unacylated ghrelin (UnAG) isoforms[17].

2.2 Ghrelin isoforms

Ghrelin is unique as it is the only known peptide that contains an eight-carbon FA (octanoate) modification. This modification allows it to form into an acyl ester[13]. The addition of the FA side chain, commonly at C8 or C10, allows the activation of ghrelin’s central receptor, GHSR-1a[14]. The production of AG is facilitated by the ghrelin-O-acyl transferase (GOAT) enzyme[18]. Although it remains to be fully established, it is likely that acylated ghrelin becomes unacylated enzymatically[19]. Acylated ghrelin has long been considered as the more bioactive
of the two isoforms; however, recent studies suggest an important role for UnAG in energy metabolism [20].

The ratio of UnAG to AG has also received considerable attention for having biologically relevant implications. Most researchers agree that the average ratio of UnAG:AG is approximately 2 to 2.5:1 [21]. UnAG is more abundant in the stomach [21][16] and in circulation [16]. Normal circulating levels of ghrelin in humans are 10-20 fmol/ml for AG, and 150-180 fmol/ml for total ghrelin [22]. Unlike digestive enzymes that are secreted into the gastrointestinal tract, ghrelin is secreted into blood vessels, which allows it to circulate throughout the entire body [23]. This fact alone suggests that ghrelin may play an important role in peripheral metabolism.

UnAG is cleared much slower from the circulation than its acylated counterpart [19]. This corresponds to the longer half-life of UnAG and its higher circulating concentrations [19]. The shorter half-life of the acylated form can be attributed to its conversion to the unacylated form and/or its binding to the GHSR-1a. Acylated ghrelin becomes unacylated in the circulation [19], likely enzymatically. Although various enzymes have been proposed, acyl protein thioesterase 1 (APT1) has received considerable attention for its potential role in unacylating the AG isoform [24]. Researchers have suggested that because APT1 lacks a secretion signal, it is likely that in vivo cells that express APT1 (liver cells, circulating platelets) secrete it into circulation [24]. Furthermore, it is probable that serum activity of APT1 may play an important role in regulating UnAG concentrations.
Acylated ghrelin is the only isoform that can bind to the ghrelin receptor (GHSR-1a), and has been suggested to be responsible for ghrelin’s orexigenic and GH releasing effects[25]. In the initial stages of ghrelin’s discovery, UnAG was considered to be either a pre-form of acyl modified ghrelin or the product of its deacylation[22]. However, more recent studies have shown that UnAG does have biological activities[20]. Unacylated ghrelin may modulate food intake[26] and various cellular responses including proliferation[27], and inflammation[28]. In obese mice[29] and humans[30], UnAG concentrations are often lower than in normal weight subjects, although AG concentrations are similar. Unacylated ghrelin has also been suggested to act as an antagonist to its acylated counterpart[31]. One study examined the difference between AG and UnAG by intravenously administering these isoforms either independently or combined [32]. When UnAG was administered alone it did not induce any changes in insulin or glucose levels; however, AG significantly decreased insulin and increased plasma glucose[32]. When co-administered, these effects were no longer present[32], suggesting that UnAG may oppose the metabolic effects of AG. In humans, UnAG co-administered with AG results in a ~60% suppression of food intake compared to the AG group alone, with no suppression of food intake by UnAG alone[33].

2.3 Ghrelin kinetics

Numerous studies have demonstrated that AG levels rise prior to entrained meal times and decrease almost immediately, within 30 to 60 min, after the ingestion of food[34][35]. The magnitude of postprandial drop in ghrelin is dependent on the macronutrient composition of the meal. Meals rich in carbohydrates have a greater suppression on circulating ghrelin than do high
fat meals[36]. High carbohydrate drinks decrease ghrelin to the greatest extent initially, but are followed by a subsequent rebound above pre-prandial levels[37].

During periods of fasting, alterations in the diurnal ghrelin patterns occur. Although some studies suggest that both AG and UnAG are elevated with fasting[18], most studies fail to consider both isoforms. Factors related to timing of the study (acute vs. chronic) as well as methodology (i.e. caloric restriction; the reduction of total calories, versus fasting; zero calories for a set period of time) influence the observed changes in ghrelin kinetics. Although not all are in agreement, studies that have examined both isoforms generally report that UnAG increases with fasting while AG decreases[35]. In the initial stages of fasting, total ghrelin levels increase[34], but over time total ghrelin levels normalize as the increase in UnAG is matched by the decrease in AG. In a study of eight male participants, following a 61.5hr fast, plasma AG decreased while UnAG remained near peak levels during the fed state[35]. Taken together, total levels of ghrelin were similar to those seen in the fed admission[35], meaning no significant differences in total ghrelin levels were found between fed and fasted states. This is consistent with other studies that have found no significant increases in total ghrelin levels during prolonged fasting (72hr)[38].

2.4 Ghrelin, insulin and leptin

Plasma ghrelin has a reciprocal relationship with insulin in vivo[34], as denoted by opposing changes in ghrelin and insulin concentrations following a 24hr time course with scheduled meals[34]. This is further supported by studies using insulin injections, which decrease circulating ghrelin in vivo[39]. In rat primary culture stomach cells, ghrelin secreting cells have been shown to express the α and β insulin receptor subunits, suggesting that ghrelin

5
and insulin may interact directly[40]. When insulin was added to these cell cultures, ghrelin secretion was decreased. When cells were placed in an environment with high insulin, however, ghrelin secretion was no longer suppressed, and after 24hr the insulin receptor expression had decreased[40]. Interestingly, individuals with type 2 diabetes (T2D) have impaired responses to insulin stimulated decreases in plasma ghrelin[41]. In healthy subjects, plasma ghrelin decreased during the administration of hyperinsulinemic clamps but in T2D, ghrelin decreased only when supraphysiological levels of insulin were administered[41]. The impairment in insulin-stimulated suppression of ghrelin release is likely due to a lower expression of insulin receptors in the ghrelin producing cells, and/or higher baseline insulin values in T2D, which requires more insulin to elicit a change.

Leptin is a satiation hormone secreted by adipose tissue, which opposes the appetite-stimulating effects of ghrelin[42]. Leptin levels do not change acutely prior to meals but have a diurnal variation, increasing throughout the day with slight drops within 1hr post meal[34]. In obesity, high circulating leptin levels and low circulating ghrelin levels have been reported[6]. Although ghrelin and leptin have opposing regulatory actions on appetite, it does not appear that leptin directly regulates ghrelin production or release. When leptin is administered to human participants, there is no change in ghrelin concentrations[38]. Further studies are required to clarify if there is a direct leptin-ghrelin interaction.

2.5 Central effects: signalling, orexigenic effects and vagal input

Two well-established central roles of ghrelin include its orexigenic effects[43] and its ability to stimulate growth hormone (GH) release[2]. The information involved in feeding behaviors are transmitted to the nucleus tractus solitarius (NTS), where it can be further
converted to a signal indicating fullness in the hypothalamus[44]. The orexigenic effects of ghrelin are exerted through the GHSR-1a, which is highly expressed in agouti-related peptide/neuropeptide Y (AgRP/NPY) neurons in the arcuate nucleus of the hypothalamus (ARC)[45]. AgRP/NPY neurons are depolarized and activated by ghrelin[46]. Orexin is an orexigenic hypothalamic neuropeptide involved in the regulation of food intake and arousal[47]. Ghrelin acts to stimulate isolated orexin neurons while glucose and leptin inhibit them[48].

Ghrelin is primarily produced in the stomach and it is likely that the vagal system contributes to relaying information to the central nervous system. Ghrelin induced food intake was blunted when rats underwent subdiaphragmatic or gastric branch vagotomy procedures[49], suggesting an important role of the vagal system in mediating ghrelin’s orexigenic effects. In humans with complete truncal vagotomy, orexigenic effects were blunted following AG infusion[50]. It has also been suggested that ghrelin and GH interact via communication of the vagal system, suggesting an important role for gastric vagal afferents in ghrelin induced GH secretion.

2.6 Ghrelin and growth hormone

Growth hormone is produced and secreted from the somatotrophs in the anterior pituitary[51]. Growth hormone is involved in many regulatory processes involved in the regulation of growth, macronutrient metabolism and water-electrolyte homeostasis[52][53]. In normal healthy individuals, GH secretion is tightly regulated. However, in diseased states GH may be in excess or limited. When GH is in excess it commonly results in acromegaly or gigantism, while GH deficiency can cause impaired growth and short stature[54]. GH release is regulated through hypothalamic factors; it is stimulated by GHRH and inhibited by
somatostatin[55]. Growth hormone secretagogue (GHSs) are synthetic compounds (peptidyl and nonpeptidyl) that possess potent GH releasing activity[23]. The activity of GHS is regulated by specific receptor subtypes (GHS-R), expressed primarily in the pituitary and hypothalamus[56], but also in other central and peripheral tissues[57]. GHS work by binding to the GHS-R, a G protein coupled receptor highly expressed in the hypothalamus and pituitary[58].

