The Regulation of Adipose Tissue and Skeletal Muscle Substrate Metabolism by Ghrelin

by

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ABSTRACT

THE REGULATION OF ADIPOSE TISSUE AND SKELETAL MUSCLE SUBSTRATE METABOLISM BY GHRELIN

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Research examining a potential role for ghrelin, the "hunger hormone", in regulating substrate metabolism beyond its classical role in regulating appetite is relatively sparse. This is surprising given that ghrelin reaches its peak circulating concentration just prior to the consumption of macronutrients. Here, we demonstrate a role for ghrelin in the direct regulation of glucose and fatty acid metabolism in isolated skeletal muscle and adipose tissue. Experiments were primarily *ex vivo* to eliminate confounding factors, such as growth hormone release following ghrelin administration *in vivo*. Metabolic measurements were coupled with cellular signalling analyses to give insight into ghrelin's cellular action.

In study one, we investigated whether ghrelin could influence the mobilization of fatty acids from adipose tissue. We speculated, in theory, that the pre-prandial rise in ghrelin would downregulate adipose tissue lipolysis in preparation for a meal. We indeed found that ghrelin inhibited the adrenergic stimulation of lipolysis, although it remains uncertain whether this has physiological mealtime implications, *in vivo*. In the second study of this thesis, the purported role in the literature for ghrelin as a regulator of glucose uptake in skeletal muscle is challenged. Ghrelin was unable to alter skeletal muscle insulin signalling or glucose uptake either independently, or in combination with insulin in isolated muscle. Finally, the third study expanded on previous work from our lab demonstrating that ghrelin stimulates skeletal muscle fatty acid oxidation. We sought to determine whether ghrelin could protect muscle against

reductions to insulin-stimulated glucose uptake and insulin signalling activation acutely caused by saturated fatty acids. While both isoforms of ghrelin appeared to protect insulin signalling, only UnAG was able to preserve insulin-stimulated glucose uptake. UnAG also stimulated palmitate oxidation more effectively than AG. To that end, the preservation of insulin action by UnAG was abolished when fatty acid oxidation was pharmacologically inhibited. Finally, ghrelin's effects on glucose uptake and palmitate oxidation were no longer evident in muscle isolated from high-fat fed rats, implying a resistance to ghrelin. Taken together, the findings in this thesis highlight important new roles for ghrelin in regulating substrate metabolism in adipose tissue and skeletal muscle.

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Abbreviations

AC	Adenylate cyclase
ACS	Acyl-CoA synthetase
ACC	Acetyl-CoA carboxylase
AG	Acylated ghrelin
AgRP	Agouti-related peptide
Akt	Akt/protein kinase B
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
ANS	Autonomic nervous system
APT-1	Acyl protein thioesterase-1
AS160	Akt substrate of 160kDa
AT	Adipose tissue
ATGL	Adipose triglyceride lipase
ATOC	Adipose tissue organ culture
ATP	Adenosine triphosphate
AUC	Area under the curve
β_1,β_2,β_3	$\beta_{1\text{-}3}$ adrenergic receptor
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Ca^{2+}	Calcium
CACT	Carnitine-acylcarnitine translocase

CaMKII	Ca^{2+}/Calmodulin-activated protein kinase 2
cAMP	Cyclic adenosine monophosphate
CD36	Cluster of differentiation 36
CL	CL 316,243
CHO	Carbohydrate
CNS	Central nervous system
CoA	Coenzyme A
CPT-1/2	Carnitine palmitoyl-transferase ½
CRF	Corticotropin-releasing factor
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDL	Extensor digitorum longus
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK-1/2	Extracellular signal-regulated kinase 1/2
FA	Fatty acid
FABP	Fatty acid binding protein
FAS	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
GH	Growth hormone

GHRH	Growth hormone-releasing hormone
GHS	Growth hormone secretagogue
GHS-R	Growth hormone secretagogue receptor
GIR	Glucose infusion rate
GLP-1	Glucagon-like peptide 1
GLUT2	Glucose transport protein 2
GLUT4	Glucose transport protein 4
GOAT	Ghrelin-O-acyl-transferase
GOS2	G_0/G_1 switch gene 2
GPCR	G-protein coupled receptor
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
HDL	High-density lipoprotein
HFD	High-fat diet
HP	High palmitate (2mM)
HSL	Hormone sensitive lipase
ICV	Intracerebroventricular
IKK-β	Inhibitor of nuclear factor kappa-B kinase subunit beta
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IMTG	Intramuscular triacylglycerol
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin receptor

IRS	Insulin receptor substrate
IV	Intravenous
iWAT	Inguinal white adipose tissue
JAK2	Janus kinase 2
K_{ATP}	ATP-sensitive potassium channel
KO	Knockout
LCFA	Long-chain fatty acid
LFD	Low-fat diet
LP	Low palmitate (0.2mM)
M199	Medium 199
MAG	Monoacylglycerol
MGL	Monoacylglycerol lipase
NaOH	Sodium hydroxide
NE	Norepinephrine
NEFA	Non-esterified fatty acid
NF-κB	Nuclear factor kappa-B
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
OMM	Outer mitochondrial membrane
PBS	Phosphate buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PDE	Phosphodiesterase
PDH	Pyruvate dehydrogenase

PDK1	Phosphoinositide-dependent kinase-1
PDK4	Pyruvate dehydrogenase kinase 4
PI3K	Phosphoinositide-3 kinase
PIP2	Phosphatidyl-4, 5-bisphosphate
PIP3	Phosphatidylinositol-3, 4, 5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2
RER	Respiratory exchange ratio
RP	Retroperitoneal
SOL	Soleus
STAT5	Signal transducer and activator of transcription 5
T2D	Type 2 diabetes
TAG	Triacylglycerol
TBC1D4 (AS160)	TBC1 domain family member 4
TCA	Tricarboxylic acid
UCP2	Uncoupling protein 2
UnAG	Unacylated ghrelin
WAT	White adipose tissue

Introduction to Literature Review

Cellular metabolism requires energy in the form of adenosine triphosphate (ATP) and the coordinated release of hormones like insulin, glucagon and catecholamines regulate the net availability of substrates for oxidation in peripheral tissues such as adipose tissue (AT), liver and skeletal muscle. Glucose is required by nearly all cells in the body and the maintenance of blood glucose homeostasis in a narrow range (~4-7mM) is of primary importance. In the absence of food (e.g. fasted and postabsorptive states), systemic glucose is replenished by liver glycogenolysis and gluconeogenesis.

The utilization of blood glucose may be spared in certain tissues such as skeletal muscle which comprises a large portion of total body mass. Skeletal muscle accounts for a significant percentage of whole-body resting energy expenditure and requires adequate substrate to meet its energetic demands. Both adipose tissue-derived and endogenous muscle lipids can provide an alternate fuel for oxidation. This is crucial as the collective demand for energy by the body's tissues would otherwise exceed that which can be provided solely by circulating glucose and the finite carbohydrate reserves in the body, particularly over a prolonged period.

Postprandially, skeletal muscle is the primary sink for insulin-stimulated glucose clearance. Insulin is an anabolic hormone secreted from β-cells of the pancreas following glucose ingestion that is responsible for governing a myriad of metabolic processes in peripheral tissues. In skeletal muscle, insulin promotes the uptake and utilization of glucose while concurrently inhibiting glucose-sparing pathways in adipose tissue e.g. lipolysis. Importantly, insulin action becomes impaired in obesity and type 2 diabetes (T2D) whereby the ensuing disturbances to glucose homeostasis carry negative long-term consequences that affect multiple organs.