In pituitary cells, ghrelin stimulates GH release in a dose dependent manner in rodents[13] and humans[2]. Ghrelin acts to induce GH release both directly, acting at the level of the anterior pituitary gland, and by enhancing GHRH release[55]. Ghrelin is a natural endogenous ligand for the GHS-R and stimulates the release of GH by increasing GHRH and decreasing somatostatin activity in the hypothalamus[59]. A study performed in a group of patients with lesions in the hypothalamic region showed impaired ability of ghrelin stimulated GH release[60].

It is well established that AG stimulates GH release and GH exerts its own effects on lipid metabolism in different tissues. In skeletal muscle, GH has been shown to stimulate FA uptake, primarily by increasing lipoprotein lipase (LPL), promoting lipid utilization[61][62]. Once taken up in skeletal muscle, FAs can be stored as triglycerides (IMTGs) or oxidized for energy[63][64]. The strong lipolytic effects of GH including altering IMTG storage[65] and lipolysis, have been established in adipose and skeletal muscle[66]. In skeletal muscle, GH studies have shown either no effect or a suppressive effect on HSL expression[67]. Taken together, GH plays an important role in peripheral lipid metabolism. Because AG can bind to the GHSR-1a, studies that use whole body ghrelin injections may fail to distinguish between effects of GH and ghrelin.
2.7 Peripheral signalling and ghrelin receptor expression in skeletal muscle

Ghrelin, once produced in the stomach, is released into blood vessels and circulates throughout the body. GHSRs are present in peripheral tissues such as the myocardium, lungs, liver, skeletal muscle etc.[57], suggesting an important role with regards to communication between central and peripheral tissues.

Ghrelin secretion in humans is stimulated by cholinergic nerves, mainly via a muscarinic mechanism[68]. In rats, stomach ghrelin secretion is mediated by both cholinergic and adrenergic branches of the autonomic nervous system in a nutrient-dependent way[69]. Ghrelin secretion in rats is stimulated by muscarinic agonists, α -and β- adrenergic antagonists, and inhibited by muscarinic antagonists and α -adrenergic agonists. The gastrointestinal tract and pancreatic islets are highly innervated with autonomic fibers, which influence the regulation of gastropancreatic hormone secretion[70]. Following vagotomy, the immediate inhibition of stomach ghrelin secretion was observed, which supports the muscarinic effects observed in the pharmacological studies[69].

Growth hormone secretagogue receptor mRNA is present in various tissues including the brain, ventricles, lungs, pancreas, small and large intestines, and is most abundant in the stomach[15]. When ghrelin gene and protein expression were compared in various tissues, unsurprisingly, the stomach had the greatest ghrelin gene expression followed by the intestines, adrenal gland and pancreas[71]. Ghrelin protein concentration was highest in the lungs, followed by the brain, pituitary, skeletal muscle, stomach, intestines, salivary glands and others[71]. In skeletal muscle, binding sites for GHS have been established using competitive binding of radioligands[57]. Another study examined the distribution of GHSR-1a, GHSR-1b and ghrelin
mRNA expression in skeletal muscle and found that GHSR-1a was not detectable but the truncated GHSR-1b was[72]. C2C12 skeletal muscle cells also do not express GHSR-1a, but do contain high affinity binding sites that are recognized by both AG and UnAG[73]. Together, results from such studies suggest the presence of an unknown binding site/receptor through which ghrelin isoforms work in skeletal muscle.

3.0 Ghrelin’s role as an energy sensor and regulator of substrate utilization

3.1 Ghrelin and energetic homeostasis

Ghrelin has been shown to regulate food intake centrally[74], and in turn adiposity, as well as other factors involved in substrate utilization and energy balance. This is supported by studies using central administration methods to examine the effects of ghrelin on appetite and adiposity. Intracerebroventricular (ICV) injections of AG strongly stimulated feeding in rats and induced subsequent weight gain[43]. Chronic ICV infusion (via minipump) of AG during a 12 day period also increased food intake and body weight in rats[43]. In GH deficient rats, AG injections still stimulated food intake, suggesting that ghrelin mediated feeding is not dependent on GH[43]. These effects are thought to be regulated by NPY and AgRP because when these peptides are inhibited, ghrelin induced feeding is suppressed[43].

The effects of UnAG ghrelin on appetite and adiposity remain unclear. One study found that UnAG administration did not increase food intake and decreased feeding behavior in food deprived mice[26]. In a separate study, UnAG administration significantly increased food intake in male Wistar rats, although not as profoundly as AG[75].
In humans, AG levels have been shown to rise nearly two-fold prior to entrained meal times[34]. These studies suggest that ghrelin may be an important player in regulation of feeding and body weight. Researchers have also investigated the levels of circulating ghrelin in various human phenotypes in order to gain a better understanding of this hormone's role in energy homeostasis. It was found that obese individuals have lower circulating ghrelin levels compared to lean subjects[6]. In a study examining different ethnic groups, lean Caucasians had the highest circulating ghrelin levels (155 ± 25 fmol/ml), followed by obese Caucasians (106 ± 23 fmol/ml), lean Pima Indians (95 ± 13 fmol/ml) and obese Pima Indians (80±36 fmol/ml)[6]. In a separate study in a Caucasian population, following the ingestion of a meal, circulating AG levels fell significantly (~40%) in the lean group, while in the obese group there was little to no decrease in AG levels[76]. These findings suggest that in human obesity, there is an impaired ability to decrease circulating ghrelin levels following food consumption, potentially contributing to an increase in energy intake.

Ghrelin levels have also been shown to fluctuate in response to macronutrient composition of a meal. Rats fed a high fat diet (48% calories from fat, 16% protein, 34% carbohydrate) had lower plasma AG levels compared to those fed a normal diet[77]. When rats were fed a low protein (5%) high carbohydrate (83%) diet, AG levels increased[77]. In healthy females who consumed either a high-carbohydrate meal (13% calories from fat, 10% protein, 77% carbohydrates) or an isocaloric high-fat meal (75% calories from fat, 10% protein, 15% carbohydrates), circulating ghrelin fell abruptly after both meals[36]. Following the high carbohydrate meal, the maximum percent decrease was significantly greater than the high fat meal[36]. In a separate study, in which male and female subjects consumed one of three
isocaloric drinks (80% carbohydrate, 80% protein or 80% fat)[37], total ghrelin and AG were suppressed most consistently with the high protein drink[37], while the high fat drink was the least effective at suppressing both AG and total ghrelin[37]. The high carbohydrate drink suppressed AG and total ghrelin the most robustly, but this was accompanied by a subsequent increase in circulating levels much greater than those observed with the other two drinks[37]. From these studies, it is possible to conclude that protein and carbohydrates are most effective at suppressing post meal ghrelin levels compared to fat. Protein consumption elicits an effective suppression with little to no rebound effect in circulating ghrelin compared to that observed with carbohydrates.

Ghrelin has also been suggested to be an important regulator of energy homeostasis during periods of fasting and caloric restriction. Ghrelin-O-acyltransferase (GOAT), the enzyme responsible for synthesis of AG, has been shown to play a critical role in starvation. Although GOAT−/− mice grow and maintain body weight similar to their wild-type (WT) counterparts on normal or high fat diets (HFD), when calorie restricted (to 60% kcal), GOAT−/− mice are unable to maintain their blood glucose[18]. The impairment in blood glucose regulation is so profound that approximately 7 days of caloric restriction in GOAT−/− mice will lead to death[18]. This highlights ghrelin’s critical role in substrate utilization as denoted by its ability to spare glucose in times of metabolic demand. It also represents an important switch point between carbohydrate and fat metabolism, which appears to be essential for survival.

3.2 Peripheral glucose metabolism

Skeletal muscle accounts for approximately 80% of glucose uptake in humans[78]. The glucose transport proteins GLUT1 and GLUT4 facilitate glucose transport across membranes of
insulin-sensitive cells such as skeletal muscle[79]. Glucose uptake in peripheral tissues is
stimulated by insulin[80][81]. Impairments in insulin-mediated glucose uptake in the liver,
adipose tissue and skeletal muscle have been observed with metabolic disorders including
obesity and type 2 diabetes[82]. Many studies have focused on the insulin-signalling cascade,
defined briefly as the activation of the insulin receptor tyrosine kinase by insulin, which
phosphorylates and recruits different substrate adaptors[83]. There are many elements thought to
play a role in the insulin-signalling cascade. Kinases can be insulin dependent, like protein
kinase b (PKB or Akt)[84] , or insulin independent, such as AMP-activated protein kinase
(AMPK)[85]. Both are positive regulators of insulin action[85]. The activation of Akt and
AMPK is thought to lead to an inhibition of AS160, a downstream effector that normally
prevents GLUT4 translocation to the outer membrane[86]. Impairments in this signalling
pathway have been observed when individuals consume a high fat diet, as well as in obesity[87].

3.3 Ghrelin and glucose metabolism

Ghrelin and its receptors are widely expressed throughout the body, and ghrelin levels
increase prior to entrained meal times. Therefore, it seems likely that ghrelin may play a role in
the regulation of macronutrient metabolism, such as glucose and FAs. Recently ghrelin has been
suggested to regulate central and peripheral glucose metabolism[88].