There is ongoing interest in discovering the therapeutic potential of pharmacological or hormonal compounds to mitigate the dysregulation of glucose and FA metabolism in peripheral tissues that become unresponsive to insulin. Interestingly, peripheral tissue substrate uptake and utilization following a meal is typically attributed to the postprandial rise in insulin. However, a growing body of evidence suggests that other hormones may also act as important regulators. Ghrelin, coined the "hunger hormone", has been recently implicated through its ability to influence substrate metabolism, beyond its classical roles as an orexigenic and growth hormone-stimulating peptide. In humans, ghrelin reaches its peak circulating concentrations immediately prior to food intake, which has led researchers to consider ghrelin as a preparatory signal and regulator of metabolism of ingested nutrients in several metabolic tissues. To that end, this literature review will delve into classical ghrelin literature and expand on the more recent findings regarding ghrelin's ability to alter substrate handling in the periphery, namely skeletal muscle and adipose tissue.

Chapter 1: Literature Review

1.0 Stomach hormone: ghrelin

Ghrelin was first isolated from the stomach of rats in 1999, and its name is of Proto-Indo-European derivation, whereby “ghre” means grow and “relin” refers to release (125). Accordingly, ghrelin was first documented to stimulate GH release, prior to establishing its effects on food intake and metabolism. Ghrelin circulates in two forms: acylated (AG) and unacylated (UnAG) ghrelin. Although UnAG was first believed to be devoid of biological activity, a plethora of work (30, 79, 154, 168) including some of our own (32, 34, 131) has demonstrated otherwise.

1.1 Discovery of Ghrelin

Orphan receptors i.e. receptors to which a ligand has not yet been identified, are a critical tool utilized in the discovery of bioactive molecules and in pharmacology (41, 105, 242). For G protein-coupled receptors (GPCRs), a cell line expressing the orphan GPCR is first established. A peptide is then applied to the cell while monitoring secondary messenger (e.g. cAMP, Ca^{2+}) responses. Several years ago, among numerous GPCRs awaiting investigation, the growth hormone secretagogue receptor (GHS-R) was of interest as its endogenous ligand could offer therapeutic value in the treatment of GH-deficiency. Kojima and Kangawa were successful in discovering a potent ligand for the GHS-R subtype 1a (GHS-R1a), the innate gastric hormone, ghrelin (125).

1.2 Source of Ghrelin and Ghrelin Receptor (GHS-R) Distribution

Endogenous ghrelin originates from endocrine P/D1 cells in humans and A-like cells (often termed X/A-like cells) in rats (182). In adults, this cell type represents about 20% of submucosal cells of the gastric oxyntic gland and they develop early in fetal life (51, 182) Ghrelin is also produced to a much lesser extent in other parts of the intestinal tract (192). Careful analysis of these gastric endocrine cells has revealed that ghrelin is largely secreted directly into blood vessels supplying systemic circulation rather than in the GI tract (51).

Two ghrelin receptor (GHS-R1) subtypes exist: GHS-R1a (full-length) and GHS-R1b (truncated), generated by the alternative splicing of a single gene (151). GHS-R1a is the functionally active receptor whereas GHS-R1b lacks the capacity for high-affinity ligand binding and signal transduction (151, 158). However, recent work has shown that GHS-R1b may be essential in the regulation of ghrelin-induced GHS-R1a trafficking and signalling (151, 158). GHS-R1a is predominantly expressed in the hypothalamus and the nearby pituitary gland, accounting for ghrelin's role in appetite and GH stimulation, respectively. However, GHS-R1 mRNA is detectable in several peripheral tissues, including the heart, adipose tissue, liver and skeletal muscle, and binding of endogenous and synthetic agonists appears to be both reversible and saturable (117, 228). Interestingly, changes to GHS-R1 content occurs with aging, lending to the idea that ghrelin action may be altered in different physiological states (141). The ability for chronic dietary manipulation to alter GHS-R1 expression remains inconclusive (33).

1.3 Post-translational modification of ghrelin

Within the human genome, the gene coding for ghrelin is located on chromosome 3 and the post-translational protease cleavage of pro-ghrelin produces the 28-amino acid ghrelin (126). Ghrelin can be further modified by acylation at Ser^3 by an n-octanoyl group to produce the

acylated form (209). The enzyme ghrelin-O-acyl-transferase (GOAT; a membrane-bound *O*-acyltransferase) catalyzes this post-translational modification with the provision of octanoyl-CoA, a product of dietary caprylic acid (medium chain FA) metabolism (88, 137, 249). This acylation reaction occurs in the lumen of the endoplasmic reticulum (ER) (212). In humans, ghrelin is the only known peptide hormone in which the hydroxyl group on one of its serine residues is acylated by a FA (126).

Ghrelin is a highly conserved protein across species, pointing towards an important evolutionary role, although, the amino acids at positions 11 and 12 as well as the ratio of circulating unacylated to acylated ghrelin are strikingly different in rats and humans. The UnAG/AG ratio is ~3:1 in humans but nearly 9:1 in rats (219). The higher concentrations of UnAG likely owe to its longer half-life, the systemic conversion of AG to UnAG as well as binding of AG to GHS-R1a *in vivo*. Concentrations of AG range from ~50-1600pg/ml in humans but nearly ~1000-8000pg/ml in rats, depending on age, sex, strain and method of collection and analysis (48, 143, 223, 245). UnAG appears to circulate in plasma mainly unbound, whereas AG has been shown to bind larger plasma proteins, particularly high-density lipoprotein (HDL) species, at least *in vitro* (15).

1.4 Regulation of circulating ghrelin isoform concentrations

Although not the primary focus of this literature review, it is important to consider that ghrelin's potential metabolic role may stem from the concentrations of its circulating isoforms. Since there has been no evidence for ghrelin acylation in serum (219), its concentrations appear to be controlled by a finely-tuned balance between ghrelin synthesis and release from gastric cells, acylation by GOAT and deacylation by circulating enzymes. In short, ghrelin kinetics can be described as a large pre-prandial rise and immediate postprandial return to baseline, and a

multitude of factors have been shown to impact these patterns (48, 143). Complicating matters, the autonomic nervous system (ANS), vagal activity, gastric emptying rate and postprandial changes to intestinal osmolarity have all been implicated in the regulation of ghrelin mealtime kinetics (23, 73, 129).

1.4.1 Acute and chronic conditions affecting ghrelin concentrations

Ghrelin promoter activity appears to be stimulated in gastric cells by the fasting hormones glucagon (human) and NE (rat) (73, 121). Additional work substantiates these claims by demonstrating that ghrelin secretion from mouse ghrelinoma cells involves β_1AR-stimulation (253). Collectively, these findings suggest that ghrelin synthesis and release are largely driven in scenarios of energy deficit. In conditions of prolonged energy deficit (e.g. anorexia, cachexia) as well as in energy surplus i.e. obesity, total circulating ghrelin concentrations appear to be increased and decreased, respectively (161, 224). In the obese, the reduction in total ghrelin concentrations is largely attributable to a decline in UnAG (71).

1.4.2 Fasted ghrelin release

Challenging the notion that ghrelin is a fasting hormone is data from continuous intravenous (IV) sampling in humans which has shown that concentrations of AG decline when participants are fasted ~42.5 h i.e. a scenario of elevated glucagon and catecholamine concentrations (143). However, UnAG concentrations remained similar to pre-prandial levels, resulting in comparable concentrations of total ghrelin between fed and fasting admissions (143). This is in line with other findings in 2 d fed and 3 d fasted humans where no significant difference in total ghrelin was observed (35). It may be that during fasting, gastric medium chain FAs are absent or insufficient for ghrelin acylation, although this fails to explain the attenuated rise in UnAG. Taken together, these findings suggest that ghrelin may not be a fasting hormone

per se. Moreover, ghrelin acylation and secretion are likely, to some extent, controlled independently.

1.4.3 Postprandial decline – macronutrients and energy intake

The exact mechanisms accounting for the postprandial decline in ghrelin remain under-explored, though several nutrient and hormonal factors have been suggested. At physiological levels, neither glucose nor insulin reduce ghrelin concentrations following an overnight fast (73, 195). In healthy, non-obese humans, ghrelin levels drop following nutrient ingestion in a manner that is proportional to the total ingested caloric load (129). By providing participants with isoenergetic meals, some research has also demonstrated that the decline in ghrelin may depend on the predominant macronutrient being consumed (129, 144, 184). In healthy individuals, carbohydrate (CHO) feeding appears to be most effective in suppressing ghrelin levels, possibly due to a greater rate of gastric emptying (144). In contrast, in the obese, the postprandial decline in ghrelin may be more responsive to high protein/fat than to CHO. Importantly, protein feeding appears to suppress ghrelin levels much longer than CHO, highlighting the complexity of measurements of satiety (20, 129). Perhaps more important, is the potential for a distinct overshoot in ghrelin levels in response to acute carbohydrate, but not fat or protein feeding (69).

1.4.4 GOAT, acylation and deacylation

GOAT is a key regulator of acylation, and therefore the acylated to unacylated ghrelin ratio in circulation. In general, the expression and activity of human GOAT is widespread, partially resembling the pattern of ghrelin expression (88, 140), with elevated levels being observed in tissues such as the stomach and adrenal gland but also peripheral tissues such as muscle and adipose tissue (140). Energy deficit appears to be a key signal to increase GOAT expression (83). The pursuit of GOAT inhibitors with the potential to regulate AG concentration

in circulation have been of significant clinical interest in parallel with the discovery of the orexigenic and various metabolic actions of ghrelin (13, 68, 119).

The amount of UnAG directly released from the stomach has been shown to be comparable to that of AG (152). Thus, since most circulating ghrelin is unacylated, it is reasonable to suggest that ghrelin is easily deacylated in circulation. The enzymes responsible appear to be less specific to ghrelin *per se*, and include various butyrylcholinesterases and several platelet-esterases (194, 238). Acyl protein thioesterase-1 (APT-1) has recently surfaced as a strong candidate enzyme for ghrelin deacylation, but overall, deacylation enzymes specific to ghrelin have yet to be identified and the regulation of ghrelin deacylation has similarly been relatively unexplored (193).

1.5 Growth hormone secretion

GHS-R1a is highly expressed in the pituitary gland and represents a major target for ghrelin. Prior to discovering that AG stimulates appetite, it was revealed that AG potently stimulates *in vivo* GH secretion via GHS-R1a in rats (125) and humans (211). Briefly, the acute administration of GH leads to a net increase in substrate mobilization and whole-body insulin resistance (162, 164, 210). Although the exact mechanisms are unclear, the effects of GH on metabolism are multi-faceted and involve anti-lipogenic action in AT and increases to hepatic glucose production (HGP) (150, 162, 237). These widespread effects should be considered when placing ghrelin's metabolic action into physiological context and collectively render it difficult to interpret ghrelin's metabolic effects *in vivo*.

1.5.1 Signalling for growth hormone release

Growth hormone secretagogues (GHSs), like AG, signal through GPCR's. In contrast to growth hormone-releasing hormone (GHRH) which binds the GHRH receptor and increases

intracellular cAMP, GHSs bind distinct receptors (GHS-Rs), and increase intracellular Ca^{2+} through an inositol 1,4,5-triphosphate (IP3) signal transduction pathway (31, 124). AG-bound GHS-R1a activates phospholipase C (PLC) tethered to inner membrane receptors (38). PLC catalyzes the hydrolysis of membrane phospholipids such as phosphatidylinositol 4,5-diphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG) (38). IP3 can bind the IP3-receptor, a ligand-gated Ca^{2+} channel of the endoplasmic reticulum, which triggers Ca^{2+} release into the cytosol (124). Ca^{2+} causes fusion of GH secretory vesicles to the plasma membrane, followed by exocytosis. It is possible that ghrelin's effects on peripheral tissues are also mediated by this rise in intracellular Ca^{2+}, but this has yet to be determined. To date, there has been little work examining whether UnAG can modulate GH secretion.

1.6 Central nervous system effects

Ghrelin is the only known orexigenic, endocrine hormone (48). In animals, both peripheral IV and central intracerebroventricular (ICV) administration of AG can stimulate food intake (245, 246). Providing exogenous AG to humans also acutely increases caloric intake (244). In rodents, the appetite-stimulating effects of AG can be abolished by atropine administration or vagotomy (53), indicating that ghrelin may regulate these physiological processes, in part, through the vagal system. In the hypothalamic arcuate nucleus, AG directly activates neuropeptide Y (NPY) and Agouti-related protein (AgRP) neurons to stimulate appetite (54). Ghrelin's action in the CNS opposes that of leptin, an endocrine hormone that signals for satiety, in addition to other important metabolic functions (123). Interestingly, ICV administration of UnAG may counteract the ability for AG to stimulate appetite, although whether this occurs physiologically is uncertain (66, 108). A single study indicates that UnAG may induce food intake independent of GHS-R1a, although this has not been replicated (222).

2.0 Hormonal regulation of glucose and lipid metabolism: a role for ghrelin

2.1 Glucose Homeostasis

Circulating glucose concentrations are tightly regulated and glucose provides an energy substrate for most cells in the human body. Glucose homeostasis is achieved in a complex but coordinated manner. A network of neuronal and hormonal interplay collectively mitigate disturbances to energy balance (e.g. mealtime, exercise) and ensure the provision of glucose to vital organs like the brain (191). Insulin and glucagon are pancreatic hormones responsible for maintaining glucose homeostasis through their widespread effects on peripheral tissues.

Glucagon is secreted from pancreatic α-cells during fasting and can directly increase HGP; the sum of liver glycogenolysis and gluconeogenesis (76). Glucagon also stimulates the hydrolysis TAG stores from AT to supply the liver with gluconeogenic precursors e.g. glycerol (96, 170). In contrast, insulin is released in the postprandial state from pancreatic β-cells following increases to extracellular glucose concentration (186). Insulin is released into portal circulation, first encountering the liver, where it can both directly and indirectly downregulate HGP (80). Indirectly, insulin reduces the provision of gluconeogenic precursors to the liver by inhibiting AT lipolysis and skeletal muscle proteolysis, while concurrently lowering glucagon secretion from the pancreas (45, 62, 231). Finally, insulin also stimulates the uptake and storage or utilization of glucose in adipose tissue and skeletal muscle (55). Insulin-stimulated glucose disposal in skeletal muscle accounts for upwards of ~80% of glucose clearance from blood during an OGTT (55). Insulin's role in the maintenance of systemic glucose homeostasis is indispensable. Insulin action is altered in several pathologies. In the pathogenesis of T2D,

decreases in muscle glucose uptake and insulin signalling protein phosphorylation are key, early defects (189).

2.1.1 Insulin secretion

In pancreatic β-cells, glucose is transported into the cytosol via GLUT2 and oxidized to yield ATP, resulting in the subsequent closure of K_{ATP} channels, halting potassium efflux (186). The resultant depolarization and opening of voltage-gated Ca^{2+} channel's leads to the trafficking and exocytosis of insulin-containing granules (186). Glucose-stimulated insulin secretion is one element of glucose metabolism that becomes impaired in type 2 diabetics (149).