Various models and techniques have been used in order to elucidate ghrelin’s role on
glucose metabolism. Results from such studies have been inconclusive; with some finding that
one isoform preferentially improves glucose tolerance over the other, and others showing no
change or impairment in glucose metabolism with ghrelin.
Some studies demonstrate a stimulatory effect of AG on muscle glucose uptake. Using C2C12 muscle cells, 24hr incubation with AG led to an increase in GLUT4 translocation to the cell surface and glucose uptake[89]. In another study using rat myoblasts, AG incubation prevented palmitic acid induced decreases in glucose uptake[90]. In rats, following 4 days of subcutaneous injections of AG, an increase in phosphorylation of Akt and GLUT-4 transcript levels in the soleus muscle were observed[91], suggesting, although not proving, improved glucose uptake and utilization. Other studies have suggested an inhibitory effect of AG on glucose metabolism. Rats injected with UnAG for 4 days had increased phosphorylation of Akt and increased insulin stimulated glucose uptake[92]. The improvements in insulin signalling markers were also observed in vitro, when C2C12 cells were incubated with UnAG for 48hr but these effects were no longer present with AG[92].

Researchers have also examined whether UnAG has any effect on glucose metabolism, with more consistent outcomes. One study performed using a leptin receptor deficient mouse model (db/db; type 2 diabetic phenotype), examined the effects of 10 days of UnAG injections on glucose metabolism[93]. With UnAG treatment, Akt phosphorylation and GLUT-4 membrane fraction protein content were increased in diabetic gastrocnemius muscle compared to the saline group[93]. Fasting blood glucose was lowered by 28% in db/db+ UnAG mice compared to db/db+saline[93]. In a separate study, when rats were placed on a HFD (60% calories from fat), UnAG injections improved both insulin stimulated glucose uptake and Akt phosphorylation in the gastrocnemius[92]. Together, these studies suggest that UnAG may improve insulin signaling and mitigate the effects of a HFD in skeletal muscle.
In humans, the acute administration of AG appears to generally have a negative effect on insulin-stimulated glucose uptake. In healthy males, AG infusion decreased both basal and insulin-stimulated whole-body glucose clearance, while endogenous glucose production remained unaffected[94]. Impairments in insulin sensitivity were found to be independent of GH release when the same researchers examined otherwise healthy men who had underwent pituitary surgery with GH deficiency[3]. Following a 5hr infusion of AG, plasma glucose levels were elevated compared to control, and glucose clearance was decreased during a hyperinsulinemic euglycemic clamp [3]. There were no changes in skeletal muscle AMPK or AKT phosphorylation[3]. The same group later used a microdialysis technique to assess tissue specific responses to AG infusion. In contrast to their previous studies, when excluding systemic effects, they found a decrease in interstitial glucose content in skeletal muscle during hyperinsulinemia in response to AG administration, suggesting improvements in glucose uptake[95]. Taken together, it is difficult to deduce from these studies ghrelin’s direct effect on glucose uptake at the level of the muscle. However, a recent study from our own laboratory, examining the direct effects of ghrelin isoforms on isolated rat skeletal muscle, found no changes in insulin and non-insulin stimulated glucose[96].

3.4 Regulation of fatty acid metabolism

Long chain fatty acids (LCFA) are important components of cellular membranes, and are a main source of energy for the human body[97]. In skeletal muscle, lipids can be stored as intramyocellular or extramyocellular triglycerides (IMTGs, EMTGs), or broken down via lipolysis to use as energy through FA oxidation[63][64].
Fatty acid uptake in skeletal muscle is facilitated by the FAT/CD36 transporter, a FA binding protein which can translocate to the sarcolemma in the presence of insulin or at the onset of muscle contractions[98]. Once in the cytosol, the FA is converted to fatty acyl CoA via the ligase enzyme acetyl-CoA synthetase (ACS). In order to enter the mitochondria to undergo beta-oxidation, fatty acyl CoAs are converted to fatty acyl carnitine derivatives, via carnitine acetyltransferase (CAT)) and carnitine palmitoyltransferase 1 (CPT1) enzymes. Once through the outer membrane, the carnitized fatty acyl can be transported across the inner mitochondrial membrane by the carnitine shuttle system via carnitine–acyl carnitine translocase (CACT). In the mitochondrial matrix, carnitine palmitoyltransferase 2 (CPT2) converts fatty acyl carnitine back to fatty acyl-CoA, which enters beta-oxidation, and to free carnitine, which returns to the inner mitochondrial space or cytosol. Acetyl-CoA generated from beta-oxidation is able to enter the citric acid cycle and generate adenosine triphosphate (ATP).

Fatty acid oxidation can be regulated by hormones (e.g. leptin, which stimulates oxidation of FAs[99]), nutritional status (increases with increased FA availability, decreases with increased glucose availability), activity level, and training status. Certain kinases have also been shown to alter FA oxidation, such as AMPK. Studies have shown that AMPK is able to potently stimulate FA oxidation by inhibiting acetyl-coA carboxylase (ACC)[100], which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. Malonyl-CoA inhibits CPT1 and prevents FA-carnitine entry into the mitochondria. Once ACC is phosphorylated/deactivated, the production of malonyl-CoA decreases, leading to less inhibition on CPT1. This allows for CPT1 to increase transport of FAs to the mitochondrial matrix, and in turn increase beta-oxidation.
Fatty acids are stored as triacylglycerol (TAG) in lipid droplets within skeletal muscle called intramyocellular triacylglycerol (IMTG)[101]. During moderate exercise, IMTGs can provide up to 25% of total energy[102]. Furthermore, IMTG pools can be adaptively increased in type 1 oxidative fibers with training[103] or in individuals with type 2 diabetes/obesity[104]. Triacylglycerol breakdown is mediated by various lipases, including adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MGL)[105][106]. In skeletal muscle, HSL and MGL are highly expressed, while HSL is expressed predominantly in type 1 oxidative fiber[107]. Once hydrolyzed, TAGs can be converted to diacylglycerols (DAGs), and further to monoacylglycerol (MAG), FAs, and glycerol[108][109]. In genetic ATGL null mice, an accumulation of TAG is observed[110], suggesting that ATGL is necessary for the conversion of TAG to DAG. In HSL null mice, TAG levels are normal but DAG content is elevated [111], denoting a role for HSL in the conversion of DAG to MAG. Together, these findings suggest that different lipases regulate the conversion of specific glyceride forms.

Lipolysis is a highly regulated catabolic process, responsible for the release of TAGs from cellular lipid droplets stores[112]. Lipases facilitate the hydrolysis of TAG until glycerol and FAs are released[106]. Some of the FAs released during lipolysis can be re-esterified into TAGs, while glycerol can be used as a gluconeogenic substrate[113]. In humans, all three beta-adrenoceptors are functional in adipose tissue, B₁ and B₂ being the most active, while in rodents, B₃ is the most abundant beta-adrenoceptor in adipose tissue[114]. In skeletal muscle, only the B₂ subtype is of importance in the regulation of catecholamine induced lipolysis and is highly expressed in both rodents and humans[115]. The effect of catecholamine’s on HSL in both adipose tissue and skeletal muscle is regulated by beta-adrenoceptors and protein kinase A
Hormone sensitive lipase is regulated by reversible phosphorylation of various serine sites, including five serine residues in rats in vitro (563, 565, 600, 659, 660)[116]. Studies examining rat HSL have shown that AMPK can phosphorylate the inhibitory serine site 565, while PKA has been suggested to phosphorylate the stimulatory serine residues 660 and 563[116]. Skeletal muscle HSL activity is intensity dependent and can be increased with contraction and exercise by 50-100%[117]. Epinephrine, a hormone produced by the adrenal glands, has also been shown to regulate skeletal muscle HSL activation in resting and contracting muscle[107]. Through beta-adrenergic receptor stimulation and PKA activation[118], epinephrine increases HSL phosphorylation and in turn FA and glycerol release. Previous studies have shown that epinephrine is able to directly stimulate skeletal muscle lipolysis in oxidative muscle[119].

Adipose triglyceride lipase (ATGL) may also play an important in skeletal muscle lipolysis. In the absence of HSL activity, studies have shown that the breakdown of IMTG continues to occur in contracting rat and mouse skeletal muscle[120]. Studies with ATGL deficient mice found an accumulation of TAG in skeletal muscle[121], which suggests a reduction in TAG hydrolysis.

3.5 Ghrelin and peripheral fatty acid metabolism

It has been proposed that ghrelin can influence FA metabolism in peripheral tissues. Alterations in substrate partitioning, for example impairments in FA oxidation/utilization, may influence the development of obesity[122]. Although the majority of the literature indicates that ghrelin injections in rodents lead to adiposity[123], the precise mechanism through which this occurs remains up for debate. It is possible that weight gain associated with ghrelin may be due
to its central orexigenic effects, its ability to stimulate GH release, or modifications in substrate utilization. One study showed that daily peripheral AG injections in rats led to weight gain by reducing fat utilization, as evidenced by increases in respiratory quotient[123], while another study showed that 4 days of subcutaneous AG injections in rats led to reduced TAG content in mixed gastrocnemius muscle and unchanged in oxidative (soleus)[124]. The change observed in mixed muscle is further supported by a study in rat myoblasts treated with AG, which, also reduced TAG accumulation[90]. In rat hepatocytes, both AG and UnAG increased AMPK and ACC phosphorylation, and AG (added at physiological concentrations) increased CPT1 mRNA[125], suggesting that ghrelin increases FA oxidation, although this was not directly assessed. Overall, ghrelin appears to alter peripheral FA metabolism. It may be tissue specific and the precise mechanism through which it works is unknown.