2.1.2 Regulation of insulin secretion by ghrelin

In both rats and humans, AG can modulate insulin release from the pancreas, although findings have been equivocal (22, 52, 61). Ghrelin has been observed to co-localize with glucagon in pancreatic α-cells, in both rats and humans (52). Interestingly, AG may increase cytosolic Ca^{2+} and participate in the stimulation of insulin secretion in isolated pancreatic islets from rodents, but only at "stimulatory" levels of glucose e.g. ~8mM (52). Other's have supported these findings with work in isolated pancreatic cells from both healthy and diabetic rats (3). However, when rat pancreas is perfused *in situ*, ghrelin has been observed to blunt glucose-stimulated insulin secretion, highlighting its complexity as a hormonal regulator (61, 84). To that end, acute ghrelin infusion in humans also leads to reductions in insulin secretion, but not when co-infused with UnAG (22, 24). In conclusion, interpreting the effects of ghrelin on insulin secretion and subsequent action is difficult. The discrepancies between studies may be due to the dose and form(s) of ghrelin administered, the concentrations of glucose, and the experimental model. Logically, it would appear catastrophic for ghrelin to directly signal for insulin release in the event a meal is missed, although more work is warranted.

2.2 Skeletal Muscle Glucose Metabolism

2.2.1 Insulin signalling

The hallmark effect of insulin is its regulation of glucose transport protein 4 (GLUT4) trafficking and glucose uptake in insulin-responsive tissues. Activation of the insulin signalling cascade involves a ligand-receptor interaction and downstream signalling protein events that have been extensively studied since insulin's discovery (115). Muscle insulin action can be inferred through intermediary insulin signalling protein activation and the effectiveness by which insulin stimulates glucose uptake.

Insulin initiates its effects in all insulin-responsive tissues by binding the insulin receptor (IR) on the cell surface. Insulin binds the α ligand-binding subunit and subsequent activation of the β tyrosine protein kinase-subunit leads to its autophosphorylation and activation of the receptors intrinsic tyrosine kinase activity (87, 115). The activated tyrosine kinase phosphorylates insulin-receptor substrate (IRS) protein. IRS proteins then recruit phosphatidylinositol-3-kinase (PI3K) which catalyzes the formation of phosphatidylinositol (3,4,5)P3 (PIP3) from PIP2 near the plasma membrane (190). PIP3 serves as a docking site for phosphoinositide-dependent kinase-1 (PDK1) and the serine/threonine kinase Akt (or protein kinase B; PKB). Akt can be phosphorylated at residues Ser^{473} (PI3K) and Thr^{308} (PDK1) to upregulate its activity. Most, if not all of insulin's metabolic effects appear to involve Akt, making this protein a useful cellular readout for insulin action (190). Akt activation leads to the Akt-dependent phosphorylation of several proteins, including the Rab GTPase activating protein Akt-substrate of 160kDa (AS160/TBC1D4) which links insulin signalling events through Akt with membrane trafficking events for GLUT4 translocation (163, 190).

2.2.2 Regulation of insulin signalling by ghrelin

The direct effects of ghrelin on insulin signalling remains sparse. A handful of studies have examined key proteins of the insulin signalling cascade (IRS-1, Akt, AS160) in response to ghrelin administration, although these are often measured following chronic ghrelin injection or infusion in the absence of insulin, questioning the physiological relevance of these findings (11, 79, 232). Ghrelin levels would normally be declining in the circulation as peripheral tissues are beginning to see insulin and have substrates delivered from a meal, such that potential overlapping cellular effects by these hormones should be considered. In general, ghrelin's regulation of muscle insulin signalling differs depending on the experimental model and route of administration and studies that administer UnAG have more consistently produced beneficial effects on insulin action.

Four days of subcutaneous AG injection has been shown to increase levels of phosphorylated Akt (11). In a different study using UnAG, which implemented the same 4d injection protocol, increased activation of both IRS-1 and Akt was observed in rat gastrocnemius muscle (30). However, when directly administered to isolated muscle, data from our own lab have not revealed any acute regulation of Akt by ghrelin isoforms (32).

In humans, two studies by the same researchers have demonstrated that the acute infusion of AG has no effect on insulin-stimulated Akt or AS160 phosphorylation, although the independent i.e. non-insulin-stimulated regulation of these proteins by ghrelin was only assessed in one of these studies (232, 235). Interestingly, AG infusion causes peripheral insulin resistance in hypopituitary patients, as evidenced by a reduced glucose infusion rate (GIR) during a hyperinsulinemic-euglycemic clamp (235). These individual's lack the AG-mediated increase in GH suggesting that the insulin resistance was directly consequent to AG action. Reductions to

insulin action following AG infusion *in vivo* are often attributed to the secondary rise in GH, and subsequently FAs which acutely impair insulin action (176). To that end, there is evidence to suggest that the pharmacological blockade of GH-induced rises in FAs (using acipimox) can preserve insulin action (164). Surprisingly, these reductions to whole-body insulin action were not paralleled by a decline in Akt (Ser^{473} or Thr^{308}) activation, although other markers of insulin action were not examined (235). It is worth noting that AG infusion has also been shown to reduce insulin action in healthy participants, but insulin signalling was not assessed (233).

2.2.3 Insulin-stimulated glucose uptake

GLUT4 was initially discovered as being highly expressed within adipose tissue and muscle (112). Over the years, pivotal work first in adipocytes, then later in skeletal muscle, has deepened our knowledge of GLUT4 by demonstrating that it is trafficked intracellularly to the plasma membrane/sarcolemma (49, 122). Most GLUT4 resides in vesicle pools that are recruited to the sarcolemma and t-tubules by contraction and insulin-stimulation (in muscle), pathways that are mediated by independent signalling cascades (113). In both skeletal muscle and AT, this insulin pathway involves the activation of Akt and subsequently AS160 to rapidly but transiently increase in the rate of glucose uptake (113). Interestingly, only a small portion of total cellular Akt and downstream AS160 need to be activated to elicit maximal GLUT4 translocation (98, 163). Insulin also activates pyruvate dehydrogenase (PDH), the terminal glycolytic enzyme responsible for irreversibly committing pyruvate to the tricarboxylic acid (TCA) cycle through its conversion to acetyl-CoA. Insulin activates PDH by increasing pyruvate dehydrogenase phosphatase activity, which keeps PDH in its active form (57). Thus, insulin collectively signals for GLUT4 translocation, increases skeletal muscle glucose uptake, glycolytic flux and glucose oxidation.

2.2.4 Regulation of glucose uptake by ghrelin

Ghrelin surges before mealtime suggesting that it may facilitate skeletal muscle's postprandial clearance of glucose in a coordinated effort with insulin, which rises postprandially. In C2C12 myocytes, both AG and UnAG stimulate glucose uptake in a dose-dependent manner (79). However, in this study the muscle cells were cultured with ghrelin for upwards of 24h which questions whether this would occur *in vivo* (79). Also, the direct stimulation of muscle glucose uptake by ghrelin *in vivo* would seem perplexing in the event that a meal is missed, leading to potential ghrelin-induced hypoglycemia. In fact, emerging evidence suggests that AG is part of a counterregulatory response to defend against hypoglycemia (198) and AG continually rises in response to a single missed meal, in obese individuals (40). Two studies have provided evidence that chronic muscle exposure to AG may increase GLUT4 gene expression (11, 79).