The effects of ghrelin on lipolysis are controversial. Some studies have shown that both ghrelin isoforms inhibit isoproterenol-induced lipolysis in rat adipocytes[126][127]. Conversely, in vivo, acute peripheral AG infusion for 4hr in healthy human males increased circulating FA by 80%, suggesting increased lipolysis[3]. These effects have been replicated in hypopituitary patients, suggesting that the increase in FA works through a mechanism independent of GH[3]. In a recent study examining the effects of AG, UnAG and AG+UnAG (~3.5hr infusion) on serum FA in humans, both AG and AG+UnAG increased FA concentration while saline and UnAG did not[128]. Although many studies have examined the effects of ghrelin on whole body and adipose tissue lipolysis, studies in skeletal muscle are limited. One of the few studies that did examined this tissue measured interstitial concentrations of glycerol and found that lipolysis was
not changed in skeletal muscle with ghrelin treatment[95]. Collectively, research suggests that ghrelin isoforms may alter lipolysis independent of GH.

3.6 Conclusion

In summary, the regulation and maintenance of whole-body glucose and FA homeostasis is critical. Alterations in glucose and FA metabolism contribute to metabolic disorders including obesity and T2D. Ghrelin is recognized for its central orexigenic effects, which may contribute to the development of adiposity, but has also been suggested to alter peripheral tissue metabolism. Although many studies have attempted to determine the precise role of ghrelin in peripheral tissues, the results remain inconclusive. Furthermore, research on the direct effects of ghrelin on FA metabolism in adipose tissue and skeletal muscle remain scarce. In order to understand this hormone’s function in peripheral tissue energy metabolism, further research is warranted. Once ghrelin’s role on substrate utilization has been established, it may be possible to identify the underlying mechanisms behind such changes.
Chapter 2: Thesis Objectives

Although the actions of ghrelin on skeletal muscle glucose uptake are rather well defined, the direct effects of ghrelin isoforms on skeletal muscle FA metabolism remain unknown. Given that ghrelin levels rise prior to entrained mealtimes and decrease shortly after it is likely that it may act as a preparatory signal, priming tissues to facilitate uptake and metabolism of substrates such as FA. Studies have shown that peripheral AG injections lead to reductions in IMTG content in mixed gastrocnemius muscle; however, this was not observed in oxidative soleus muscle[124]. In healthy humans, acute peripheral AG injections have increased circulating FAs by 80%, suggesting that ghrelin may work to increase lipolysis[3]. Taken together, it appears that ghrelin can alter FA metabolism. However, methodological limitations associated with ghrelin injections make it difficult to identify the independent effects of ghrelin. Therefore, the objective of this thesis was to identify the direct effects of ghrelin isoforms on skeletal muscle FA metabolism. Furthermore, we sought to gain insight as to whether these effects were dependent on pre (5 mM) or post (10 mM) prandial glucose levels. The rise of circulating ghrelin levels pre-meal[35] has lead us to speculate that ghrelin may act in a protective manner, promoting the oxidation and lipolysis of FAs, while reducing esterification of triglycerides to maintain blood glucose levels. Therefore, we hypothesized that ghrelin isoforms would increase FA oxidation and lipolysis while down-regulating FA incorporation into lipid pools in oxidative and glycolytic muscles at pre-prandial glucose levels.
Chapter 3: Ghrelin Increases Fatty Acid Oxidation and Blunts Epinephrine Stimulated Lipolysis in Skeletal Muscle

Introduction.

Ghrelin is classically known as a central appetite-stimulating hormone, but has recently been recognized to have a significant role in peripheral tissue energy metabolism. There are two main ghrelin isoforms that exist; unacylated ghrelin (UnAG) and acylated ghrelin (AG). Acylated ghrelin has long been considered as the more bioactive of the two isoforms. However, recent studies suggest an important role of UnAG in energy metabolism[20].

Given that ghrelin rapidly increases prior to entrained mealtimes[35], it is possible that ghrelin may act as part of a preparatory response to facilitate the metabolism of ingested glucose and lipids. Skeletal muscle is a major site for glucose and lipid disposal; however, the direct effects of ghrelin on this tissue remain understudied. Furthermore, studies examining the metabolic effects of in vivo ghrelin injections fail to distinguish independent effects of ghrelin from secondary effects such as increases in growth hormone.

The rise of circulating ghrelin levels pre-meal has lead us to speculate that ghrelin may act in a protective manner, promoting the oxidation and lipolysis of FAs, while reducing esterification of triglycerides to maintain blood glucose levels. This is further supported by studies which have shown that mice lacking the ghrelin-O-acyltransferase (GOAT), the enzyme responsible for the synthesis of AG, are unable to maintain blood glucose while calorie restricted[18]. This impairment in blood glucose regulation is severe enough that it leads to subsequent death[18]. In humans, ghrelin levels are elevated in individuals with anorexia
nervosa[5], suggesting that in states of low nutrient availability ghrelin may influence substrate metabolism to promote survival.

By examining the effects of ghrelin isoforms on FA metabolism in skeletal muscle, we may better understand its role in energy maintenance and potentially obesity. Therefore, the objective of this study was to determine the direct effects of ghrelin on FA metabolism (incorporation into lipids, oxidation and lipolysis) in isolated, mature rodent skeletal muscle. Since ghrelin rapidly changes surrounding mealtime, we examined the impact of ghrelin on muscle FA metabolism at normal (premeal, 5mM) and elevated (postmeal, 10mM) glucose concentrations. Finally, we examined the potential effect of ghrelin on FA metabolism in muscles of different oxidative potential (soleus, slow oxidative; extensor digitorum longus, fast glycolytic). We have previously used these muscles, which are suitable for incubation[63].

Labelled $^{14}$C palmitate was used to assess oxidation and incorporation into endogenous pools (triacylglycerol (TAG), diacylglycerol (DAG), phospholipids (PHOS)). Lipolysis was assessed by glycerol release into the surrounding medium. Finally, we assessed the phosphorylation of enzymes recognized for their role in muscle FA metabolism, including AMP-kinase (AMPK), acetyl-CoA carboxylase (ACC), and hormone sensitive lipase (HSL).

We hypothesized that in pre-meal conditions (glucose, 5 mM) AG and UnAG would 1) increase FA oxidation and IMTG lipolysis; 2) decrease the rate of FA esterification into TAGs; and 3) that these effects would no longer be present in the post-meal condition (glucose, 10 mM). Furthermore, we hypothesized that ghrelin would alter FA metabolism through 4) up-regulation of phosphorylation of AMPK/ACC and HSL.
Methods.

Materials and reagents.

Reagents, molecular weight markers, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON, Canada). Western Lightning Plus enhanced chemiluminescence (ECL) was purchased from Perkin Elmer (NEL105001EA). The following primary antibodies were purchased from Cell Signaling Technology: phospho-HSL (Ser\textsuperscript{563} catalog no. 4139, Ser\textsuperscript{660} catalog no. 4126, Ser\textsuperscript{565} catalog no. 4137), total HSL (catalog no. 4107), AMPK-\(\alpha\) (catalog no. 2532), p-AMPK Thr\textsuperscript{172} (catalog no. 2531), phospho-ACC (Ser\textsuperscript{79} catalog no. 3661) and total ACC (catalog no. 3662). Horseradish peroxidase-conjugated donkey anti-rabbit antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) (711-035-152). Free glycerol was measured using a commercially available fluorometric kit from BioVision (catalog no. K630–100). Medium 199 (product no. M3769), benzethonium hydroxide solution (product no. B2156), epinephrine hydrochloride (product no. E4642), glycerol standard (product no. G7793), and free glycerol reagents (product no. F6428) were purchased from Sigma-Aldrich Canada Co. AICAR was purchased from Cayman Chemical Co. (10010241-100). Palmitic acid [1-14C] was purchased from American Radiolabeled Chemicals (product no. 0172A). Acylated (Cat. #H-4862) and unacylated (Cat. #H-6264) rat ghrelin were sourced from Bachem (Torrance, CA).

Animals.

All procedures were carried out in accordance with the recommendations of the Canadian Council of Animal Care, and were approved by the Animal Care Committee at the University of
Guelph (AUP 3270). All surgeries were performed under sodium pentobarbital anesthesia (6mg/100g body wt) and all efforts made to prevent discomfort and suffering. Male Sprague-Dawley rats approximately 7 weeks in age (200-250g) were purchased from Charles River Laboratories. All animals were group housed (2-4 per cage) and kept in a 22-24°C environment. Animals were on a 12:12-h reverse light-dark cycle and fed standard rodent chow (16% protein Teklad laboratory diet, Envigo) ad libitum. Animals were given a minimum of 1 week to acclimate to their environment before all procedures.

In vitro experiments.