Data from our own lab provides evidence that in mature, isolated EDL and soleus skeletal muscle, ghrelin does not acutely affect basal or insulin-stimulated glucose uptake (32). In contrast, the 4d (2x daily) injection of UnAG has been shown by others to enhance insulin-stimulated glucose uptake in rat gastrocnemius, pointing towards a role for prolonged ghrelin exposure in the regulation of muscle's response to insulin (30). Interestingly, work in rat myoblasts has shown that AG prevents palmitic acid-induced reductions to insulin-stimulated glucose uptake, suggesting that the availability of FAs, which are known to affect insulin response and glucose uptake (176), impact ghrelin's regulatory role on glucose metabolism in muscle (91). These findings are consistent with data in rat soleus muscle from our lab that demonstrate a protective role for both ghrelin isoforms on FA-induced decrements to insulin-stimulated glucose uptake (33).

In humans, the infusion of AG during a hyperinsulinemic-euglycemic clamp results in a worsening of insulin action i.e. reduced GIR (234). The infusion of UnAG in humans is often purported in the literature as being beneficial towards glucose homeostasis, however this may solely be the case when co-infused with AG (77). The prevailing mechanisms in skeletal muscle during ghrelin isoform co-infusion remain uncertain but receptor competition is one possibility. Collectively, more work is required to fully elucidate ghrelin's role in muscle insulin action. The effects of chronic ghrelin administration in humans has yet to be explored. Moving forward, careful attention should be paid to the route of ghrelin administration, nutrient and hormonal conditions and the experimental model. Clearly, data from both humans and *ex vivo* work provide evidence that ghrelin isoforms are likely to directly alter insulin action in muscle i.e. independent of GH (32, 235).

Incretins are intestinally-derived hormones that regulate glucose homeostasis by potentiating insulin secretion or directly affecting muscle glucose handling (29). In lean and obese mice, the injection of AG prior to an OGTT has been shown to enhance glucose-stimulated incretin (glucagon-like peptide-1; GLP-1) and insulin release to improve glucose clearance (74). These effects were abolished with GHS-R1a antagonism and GLP-1-null mice, highlighting the importance of the incretin response to the observed beneficial effects of AG (74). These findings have been tested in humans by assessing glucose clearance following a mixed meal in healthy, young men and women. Expectedly, as others have shown using glucose infusion (234), AG worsened glucose tolerance following the mixed meal, whereas the combined administration of AG and Ex9 (a GLP-1 receptor antagonist) exacerbated these effects, when compared to either alone (166). Despite these studies that provide a strong link between the gastric and enteric systems in the regulation of mealtime glucose metabolism, the interplay between incretins and

ghrelin, especially UnAG is still very much unexplored. A more recent study challenges any involvement of ghrelin in the direct regulation of GLP-1 secretion, by demonstrating that supraphysiological levels of AG do not increase 2 h GLP-1 AUC in hypopituitary patients (negating any AG stimulated rise in GH) nor do they significantly increase GLP-1 output from perfused mouse small intestine compared to glucose alone (114).

2.3 Skeletal Muscle Fatty Acid Metabolism

Long-chain FAs serve numerous roles in cellular biology, spanning from their effects in biological membranes to their regulation of gene expression, use as a substrate for mitochondrial oxidative phosphorylation and ultimately, energy provision. It had long been thought that long-chain FAs were freely diffusible across the plasma membrane (90). However, in the last 20 years it has been demonstrated that a significant proportion are actively transported via saturable, protein-mediated mechanisms that involve several FA binding proteins (transporters) such as the fatty acid translocase (FAT)/cluster of differentiation 36 (CD36), as well as others (e.g. FATPs, $FABP_{pm}$) (225, 226). The expression of these transport proteins correlates with the oxidative capacity of the tissue i.e. higher in oxidative versus glycolytic skeletal muscle, and the discovery of a protein-mediated system for long-chain FA transport in skeletal muscle has led researchers to consider whether this system altered in pathological states of increased FA availability e.g. obesity or T2D (101).

2.3.1 Fatty acid transport and oxidation

Like GLUT4, FA transporters exist in part, within intracellular vesicle pools and their movement to the sarcolemmal membrane may be stimulated by insulin and contraction (75, 146). Insulin-stimulated translocation of FA transporters also requires the activation of the PI3K/Akt-dependent pathway (146). However, not all transport proteins are insulin-responsive (81, 111).

FAT/CD36 has gained considerable attention as it represents a highly regulated component to the muscle FA transport system. Both acute and chronic stimuli (e.g. muscle stimulation, training) increase its expression at the sarcolemma and muscle-specific FAT/CD36 overexpression leads to an increase in FA uptake and oxidation (107). A selective protein-mediated mechanism for FA uptake into skeletal muscle may ensure that FA uptake is adequate and matched to cellular metabolic demands (81). Accordingly, research is ongoing to determine whether FA transport becomes dysregulated in several metabolic pathologies.

Once FAs enter the cytosol, they are converted to fatty acyl-CoA by the enzyme acyl-CoA synthetase (ACS). However, the ability to enter the mitochondria for oxidation requires their conversion to fatty acyl-carnitine derivatives via carnitine palmitoyltransferase-1 (CPT-1). The FAs are shuttled through the outer mitochondrial membrane (OMM) and then through the inner mitochondrial membrane (IMM) via the carnitine-acyl carnitine translocase (CACT). Within the mitochondrial matrix, fatty acyl-carnitine is re-converted to fatty acyl-CoA by carnitine palmitoyltransferase 2 (CPT-2), to then enter β-oxidation which liberates carnitine allowing it to return to the inner mitochondrial space (IMS) or to the cytosol. β-oxidation yields acetyl-CoA which enters the citric acid cycle to generate ATP.

2.3.2 Lipid metabolites and insulin action

Despite short-term adaptations that increase muscle's capacity to transport and oxidize lipids during high-fat feeding (28), the mismatch between lipid availability (substrate provision) and metabolic rate (substrate demand) can result in excess FAs being deposited. During acute lipid oversupply, or following high-fat feeding, FA deposition can contribute to the expansion of various lipid pools within muscle, such as TAGs, DAGs, ceramides and membrane phospholipids (155, 204). TAGs are typically considered relatively inert, but the accumulation of

other reactive lipid intermediates like DAGs and ceramides is a finely regulated process that carries large physiological implications in cellular signalling, health and disease (155, 204). Both DAGs and ceramides have been shown to directly impair the activation of insulin signalling proteins. DAGs modulate PKC-isoforms, which in turn serine phosphorylate IRS-1 to negatively regulating its effects (37, 110). In myocytes, the effects of DAGs on insulin signalling are likely via PKC-θ, which then activate the IKKβ-NF-κB pathway (42, 110). Following lipid infusion *in vivo,* increases to DAG and NF-κB pathway activation are accompanied by PKC-β/δ activation, but not PKC-θ (110). Ceramides negatively regulate insulin's effects by activating the serine/threonine phosphatase PP2A, which can then dephosphorylate insulin signalling proteins (207). Importantly, the accumulation of these muscle lipids are altered in the obese, diabetics and by chronic aerobic training (5).