Lights went off at 6:00 am to signal the beginning of the active phase for the animals and they were given time to feed. 2 hours prior to anaesthetization food was taken away. Experimental procedures began between 8:30-10:30 am. All animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (6mg/100g body wt). Soleus (oxidative) and extensor digitorum longus (EDL; glycolytic) muscles from hind limbs were stripped longitudinally and excised, and weighed. Muscles were then preincubated for 30 min at 30°C in oxygenated (95% O2-5% CO2) medium 199 modified with 1 mM palmitate and 4% bovine serum albumin FA free in all experiments except Western blotting. 1% bovine serum albumin FA free was used in order to prevent the development of a gross non-specific band around 50-60 kDa.

For determination of FA oxidation and incorporation into lipid pools, muscles were incubated with 1mM palmitate, 0.5 uCi/mL palmitic acid [1-14C], and either 5 mM or 10 mM d-deoxy-glucose. Muscle strips from each animal were randomly assigned to either the control
group with no additional treatment, AICAR (2mM), AG (150ng/ml) or UnAG (150ng/ml) for a one-hour incubation. Previous work from our laboratory has validated the stability of both AG and UnAG in incubation media, and found that they remain stable for 2 hrs[96]. The concentration of ghrelin chosen has been shown previously to elicit significant metabolic effects; this concentration of ghrelin has been shown to significantly stimulate GH release in vivo and in vitro[13].

**Fatty Acid Oxidation.**

Muscles strips were incubated in the water bath with a sealed rubber stopper containing an Eppendorf tube. Following the 1 hour incubation, 230μl of benzethonium hydroxide was added to the Eppendorf tube inside the flasks and 2ml of sulfuric acid (1M) was added directly to the medium and muscle via syringe. The puncture holes on the top of the rubber stopper from the syringes were sealed using Dow Corning vacuum grease. The mixture sat at room temperature for 2 hours to allow for $^{14}$CO$_2$ gas to be released and trapped in the benzethonium hydroxide. Following the 2 hour period, the Eppendorf tube containing the benzethonium hydroxide was placed into a scintillation vial for quantification. Tendons were then cut and their weight was recorded and subtracted from the initial muscle weight obtained prior to incubations.

**Fatty Acid Incorporation into Lipid Pools.**

Following incubations, muscles were placed in 2:1 Chloroform:Methanol solution and homogenized with a polytron. They were then centrifuged for 10 minutes at 4°C at 4000 rpm. The supernatant was removed, 2ml of ddH2O was added, shaken gently for 10 minutes and centrifuged once again. The underlying layer of chloroform was removed, and 1ml of 100%
chloroform was added to the centrifuge tube to dissolve any remaining lipids. Samples were placed in a water bath warmed at 30°C and under a stream of nitrogen gas to facilitate evaporation of remaining liquid. Once samples were blown down, 100µl of 2:1 chloroform:methanol solution with tripalmitin and dipalmitin standards were added to the samples. 50µL of dissolved lipids spotted on a designated lane on a thin layer chromatography (TLC) plate. The TLC plate was placed in a developing tank with enough heptane:isopropylether:acetic acid (60:40:3) to reach approximately 1cm on the plate. Following 45 minutes in the tank, the resolved plates were dried horizontally in room air for ~2-3 minutes, and lightly sprayed with chlorofluorescein dye (0.02% w/v in ethanol). Lipid pools, including TAGs DAGs, and PHOS, were visualized under long-wave UV light, the individual bands were scraped off onto weigh paper, and placed into a scintillation vial with 5ml of scintillation cocktail for quantification.

**Lipolysis and glycerol determination.**

Only soleus muscle was used for the assessment of lipolysis as previous work in our laboratory has shown that EDL does not respond significantly to epinephrine (positive control for lipolysis)[119]. All incubation mediums contained medium 199, 4%BSA, 1 mM palmitate, and either 5 mM or 10 mM D-glucose. Muscle strips were pre-incubated for 30 minutes and incubated for 1 hour at 30°C in oxygenated (95% O₂-5% CO₂) in medium 199. 1 mM of palmitate and either 5 mM or 10 mM of D-glucose were also added to the incubation buffer, in order to replicate the conditions used in the oxidation experiments. Incubation conditions
included control, epinephrine (epi) (1 uM), epi+AG (1 uM, 150 ng/ml), and epi + UnAG (1 uM, 150 ng/ml).

Glycerol concentration in the incubation media was measured using a fluorometric assay on a black 96-well plate. Briefly, a glycerol standard curve was prepared by diluting a 1 mM kit standard to a range of 0.1–1.2 nmol/well. All standards and samples were loaded in triplicate and wells were adjusted to 50 µl total volume with assay buffer; 50 µl enzyme reaction mix (46 µl assay buffer, 2 µl glycerol probe, 2 µl glycerol enzyme mix) were added to samples and standards. Reactions were incubated at room temperature for 30 minutes and were protected from light. Concentrations were determined fluorometrically at excitation/emission wavelengths of 535/590 nm. Glycerol concentration was normalized to tissue weight (nmol/g wet weight).

**Signalling Experiments and Western Blotting.**

Pre-incubations were 30 min for all signalling experiments; incubations for AMPK and ACC were 30 min while incubations for HSL were 1 hr in length. All samples were incubated at 30°C in oxygenated (95% O₂-5% CO₂) medium 199. Incubation medium contained medium 199, 5 mM of D-glucose, and 1% FA free bovine serum albumin. All muscles were blotted, frozen in liquid nitrogen, and stored at −80°C until analyses were performed. Soleus and EDL muscles were homogenized in a 25:1 volume-to-weight ratios of ice-cold cell lysis buffer supplemented with PMSF and protease inhibitor cocktail (Sigma catalog nos. 78830 and 9599). Samples were homogenized in a Qiagen TissueLyser LT (cat. No 85600) for three, 3 minute intervals and then centrifuged at 1,500 g for 15 min. Protein concentration of the supernatant was determined using the bicinchoninic acid method[129] and equal amounts of protein were separated on 10% gels to
assess the protein content of p-HSL Ser\textsuperscript{660}, p-HSL Ser\textsuperscript{563}, p-HSL Ser\textsuperscript{565}, HSL, p-AMPK Thr\textsuperscript{172}, total AMPK, and 5% gels for p-ACC Ser\textsuperscript{79}, total ACC. Proteins were transferred to nitrocellulose membranes at a constant 200 mA per tank and subsequently blocked in Tris-buffered saline-0.01% Tween (TBST) supplemented with 5% nonfat dry milk for 1 hour at room temperature with gentle shaking. Membranes were incubated at 4°C overnight in primary antibodies diluted 1:1,000 in TBST with 5% BSA. The following day blots were washed with TBST and then incubated in TBST-1% nonfat dry milk supplemented with 1:2,000 horseradish peroxidase conjugated donkey ant-rabbit secondary antibody for 1 h at room temperature. Bands were visualized using ECL and quantified using Alpha Innotech software.

Following visualization, membranes were placed in a ponceau staining solution in order to locate protein bands. Protein bands were imaged and quantified using Alpha Innotech software. Western blots were normalized against ponceau stained membranes to account for differences in protein loading concentrations. Once both phosphorylated proteins and total proteins were normalized to their respective ponceau stain, phosphorylated proteins were normalized to total protein content.

**Statistical Analysis.**

A repeated measure one-way ANOVA and Fisher’s LSD was used for analysis of FA oxidation and FA deposition. A two-way repeated measure ANOVA and Fisher’s LSD was used to analyze the free glycerol assay in order to examine the interaction of ghrelin and epinephrine independently and in combination. A one-way repeated measure ANOVA and Fisher’s LSD was used for analysis of Western blotting. Statistical analysis was accepted at p<0.05.
Results.

Ghrelin directly stimulates fatty acid oxidation in glycolytic and oxidative muscle.

AICAR, which served as a positive control for the assessment of FA oxidation[130], stimulated palmitate oxidation in both soleus and EDL regardless of the glucose concentration (p<0.01). At 5 mM glucose, UnAG stimulated oxidation in both soleus (p<0.01) and EDL (p<0.03). AG also tended to stimulate palmitate oxidation in EDL (p=0.07). Contrary to our hypothesis, at 10 mM glucose, both ghrelin isoforms significantly stimulated palmitate oxidation in EDL (p<0.05) and soleus (p<0.01) (Figure 1).
Figure 1: Ghrelin stimulates fatty acid oxidation.

Unacylated ghrelin (UnAG) stimulates fatty acid oxidation at 5 mM glucose in soleus (A) and EDL (B). Both acylated (AG) and unacylated (UnAG) stimulate fatty acid oxidation in soleus (C) and EDL (D) at high glucose (10 mM) concentrations. Data is expressed as the mean ± SE.
n=10 for each group. Data was considered significant at $p \leq 0.05$. Groups not sharing a letter are statistically different from each other.

**Ghrelin does not alter fatty acid incorporation into triacylglycerol, diacylglycerol or phospholipids.**

No significant differences in the incorporation of labelled palmitate into triacylglycerol (TAG), diacylglycerol (DAG) or phospholipids (PHOS) were observed with any treatment in either the 5 or 10 mM glucose environments in soleus or EDL (Figure 2). Similarly, esterification of TAGs, DAGs and PHOS remained unaltered at high glucose concentrations (refer to appendix I). As expected, overall esterification of respective lipid pools was greatest in the oxidative muscle type (soleus).
Ghrelin does not alter fatty acid incorporation into triacylglycerol (TAG), diacylglycerol (DAG) or phospholipids (PHOS). Shown above (Figure 2) are the esterification rates of TAG, DAG and PHOS.