2.3.3 Regulation of fatty acid metabolism in muscle by ghrelin

Given its collective mass, skeletal muscle can contribute upwards of 20% of resting metabolic rate (240). As such, its selection of fuel can have significant whole-body ramifications. Reductions to whole-body fat utilization i.e. increases to RER have been linked to the seminal findings in rodents that showed increases to adiposity with central AG administration (223). In contrast, AG antagonizes acipimox-induced (antilipolytic agent) reductions to whole-body lipid oxidation i.e. increases lipid oxidation in humans, although no independent effect of AG was shown (235). The direct exposure of rat soleus muscle to both ghrelin isoforms has been shown to increase exogenous oxidation of FAs, at physiological concentrations (1-2mM) (33, 131). Collectively, it is likely that ghrelin isoforms increase FA utilization in skeletal muscle (235). Whether this occurs physiologically to stimulate the oxidation of ingested lipids remains to be seen. In addition, ghrelin's influence on FA utilization may depend on the tissue. For example,

the subcutaneous injection (4d, twice daily) of AG in rats reduces TAG content in gastrocnemius, but not soleus or liver (11). This also suggests that ghrelin's effects may be fiber-type specific (11).

2.3.4 High fatty acid availability, lipid intermediates and ghrelin

Despite evidence that ghrelin does not directly regulate glucose uptake (see Section 2.2.4), clinical evidence negatively correlates ghrelin levels, particularly UnAG, with insulin resistance in conditions of elevated FA availability, like obesity and T2D (32, 116). The first study to directly examine the metabolic action of ghrelin in muscle during elevated FA exposure was in 2015, by Han *et al.* who observed a reduction in TAG content in myocytes following exposure to AG (91). The researchers further demonstrated that AG protected myocytes from the acute palmitate-induced impairment to intracellular glucose uptake (91). Similarly, a recent study by our own lab demonstrated that UnAG could preserve insulin-stimulated signalling and glucose uptake in mature skeletal muscle from rats during the exposure of muscle to high FA concentrations that are similar to what would be observed following lipid infusion (2mM) (33). This protective effect was likely due to UnAG acting as a stimulator of FA oxidation, and importantly, this preservation of insulin action during high FA exposure was lost in muscle from high-fat fed animals (33). Whether any of the beneficial effects of ghrelin on insulin action are attributable to its regulation of lipid intermediates remains uncertain. To date, only one study has investigated the direct effects of ghrelin isoforms on FA deposition into TAG, DAG and phospholipids with no changes being observed (131). Pinpointing any additional mechanisms that may account for the beneficial effects of ghrelin on insulin action during elevated FA availability is an important avenue for future work.

2.3.5 Cellular signalling

It has recently been shown that ghrelin's orexigenic and peripheral tissue effects may act in part through AMPK activation (127, 128, 131). AMPK is a heterotrimer complex that has energy-sensing and regulating effects on cell metabolism in various metabolic tissues (205). AMPK can be activated by stressors that transiently deplete cellular ATP and increase AMP (205). Subsequent phosphorylation of its α-subunit on Thr^{172} by upstream kinases activate and transiently contribute to an increase in AMPK activity, reducing acetyl-CoA carboxylase activity (via phosphorylation) to reduce malonyl-CoA content and relieve its inhibitory effects on mitochondrial CPT-1. Other findings challenge ghrelin as a direct regulator of the AMPK/ACC axis, necessitating studies using AMPK-knockout animals (32, 233). The mechanism by which ghrelin-mediated increases to AMPK/ACC activation arise are still unclear.

Putative ghrelin receptor expression is abundant in the tissues that account for the hallmark effects of ghrelin i.e. pituitary gland and arcuate nucleus of the hypothalamus. There is uncertainty with respect to the cellular action of ghrelin in metabolic tissues like skeletal muscle and AT. There has yet to be any confirmed cellular signalling pathway for AG or UnAG effects in peripheral tissues, making it difficult to confirm ghrelin's cellular action.

2.4 Adipose Tissue Metabolism

2.4.1 Fatty acid mobilization (lipolysis)

AT is an important source of FAs for several tissues and during times of negative energy balance, AT hydrolyzes TAG reserves largely for the provision of FAs to other tissues. AT lipolysis is a highly regulated, multi-step, enzymatic pathway. Various hydrolytic enzymes can act on TAG-containing lipid droplets. These enzymes are subject to regulation by hormones and other factors that are influenced by energy balance, exercise and feeding. Briefly, three lipase

enzymes are responsible for the complete hydrolysis of one TAG molecule. TAG conversion to DAG by the enzyme adipose triacylglycerol lipase (ATGL), DAG to MAG by the enzyme hormone-sensitive lipase (HSL) and MAG hydrolysis to its constituent three FAs and single glycerol molecule by monoacylglycerol lipase (MGL) (60). More recently, other lipid droplet-associated proteins (e.g. perilipins) have been implicated in the regulation of TAG hydrolysis (109).

2.4.2 Adrenergic receptors and lipolytic enzymes

Increased whole-body rates of lipolysis are attributable to elevated levels of circulating catecholamine's like epinephrine, as well as increased glucagon. The autonomic nervous system also directly innervates AT depots and the sympathetic-mediated release of norepinephrine (NE) from nerve terminals can influence AT lipolysis (14, 185). Depending on the receptor, stimuli may be pro- ($\beta_{1\text{-}3}$-ARs) or anti- (α_2-ARs) lipolytic (133). For example, epinephrine has a high affinity for β_2-ARs on AT plasma membrane which increase the activity of adenylate cyclase and subsequently, intracellular levels of cAMP (43). cAMP can activate PKA which phosphorylates HSL at various amino acid residues, notably Ser^{660} and Ser^{563} (6). These phospho-modifications increase HSL translocation to the lipid droplet and the rate of TAG/DAG hydrolysis (102). Other kinases such as extracellular signal-related kinases (e.g. ERK-1/2) have also been shown to phosphorylate HSL (85). HSL exhibits broad substrate specificity and can hydrolyze both TAGs and DAGs (174). Since HSL can be covalently modified i.e. phosphorylated at multiple amino acid residues that collectively regulate its activity, the literature examining the regulation of TAG hydrolysis had largely diverted its attention to this enzyme, until it was observed that HSL-knockout mice only had a ~60% reduction in lipolytic rate and DAG but not TAG accumulation

in AT (89). These findings highlight that HSL is not solely responsible for the hydrolysis of TAG stores in AT and that HSL may preferentially hydrolyze DAGs.

The primary enzyme responsible for TAG cleavage was uncovered with the discovery of ATGL/desnutrin (256). ATGL activity can be upregulated by a physical interaction with co-activator protein comparative gene identification-58 (CGI-58) and attenuated by G_0/G_1 switch gene 2 (GOS2) (134, 250). ATGL has multiple phosphorylation sites (e.g. Ser^{406}) although their exact contribution to the regulation of its activity remains a matter of debate (248). Interestingly, when ATGL deletion is targeted to adipocytes *in vivo*, animals are not susceptible to HFD-induced increases to body weight or fat mass (196). Collectively, the concerted efforts of ATGL and HSL may account for upwards of ~95% of TAG breakdown in AT (197). MAG hydrolysis is catalyzed by MGL, which is not under hormonal regulation. MGL activity is likely consequent to substrate availability.

2.4.3 Insulin inhibition of lipolysis

In the postprandial state, the rise in insulin and its subsequent effects in AT downregulate lipolysis and promote the storage of FAs (50, 64). Insulin inhibits AT lipolysis through its activation of phosphodiesterase (PDE) which degrades intracellular cAMP, leading to inactivation of the PKA/HSL axis (50, 60). Insulin has also been shown to stimulate protein phosphatase-1, which directly dephosphorylates/inactivates HSL (175).