Figure 2: Ghrelin and palmitate incorporation into TAG, DAG, and phospholipids.
PHOS (nmol/g/hr) in soleus (A) and EDL (B) with 5 mM glucose. Similar results were observed at high glucose concentration (10 mM) (results not shown). Data is expressed as the mean ± SE, soleus n=7 for each group, EDL n=8 (TAG), n=6 (DAG, PHOS). Data was considered significant at \( p \leq 0.05 \).

**Ghrelin blunts epinephrine-induced lipolysis in soleus muscle.**

Previous work from our laboratory has demonstrated that epinephrine is able to significantly stimulate lipolysis in the soleus but not the EDL muscle[119]. Therefore, we examined only the soleus muscle to determine the role of ghrelin as a regulator of lipolysis. Epinephrine significantly stimulated free glycerol release, an indicator of lipolysis, at normal (\( p=0.0002 \)) and high (\( p=0.0016 \)) glucose concentration compared to control. Neither ghrelin isoform (AG, UnAG) was able to independently alter glycerol release compared to control. However, when ghrelin isoforms were combined with epinephrine, a significant blunting of epinephrine's stimulatory effect on glycerol release was observed (Figure 3).
Figure 3: Ghrelin and skeletal muscle lipolysis.

Ghrelin does not stimulate glycerol release (index of lipolysis) alone and blunts epinephrine (EPI) stimulated lipolysis at normal (A) and high (B) glucose concentrations. Data is expressed...
as the mean ± SE. n=8 (A) and n=7 (B). Data was considered significant at p ≤ 0.05. Groups not sharing a letter are statistically different from each other.

AMPK and ACC are increased in EDL but not soleus with ghrelin treatment.

We hypothesized that the increase in FA oxidation observed with ghrelin may be due to the activation/phosphorylation of AMP-activated protein kinase (AMPK) which, in turn, phosphorylates/deactivates acetyl CoA carboxylase (ACC). A trend towards increases in AMPK and ACC phosphorylation were observed in the EDL muscle (4b, 4d). No significant changes in AMPK and ACC phosphorylation were observed in the soleus muscle (Figure 4a, 4c).
Figure 4: Ghrelin and phosphorylation of AMPK/ACC.

Phosphorylation of AMPK and ACC normalized to ponceau and total AMPK, ACC content. A trend towards an increase in phosphorylated AMPK in EDL muscle was observed. Data is
expressed as the mean ±SE, 4A. n=6, 4B. ACC n=7, 4C. n=7, 4D. n=8. Data was considered significant if p < 0.05. Groups not sharing a letter are considered statistically different from each other.

The effect of epinephrine and ghrelin isoforms on HSL phosphorylation in skeletal muscle.

A trend towards an increase in HSL phosphorylation at the serine 563 (p=0.07) was observed with epi treatment alone (Figure 5). When AG was combined with epi (Epi+AG), a significant increase in serine 563 phosphorylation was observed. No significant changes in serine 563 phosphorylation was observed with Epi+UnAG. Epinephrine and AG decreased phosphorylation of the inhibitory serine 565 site, while no other significant changes were observed with epi or Epi+AG treatments (Figure 5). Taken together, it does not appear that ghrelin’s ability to blunt epinephrine stimulated-lipolysis works through reducing the phosphorylation of the stimulatory serine 563, nor does it do so by increasing the phosphorylation of the inhibitory serine 565 site.
Figure 5: The effects of epinephrine and ghrelin on skeletal muscle HSL phosphorylation.

Ghrelin does not appear to influence the ability of epinephrine to phosphorylate HSL activating site 563 (A) and inhibitory site 565 (B), n=6. Data was considered significant if p < 0.05. Groups not sharing a letter are considered statistically different from each other.
Discussion.

The purpose of this investigation was to determine ghrelin’s direct effects on FA oxidation, incorporation and lipolysis in isolated oxidative (soleus) and glycolytic (EDL) muscles. Here we show that both ghrelin isoforms are able to directly stimulate palmitate oxidation and inhibit epinephrine-induced lipolysis ex vivo. Although some have proposed that ghrelin injections lead to increases in respiratory quotient, indicative of decreased fat utilization, the direct effects of ghrelin on FA metabolism in skeletal muscle remain unknown. In this study, we utilized an isolated skeletal muscle preparation to assess changes in FA metabolism caused directly by acylated (AG) and unacylated (UnAG) ghrelin isoforms. Muscle strips were incubated with either normal (5mM) or high glucose (10 mM) concentrations in order to simulate and compare pre and postprandial states. This ex vivo preparation allowed us to directly examine the effects of ghrelin isoforms without the secondary effects or interaction of other hormones, such as GH and insulin, that normally occur in vivo.

A number of novel observations have been made in the present study: 1) AG and UnAG increased palmitate oxidation in oxidative and glycolytic muscles, and this was generally true regardless of the glucose concentration used; 2) the increase in palmitate oxidation in both muscle types was not associated with increases in AMPK and ACC; 3) neither ghrelin isoform had an effect on palmitate incorporation into any of the major lipid pools (TAG, DAG and PHOS); 4) both AG and UnAG blunted epinephrine-induced lipolysis in the soleus muscle, but did not do so independently; and 5) the blunting of epinephrine stimulated lipolysis by ghrelin did not coincide with a decrease in HSL activation at the serine 563 activation site or the serine 565 inhibitory site. Collectively, this suggests that ghrelin has a direct effect on stimulating
palmitate oxidation and reducing lipolytic activation (epinephrine induced) in muscle in the absence of any observable effect on lipid incorporation into intramuscular pools. These findings suggest that ghrelin may act to facilitate the maintenance of circulating blood glucose by promoting FA oxidation. However, it is unclear why ghrelin would act to blunt lipolysis, as this would seem contradictory given the increase in FA oxidation.

Effect of ghrelin isoforms on fatty acid oxidation

Previous work from our laboratory has demonstrated that ghrelin isoforms have no effect on glucose uptake in skeletal muscle[96], suggesting that ghrelin’s effect is selective to FA oxidation. Normal circulating levels of ghrelin in rats are approximately 0.02 ng/ml AG and 0.120 ng/ml UnAG[125]. However, many studies have used higher concentrations of ghrelin to study its effects; common injection protocols include administration of approximately 100 ug [93] or 200 ug[131] twice daily, and incubation with 100 nM ghrelin in cell cultures [89][20]. We chose to incubate the muscle strips with high AG and UnAG concentrations (150ng/ml) to maximize the chance of eliciting a response in the resting muscle. The presence of exogenous FA concentrations used (1.0 mM palmitate) has been previously shown to adequately supply the muscle with enough substrate to measure FA oxidation and incorporation into lipid pools[63].

To our knowledge, we are the first to show that ghrelin isoforms directly and independently stimulate FA oxidation in skeletal muscle. Ghrelin in its UnAG form increased palmitate oxidation by approximately 42% in the EDL when incubated with 10 mM glucose, and 39% in soleus, regardless of glucose concentration, compared with control muscles. Similarly, AG increased oxidation ~38% compared to control when muscles were placed in high glucose
media (10 mM). Although it did not reach statistical significance, AG increased palmitate oxidation by ~15% in soleus and ~23% in EDL compared to control when incubated with low glucose (5 mM). Ghrelin appears to stimulate FA oxidation significantly in conditions of high glucose, which suggests that this hormone may affect postprandial lipid metabolism in vivo.

The mechanism by which acute ghrelin (AG and UnAG) treatment increases FA oxidation in skeletal muscle is unknown. We hypothesized that AG and UnAG would stimulate FA oxidation in skeletal muscle by upregulating the phosphorylation of AMPK and the downstream effector ACC. Once phosphorylated, ACC becomes inactive which in turn decreases the production of malonyl-CoA, which normally inhibits carnitine palmitoyl transferase 1 (CPT1). The relief of inhibition on CPT1, a rate-limiting step in FA oxidation then allows FAs to be readily taken up into the mitochondria for oxidation. Previous work in our laboratory has shown that AICAR is able to stimulate AMPK-α2 and FA oxidation in resting soleus muscle following 60 minutes of treatment [132]. In the current study, AMPK and ACC phosphorylation were not significantly increased in either muscle with AG or UnAG when incubated with 5 mM or 10 mM of glucose. It is possible that increases in FA oxidation are not due to increased activity of these enzymes. Presumably, AMPK becomes activated in response to an increase in energy demand, implying that ghrelin did not alter the energy charge of the muscle (AMP, PCr levels) and therefore did not stimulate AMPK. Our findings are similar to those seen previously, with no changes in AMPK phosphorylation with isolated soleus and EDL muscles and ghrelin (AG, UnAG) treatment [96]. This is the first study to demonstrate that acute incubation with ghrelin isoforms is able to directly increase FA oxidation in skeletal muscle, although the mechanism remains undefined.
Effect of ghrelin isoforms on fatty acid incorporation

Previous studies have demonstrated that chronic AG injections for 4 days can reduce TG content in gastrocnemius, but not in the oxidative soleus muscle[124]. In the current study, neither ghrelin isoform significantly altered palmitate incorporation into TAG, DAG and P pools (see appendix I) regardless of glucose concentration in either soleus or EDL. One limitation of this technique is that it only allows us to measure the net accumulation of $^{14}$C palmitate within a given lipid pool. Without the ability to measure the movement of $^{14}$C palmitate between pools we do not know whether transfer of $^{14}$C palmitate from other lipid pools replaced a small loss of $^{14}$C palmitate from the TAG pool.

Effects of ghrelin on skeletal muscle lipolysis

Contrary to previous studies in humans that suggest an increase in lipolysis with ghrelin injection[3], we found no significant changes in glycerol release with ghrelin treatments compared to control. Previous work has shown that both AG and UnAG inhibit isoproterenol-induced lipolysis in isolated rat adipocytes[126], which is similar to what we observed in skeletal muscle in this study. Both AG and UnAG blunted epinephrine-induced lipolysis in the soleus muscle when incubated with normal and high glucose concentrations. In skeletal muscle, the $\beta_2$ adrenoreceptor subtype is critical in the regulation of catecholamine induced lipolysis and is also highly expressed in both rodents and humans [115]. The effect of catecholamines on HSL in both adipose tissue and skeletal muscle is regulated by beta-adrenoceptors and protein kinase A (PKA)[107]. However, the precise site of PKA phosphorylation on HSL in vivo remains unclear[116]. HSL is regulated by reversible phosphorylation of various serine sites, including
five serine residues in rats in vitro (563, 565, 600, 659, 660) [116]. Studies examining rat HSL have shown that AMPK can phosphorylate the inhibitory serine site 565, while PKA phosphorylates the stimulatory serine residues, 660 and 563[116]. Skeletal muscle HSL activity also increases with contraction/exercise by 50-100%, and is intensity dependent[117]. Epinephrine has also been shown to regulate skeletal muscle HSL activation in resting and contracting muscle[107]. Through beta-adrenergic receptor stimulation and PKA activation[118], epinephrine increases HSL phosphorylation and in turn free FA and glycerol release. Previous studies have shown that epinephrine is able to directly stimulate skeletal muscle lipolysis in oxidative muscle[119]. In adipose tissue, ghrelin alters lipolysis through modifications in HSL activation (Cervone, unpublished data). Therefore, we hypothesized that AG and UnAG would induce lipolysis through activation/phosphorylation of a serine site on the HSL residue. Contrary to our hypothesis we observed no change in lipolysis with either ghrelin isoform, but blunted epinephrine induced lipolysis. Phosphorylated protein content of HSL site 563 increased with epinephrine treatment as expected. However, this site does not appear to be responsible for the reductions in lipolysis observed with ghrelin treatment. Studies in humans with epinephrine and exercise have shown only mild to moderate (28%) activation of HSL[118]. Therefore, it may be reasonable to believe that we were unable to detect the slight increase in protein content of the phosphorylated serine 563 site. Measurements of alternative HSL phosphorylation sites and activity of HSL are warranted. In addition, it may be beneficial to consider the interaction of HSL with perilipins and lipid droplets, as these interactions are important in the regulation lipolysis[133].
The observed reduction in epinephrine-induced lipolysis with ghrelin treatment is intriguing. However, it remains to be determined whether this reduction in intramuscular lipolysis would still exist if ghrelin were to be injected in vivo using a whole-body model. Many studies have shown that ghrelin’s effect on peripheral metabolism in \textit{ex vivo} models\cite{127} differ from that observed \textit{in vivo}\cite{134}. In isolated adipose tissue, ghrelin blunts CL-316, 243 induced-lipolysis, and \textit{in vivo} that effect is no longer present (Cervone, unpublished data) or even the opposite effect (cite some of the other human injection studies here). Regardless, it is important to measure \textit{ex vivo}/\textit{in vitro} systems to determine whether the apparent effects of ghrelin in vivo are secondary to other metabolic regulatory points, or independent.

Taken together, the findings from this study may suggest that in isolated rat skeletal muscle, ghrelin isoforms directly stimulate FA uptake (although this remains to be directly assessed), as seen with increases in \textsuperscript{14}C palmitate oxidation, and no changes in FA incorporation into lipids. Furthermore, ghrelin does not independently alter lipolysis. Some additional sites of FA regulation that future studies should look to examine include the FAT/CD36 and CPT1 as these transporters facilitate the entry of FAs across the sarcolemma in skeletal muscle\cite{98} and catalyze the rate-limiting step of FA oxidation. This allows the transport of FAs into the mitochondria for beta-oxidation\cite{135}. Central administration of ghrelin has been shown to alter both FATCD/36 and CPT1 expression in the brain as well as in adipose tissue\cite{136}\cite{137}. Future studies should consider the expression and activity of these transporters when examining ghrelin’s effects on skeletal muscle FA metabolism.
Ghrelin pre and post prandial fatty acid metabolism

In this study, we examined the effects of ghrelin on skeletal muscle FA metabolism in normal (5 mM) and high (10 mM) glucose environments. This allowed us to determine if ghrelin’s effects were specific to pre or post prandial states. This is important as the regulation of glucose and FA metabolism are often reciprocal and it is important to study FA metabolism in the context of different glucose availabilities.

Regulation of fatty acid metabolism at mealtime

It is well established that ghrelin can act as a meal initiation signal, however, other hormones such as leptin and insulin also play an important role in regulating muscle FA metabolism particularly at mealtime. It is likely that in vivo, ghrelin’s effects are influenced by the interaction with these signaling molecules. Plasma ghrelin has a reciprocal relationship with insulin in vivo[39] and in cell cultures ghrelin secreting cells have been shown to express the α and β insulin receptor subunits, suggesting that ghrelin and insulin may interact directly[40]. When cells are incubated with insulin, ghrelin secretion is decreased[40]. However, cells placed in an environment with high insulin do not exhibit suppression in ghrelin secretion, and after 24h the insulin receptor expression is decreased[40]. Interestingly, individuals with T2D have impaired responses to insulin stimulated decreases in plasma ghrelin[41]. Unlike ghrelin, leptin levels do not change acutely prior to meals but have a diurnal variation, increasing throughout the day with slight drops within 1h post meal[34]. Leptin, has been shown to increase oxidation of FAs in lean skeletal muscle[138]. In obese skeletal muscle, the increase in oxidation of FAs with leptin is no longer present[138]. Low circulating ghrelin and high circulating leptin have
been reported with obesity [6]. The interactions between ghrelin, insulin and leptin in vivo may play a key role in the development of metabolic disorders associated with obesity. In order to gain a more complete understanding of ghrelin’s effects investigators should consider the effects of ghrelin in the presence of insulin and leptin.

Limitations and Considerations

Because plasma ghrelin (UnAG) levels increase during fasting and decrease following food intake[139], the nutritional state of the animal is an important consideration. Researchers should consider light and dark[33] phases as well as control for time of meal ingestion and standardize macronutrients as these have also been shown to influence the level of decrease and/or subsequent increase in ghrelin[37]. Although we’ve tried to the best of our ability to control for these factors is it difficult to assure consistent baseline ghrelin levels in the animals at the time of the experimental procedures. Another limitation to this study is our exclusively male rodent cohort. Research has shown that mean circulating ghrelin levels differ significantly between male and females, in both humans and rodents[38][140]. Higher mean circulating levels in females may alter the effect of ghrelin on fatty acid metabolism in skeletal muscle.

The rodents in our study were group housed with adequate bedding at a temperature of 25 degrees Celsius. Future studies should follow a similar practice as housing conditions may play a role in ghrelin regulation. Single housing, which is often used to measure food intake and/or energy expenditure, has been shown to alter adrenal and gonadal function[141]. Both ghrelin and GHSR are expressed in the rat adrenal cortex[142] and changes in adrenal function likely alter ghrelin levels and in turn specific endpoints of interest in whole body rodent models. Housing
temperature of rodents should also be taken into consideration when examining the effects of ghrelin on metabolism. Thermoneutrality can be defined as the range of ambient temperature that does not require an animal to combat the cold by 1) raising heat production and/or 2) evaporative heat loss, in which 3) normal thermoregulation mechanisms are absent[143]. An inverse effect of ambient temperature and food intake were found in animals housed at 30° and 32° C compared to those housed at 20°, 22°, 24° and 26° C[144]. Mice housed in the cooler environment consumed ~37% more food relative to the group housed in the warmer conditions[144]. This is important especially in studies examining the orexigenic effects of ghrelin.