2.4.4 Depots

Adipose tissue can be categorized into three distinct depots, distinguished by their anatomical, morphological and functional characteristics: brown adipose tissue (BAT), beige adipose tissue and white adipose tissue (subcutaneous and visceral; WAT). WAT is a large sink for FAs, acting as a storage site for upwards of ~80 000 kcal in excess lipid (103). Considerable

differences exist between AT depots, including: adrenergic receptor density, uncoupling protein expression and thermogenic potential as well as mitochondrial content, which collectively contribute to their roles in physiology (136). For example, in human-derived adipocytes, insulin's anti-lipolytic effects are more pronounced in subcutaneous regions of AT, when compared to visceral tissue (19). This may be due to the lower protein expression for insulin-receptor as well as IRS-1 phosphorylation/PI3K activity following insulin-stimulation in visceral AT (255). In contrast, both basal and stimulated (e.g. by isoproterenol, cAMP or forskolin) rates of lipolysis are elevated in visceral WAT and less effective in subcutaneous adipocytes and AT explants (181, 239). β-adrenergic responsiveness is likely higher in visceral WAT due to its increased β-AR expression (181). The responsiveness of AT is quite heterogenous across different regions of AT and species i.e. rodents versus humans.

2.4.5 Regulation of lipolysis by ghrelin

Given that WAT is a large site for FA clearance following a meal, it would appear intuitive for ghrelin to have a potential metabolic role in this tissue, perhaps in concert with other regulators of AT turnover. Infusing AG *in vivo* leads to increases in basal and hyperinsulinemic rates of abdominal and subcutaneous interstitial glycerol, which indicate an increase in AT lipolysis or a potential counter to insulin's antilipolytic effects (233). Given its pre-prandial rise, a pro-lipolytic role for ghrelin is perplexing, considering that a meal is about to be consumed, and blood-glucose would no longer need to be spared. While effort is sometimes made to minimize the secondary rise in GH (using somatostatin co-infusion) following AG infusion, its own pro-lipolytic effects cannot be entirely discounted. Interestingly, the co-infusion of both ghrelin isoforms results in a lowering of systemic FAs more effectively than UnAG alone, suggesting that UnAG may counteract the pro-lipolytic effects of AG or possibly GH (77).

To assess the direct effects of ghrelin, isolated models have been used to study ghrelin's regulation of lipolysis, although findings have varied, and measures of subcellular signalling events are severely lacking. In primary adipocytes, AG blunts the non-selective adrenergic-induction (isoproterenol; β-agonist) of glycerol release (154). However, these effects were lost with a physiological concentration of AG and the effects of UnAG were not pursued. Data from our own lab expanded these findings by demonstrating that AG and UnAG both attenuate β_3-stimulated (CL 316,243) lipolysis and decreased CL-mediated activation of HSL at its stimulatory phospho-sites ($Ser^{563/660}$) (34). These antilipolytic effects of ghrelin were consistent across subcutaneous (inguinal WAT; iWAT) and visceral (retroperitoneal; RP) AT depots (34). There has yet to be any assessment of simultaneous AG and UnAG administration in isolated AT which could provide insight into their concurrent cellular action, as would be observed *in vivo*.

2.4.6 Lipogenic action of ghrelin in AT

Adipocytes are the primary storage site for TAGs. TAG accumulation in AT is dependent on the balance between its synthesis (lipogenesis) and breakdown (lipolysis). Both are under nutritional and hormonal regulation with the latter being potently mediated by insulin (118). Researchers have postulated whether ghrelin's inhibitory effects on lipolysis substantiate the claim that it is lipogenic. Some literature supports a lipogenic role for ghrelin, but these findings are often complicated by its orexigenic effects i.e. increasing energy intake. When animals are pair-fed during 6d of ghrelin ICV administration, increases to both body and fat pad mass are similar to control, suggesting that increased food intake is primarily responsible for ghrelin-induced increases in fat mass (213). Furthermore, some evidence suggests that UnAG may even be anti-lipogenic (56, 223). Further underlying mechanisms should be pursued to determine whether ghrelin direct regulates lipogenesis in AT.

2.4.7 Fatty acid re-esterification

Decades ago, it was observed that most liberated FAs from the increased rates of AT lipolysis are re-trafficked back into the adipocyte, termed re-esterification. It has been approximated that, depending on energy status, ~50-70% of hydrolyzed FAs can be re-esterified, a process that is energetically costly to the adipocyte (78, 197). Re-esterification of FAs in AT require adequate availability of glycerol-3-phosphate (G3P) derived primarily from glyceroneogenesis, that can then be used for FA esterification (138). Respectively, PDK4 and PEPCK coordinate the regulation of glyceroneogenesis by inhibiting PDH and catalyzing the decarboxylation of oxaloacetate to form phosphoenolpyruvate which is then converted to G3P (27, 70).

2.4.8 Regulation of fatty acid re-esterification by ghrelin

Ghrelin's effect on FA re-esterification in AT has yet to be measured, aside from a single study from our own lab that indirectly assessed primary re-esterification i.e. the observed FA release subtracted from theoretical maximum for FA release (34). Interestingly, in AT organ culture, the FA concentration in media was maintained during adrenergic stimulation, even in the presence of ghrelin isoforms which blunted lipolysis (34). This suggests that they must have inhibited FA re-esterification in AT. The mechanisms by which ghrelin may affects this pathway are unknown. More direct measures of FA re-esterification should be implemented using radiolabeled FA tracers during ghrelin administration. Nonetheless, these findings imply that ghrelin may help to maintain circulating FA levels, even in scenarios where lipolysis is blunted.

3.0 Diet-induced obesity

Obesity is a growing epidemic in the modernized world. While the etiology of the disease is still unraveling, it is critical that researchers model animal work so that the findings carry translational value to humans. Obesity and metabolic syndrome are multi-faceted such that the value of genetic animal models of obesity that target a specific pathology ought to be weighed against nutritional and lifestyle modifications that induce obesity and metabolic dysfunction. Considering the short lifespan of a rodent, dietary approaches i.e. high-fat diets (45-60% total kcal) are often utilized to cause rapid metabolic disturbances and insulin resistance. Dysfunctional skeletal muscle and whole-body glucose metabolism develop in a matter of weeks (167, 206, 227).

3.1 Ghrelin in obesity

Tissues may become resistant to the effects of several hormones in obesity (47, 183), however potential changes to ghrelin's effects in the CNS and in peripheral tissue metabolism are largely unknown. Studies of diet-induced obesity have observed AG resistance in the CNS following peripheral AG administration (21, 159). Central ghrelin resistance may manifest differently than the peripheral sensitivity to ghrelin, since as little as ~12h HFD over-feeding impairs the orexigenic action of ghrelin in mice (171). With respect to ghrelin signalling in the periphery, initial work by Gershon *et al.* using a CRF-2R inhibitor (anti-sauvagine), observed that the stimulatory effects of AG on glucose uptake in C2C12 myocytes could be attenuated, suggesting that AG may act through this receptor to exert some of its metabolic effects (79). We have shown in our lab that CRF-2R content is reduced in soleus muscle from 6-week high-fat fed rats, which coincides with the loss of ghrelin stimulation of palmitate oxidation and its preservation of insulin-stimulated glucose uptake (33). These novel findings further implicate

this receptor in mediating ghrelin action in muscle and suggest that ghrelin action may be altered in obesity, although more work is warranted.

4.0 Conclusion

In summary, whole-body substrate metabolism is governed by a myriad of neural and hormonal factors that contribute to the maintenance of glucose homeostasis. The dysregulation of peripheral tissue glucose and lipid metabolism can contribute to metabolic disorders including obesity and T2D. The neural effects of acylated ghrelin may contribute to the regulation of appetite, energy intake and ultimately adiposity. However, more recent findings, though inconclusive, provide evidence for both acylated and unacylated ghrelin as modulators of peripheral tissue substrate metabolism. Interestingly, emerging research suggests that ghrelin action in skeletal muscle may be altered in the context of obesity like that of insulin. Further research on the peripheral effects of ghrelin is warranted, particularly as they relate to its rhythmic fluctuations in circulation. The mechanisms that underlie ghrelin's effects in tissues like adipose tissue and skeletal muscle remain poorly defined.