Summary

From the isolated skeletal muscle preparation used, we were able to examine ghrelin’s direct effects on FA metabolism at normal and high glucose concentrations in oxidative and glycolytic muscles. We have demonstrated that AG and UnAG increase FA oxidation in soleus and EDL muscle, but do not appear to alter incorporation of FAs into lipid pools or lipolysis independently. Thus, ghrelin may be an important factor in regulating FA oxidation to provide fuel for tissues and possibly entire systems in vivo. We speculate that the decreases in circulating ghrelin levels observed with obesity may also contribute to impairments in skeletal muscle metabolism. Research examining the effects of ghrelin on skeletal muscle FA metabolism from lean and obese animals is warranted.
REFERENCES


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Appendix I: Fatty acid incorporation into triacylglycerol, diacylglycerol and phospholipids

Table 1 $^{14}$C incorporation into major lipid pools at normal or high glucose concentrations in the soleus muscle

<table>
<thead>
<tr>
<th></th>
<th>SOL 5 mM Glucose</th>
<th>SOL 10 mM Glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>AICAR</td>
</tr>
<tr>
<td>TAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/g/hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>59.8 ± 5.2$^a$</td>
<td>54.4 ± 7.5$^a$</td>
</tr>
<tr>
<td>DAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/g/hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.5 ± 2.9$^b$</td>
<td>24.5 ± 5.0$^b$</td>
</tr>
<tr>
<td>PHOS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/g/hr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>37.6 ± 2.6$^c$</td>
<td>36.7 ± 2.7$^c$</td>
</tr>
</tbody>
</table>

Data is expressed as the mean ± SE. n=7 TAG, DAG, PHOS (5 mM glucose) and n=6 TAG, n=7 DAG, n=7 PHOS (10 mM glucose). Data was considered significant if p < 0.05. Groups not sharing a letter are considered statistically different from each other.
Table 2 \(^{14}C\) palmitate incorporation into major lipid pools at normal or high glucose concentrations in the EDL muscle

<table>
<thead>
<tr>
<th></th>
<th><strong>EDL 5 mM Glucose</strong></th>
<th></th>
<th><strong>EDL 10 mM Glucose</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
<td>AICAR</td>
<td>AG</td>
</tr>
<tr>
<td><strong>TAG</strong> (nmol/g/hr)</td>
<td>38.1 ± 5.0(^a)</td>
<td>30.2 ± 3.2(^a)</td>
<td>38.3 ± 6.2(^a)</td>
</tr>
<tr>
<td><strong>DAG</strong> (nmol/g/hr)</td>
<td>16.6 ± 2.4(^b)</td>
<td>15.2 ± 2.4(^b)</td>
<td>16.8 ± 2.6(^b)</td>
</tr>
<tr>
<td><strong>PHOS</strong> (nmol/g/hr)</td>
<td>32.5 ± 3.0(^c)</td>
<td>31.0 ± 3.6(^c)</td>
<td>33.4 ± 3.5(^c)</td>
</tr>
</tbody>
</table>

Data is expressed as the mean ± SE. n=6 TAG, n=7 DAG and PHOS (5 mM glucose) and n=8 TAG, n=6 DAG, n=7 PHOS (10 mM glucose). Data was considered significant if p < 0.05. Groups not sharing a letter are considered statistically different from each other.
Appendix II: Epinephrine stimulated lipolysis is blunted by AG and unAG

Table 3 The effect of ghrelin isoforms on lipolysis with or without epinephrine

<table>
<thead>
<tr>
<th>Glycerol Release (nM/g tissue/hr)</th>
<th>CON</th>
<th>EPI</th>
<th>AG</th>
<th>UnAG</th>
<th>EPI+AG</th>
<th>EPI+UNAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus 5 mM glucose</td>
<td>15.2 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.8 ± 2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.8 ± 1.7&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>13.0 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.7 ± 2.0&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>15.2 ± 1.0&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soleus 10 mM glucose</td>
<td>17.2 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.7 ± 2.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>20.0 ± 1.4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>19.1 ± 2.0&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>18.5 ± 2.6&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data is expressed as the mean ± SE. n=8 (5 mM glucose) and n=7 (10 mM glucose). Data was considered significant if p < 0.05. Groups not sharing a letter are considered statistically different from each other.
### Appendix III: Diet composition

**Teklad Global 16% Protein Rodent Diet**

**Product Description:** 2016 is a fixed formula, non-irradiated diet manufactured with high quality ingredients and designed to support growth and maintenance. It contains no alfalfa or soybean meal, minimizing the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. For autoclavable diet, refer to 2016S (Sterilizable).

**Ingredients:** (in descending order of inclusion) Ground wheat, ground corn, wheat middlings, corn gluten meal, calcium carbonate, dicalcium phosphate, soybean oil, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganese oxide, ferrous sulfate, zinc oxide, niacin, calcium panthothenate, copper sulfate, pyridoxine hydrochloride, dicalcium phosphate, threonine mononitrate, vitamin A acetate, calcium iodate, vitamin B<sub>12</sub> supplement, folic acid, biotin, vitamin D<sub>3</sub> supplement, cobalt carbonate.

**2016**

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>Standard Product Form: Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>% 16.4</td>
</tr>
<tr>
<td>Fat (ether extract)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>% 4.0</td>
</tr>
<tr>
<td>Carbohydrate (available)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>% 49.5</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>% 3.3</td>
</tr>
<tr>
<td>Neutral Detergent Fiber&lt;sup&gt;c&lt;/sup&gt;</td>
<td>% 15.2</td>
</tr>
<tr>
<td>Ash</td>
<td>% 4.9</td>
</tr>
<tr>
<td>Calories from Protein</td>
<td>kcal/ (kJ)</td>
</tr>
<tr>
<td>Calories from Fat</td>
<td>% 22</td>
</tr>
<tr>
<td>Calories from Carbohydrate</td>
<td>% 12</td>
</tr>
<tr>
<td>Carbohydrate (available)</td>
<td>% 96</td>
</tr>
<tr>
<td>Minerals</td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>% 1.0</td>
</tr>
<tr>
<td>Phosphorous</td>
<td>% 0.7</td>
</tr>
<tr>
<td>Sodium</td>
<td>% 0.2</td>
</tr>
<tr>
<td>Potassium</td>
<td>% 0.6</td>
</tr>
<tr>
<td>Chloride</td>
<td>% 0.4</td>
</tr>
<tr>
<td>Magnesium</td>
<td>% 0.2</td>
</tr>
<tr>
<td>Zinc</td>
<td>mg/kg 70</td>
</tr>
<tr>
<td>Manganese</td>
<td>mg/kg 100</td>
</tr>
<tr>
<td>Copper</td>
<td>mg/kg 15</td>
</tr>
<tr>
<td>Iodine</td>
<td>mg/kg 6</td>
</tr>
<tr>
<td>Iron</td>
<td>mg/kg 200</td>
</tr>
<tr>
<td>Selenium</td>
<td>mg/kg 0.23</td>
</tr>
</tbody>
</table>

| Amino Acids     |                              |
| Aspartic Acid   | % 1.0                         |
| Glutamic Acid   | % 3.3                         |
| Asparagine      | % 0.5                         |
| Glutamine       | % 0.7                         |
| Threonine       | % 0.6                         |
| Valine          | % 1.5                         |
| Isoleucine      | % 0.8                         |
| Leucine         | % 1.9                         |
| Isoleucine      | % 0.7                         |
| Valine          | % 0.8                         |
| Phenylalanine   | % 0.9                         |
| Tryptophine     | % 0.5                         |
| Histidine       | % 0.5                         |
| Arginine        | % 0.8                         |
| Lysine          | % 0.3                         |
| Methionine      | % 0.8                         |
| Cystine         | % 0.8                         |
| Ornithine       | % 0.3                         |
| Tryptophan      | % 0.2                         |

**Vitamins**

| Vitamin A<sub>a</sub> | IU/g 15.0                        |
| Vitamin D<sub>3</sub><sup>a</sup> | IU/g 1.5                      |
| Vitamin E          | IU/g 11.6                      |
| Vitamin K<sub>1</sub> (menadione) | mg/kg 50             |
| Vitamin B<sub>1</sub> (thiamin) | mg/kg 17                  |
| Vitamin B<sub>2</sub> (riboflavin) | mg/kg 15               |
| Vitamin B<sub>3</sub> (niacin) | mg/kg 75                    |
| Vitamin B<sub>5</sub> (pyridoxine) | mg/kg 18               |
| Pantothenic Acid   | mg/kg 33                      |
| Vitamin B<sub>12</sub> (cyanocobalamin) | mg/kg 0.08 |
| Folate             | mg/kg 4                       |
| Choline            | mg/kg 1030                    |

| Fatty Acids |                              |
| C16:0 Palmitic | % 0.5                         |
| C18:0 Stearic | % 0.1                         |
| C18:1ω9 Oleic | % 0.7                         |
| C18:2ω6 Linoleic | % 2.0                      |
| C18:3ω3 Linolenic | % 0.1                  |
| Total Saturated | % 0.8                      |
| Total Monounsaturated | % 0.7                   |
| Total Polyunsaturated | % 2.1                   |

| Other            |                              |
| Cholesterol      | mg/kg —                      |

<sup>a</sup> Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

<sup>b</sup> Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

<sup>c</sup> Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

<sup>d</sup> Energy density is a calculated estimate of metabolizable energy based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrates.

<sup>e</sup> Indicates added amount but does not account for contribution from other ingredients.

<sup>f</sup> 1 IU vitamin A = 0.3 µg retinol

<sup>g</sup> 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

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We hypothesize that the increase in fatty acid oxidation (FAO) observed with ghrelin may be due to the activation/phosphorylation of AMP-activated protein kinase (AMPK) which, in turn, phosphorylates/deactivates acetyl CoA carboxylase (ACC).