CHAPTER 2: Thesis Objectives

The study of ghrelin as a novel regulator of peripheral tissue metabolism is an evolving area that extends our knowledge of ghrelin to other aspects of physiology, beyond its known effects on appetite and growth hormone release. The purpose of this thesis was to explore whether ghrelin directly regulates skeletal muscle glucose and lipid metabolism and adipose tissue lipolysis. Another major objective was to elucidate any potential ghrelin isoform-specific effects. Isolated experiments were aimed at replicating, in part, the nutritional and hormonal milieu that coincide with ghrelin release into circulation and pertinent metabolic processes. A key aspect of the experimental design of these studies was to eliminate secondary confounding factors that arise through the administration of ghrelin, particularly the acylated form, *in vivo*.

The basis of the first study was to test the hypothesis that ghrelin's pre-prandial rise could oppose the action of adrenergic stimuli to attenuate the mobilization of fatty acid substrates from adipose tissue. Fatty acids substrates are no longer required to spare blood glucose such that ghrelin may signal for appetite, and presumably glucose consumption, whilst also preparing adipose tissue for the storage of ingested nutrients.

The second study of this thesis focused on skeletal muscle, the major sink for postprandial glucose clearance. The rationale was that ghrelin's pre-prandial rise suggests that it prepare skeletal muscle for ingested carbohydrates i.e. glucose provision. Since insulin is the major postprandial hormone responsible for glucose clearance, the overlapping effects of ghrelin and insulin were evaluated in skeletal muscle. It was hypothesized that ghrelin isoforms would increase glucose uptake into muscle in the presence of insulin, but not independently.

The foundation for the third and final study of this thesis were, in part, laid by previous work within our laboratory which demonstrated that ghrelin isoforms stimulate fatty acid

oxidation in skeletal muscle. A plethora of literature demonstrates that fatty acids can acutely impair insulin action in muscle. The novel role for ghrelin in the stimulation of fatty acid oxidation provided an avenue to explore whether ghrelin could preserve muscle insulin action following acute lipid oversupply and impairment to insulin action. We hypothesized that ghrelin would maintain muscle's ability to uptake glucose in response to insulin following exposure to the saturated fatty acid challenge.

CHAPTER 3: Acylated and unacylated ghrelin directly regulate β-3 stimulated lipid turnover in rodent subcutaneous and visceral adipose tissue *ex vivo* but not *in vivo*

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Abstract

Ghrelin has garnered interest as a gut-derived regulator of lipid metabolism, beyond its classical roles in driving appetite and growth hormone release. Ghrelin's circulating concentrations follow an ultradian rhythm, peak immediately before a meal and point towards a potential metabolic role in reducing the mobilization of fatty acid stores in preparation for the storage of ingested food. Here, we demonstrate that both acylated and unacylated ghrelin have physiological roles in attenuating lipolysis in mature subcutaneous and visceral adipose tissue depots of rats. Ghrelin blunted the ß3-induction (CL 316, 243) of glycerol release (index of lipolysis) which coincided with a reduced activation of the key lipid hydrolase HSL at two of its serine residues ($Ser^{563/660}$). Furthermore, ghrelin appeared to inhibit fatty acid re-esterification in the presence of CL such that fatty acid concentrations in the surrounding media were maintained in spite of a reduction in lipolysis. Importantly, these effects were not observed following ghrelin injection *in vivo*, as there was no attenuation of CL-induced glycerol release. This highlights the importance of exercising caution when interpreting the effects of administering ghrelin *in vivo*, and the necessity for uncovering the elusive mechanisms by which ghrelin regulates lipolysis and fatty acid re-esterification. We conclude that both acylated and unacylated ghrelin can exert direct inhibitory effects on lipolysis and fatty acid re-esterification in adipose tissue from rats. However, these effects are not observed *in vivo* and outline the complexity of studying ghrelin's effects on fatty acid metabolism in the living animal.

Introduction

Ghrelin is a gut-derived hormone classically defined by its central effect as a potent appetite stimulant (245, 246). Beyond this, given that its pulsatile secretion coincides with anticipated meal times (143), ghrelin has recently harbored interest as a potential regulator of carbohydrate and lipid metabolism. Although convincing evidence exists to suggest that ghrelin administration may influence peripheral tissue metabolism in skeletal muscle and adipose tissue (10, 233), acute and chronic ghrelin administration are confounded by secondary effects. Acutely, ghrelin can cause growth hormone (GH) and glucagon-like peptide (GLP-1) release, whereas chronic ghrelin administration leads to alterations in body and fat mass due to changes in feeding behavior (125, 223, 245). The assessment of the direct metabolic effects of acylated (AG) and unacylated (UnAG) ghrelin on tissues such as muscle and adipose is relatively sparse.

Adipose tissue (AT) lipolysis releases free fatty acids for utilization by metabolically active tissues, including skeletal muscle, during states of fasting allowing for glucose to be spared for the brain and other glucose-dependent tissues. When a meal is consumed, lipolytic activity in AT is attenuated to prioritize the clearance and storage of plasma fatty acids (139). Ghrelin reaches its peak circulating concentration immediately prior to meal ingestion, which points towards a potential role in contributing to the downregulation of AT lipolysis. Ghrelin may facilitate this transition following a meal, i.e. suppression of lipolysis, when there would no longer be a requirement to preferentially use fatty acids and spare blood glucose. Interestingly, during the prolonged absence of food, the normal rise and fall in ghrelin is largely abolished, at a time when it would be beneficial to sustain elevated AT lipolysis (143).

Whole-body and isolated tissue models assessing ghrelin's effects on AT lipolysis have been conflicting. Vestergaard et al. demonstrated that the administration of ghrelin in humans stimulated glycerol release (index of lipolysis) into the interstitial space of subcutaneous AT and skeletal muscle (233). However, these findings are potentially confounded by the influence of ghrelin on GH and incretin response. Growth hormone, specifically, has been demonstrated by some to stimulate lipolysis when administered *in vivo* (173). More direct approaches using primary adipocytes have yielded results that contrast with the aforementioned findings, and demonstrate an inhibitory role for ghrelin on the β-adrenergic stimulation of lipolysis (154). To date, this has not been assessed in mature subcutaneous and visceral adipose tissue depots, and there has been very little determination of UnAG's role in adipose tissue lipolysis, particularly *in vivo*. This is important, given the growing evidence suggesting that UnAG may also have metabolic effects (30). Moreover, an examination of underlying cellular signalling events to clarify the regulatory role of both ghrelin isoforms on lipolysis is lacking.

Therefore, in the current study, we aimed at extending previous findings to elucidate whether AG and UnAG could regulate lipolysis directly (*ex vivo*) in mature subcutaneous and visceral adipose tissue depots from rodents, while also assessing concomitant changes in cellular signalling. In addition, we examined whether any of the observed lipolytic or signalling effects observed *ex vivo* would also be evident following acute *in vivo* ghrelin administration with the β3-receptor specific pharmacological agonist CL 316,243. The β3-receptor is highly expressed in adipose tissue, but not skeletal muscle or liver (132).

Methods

Animals

All procedures were approved by the Animal Care Committee at the University of Guelph and followed Canadian Council of Animal Care guidelines. Male Sprague-Dawley rats were obtained from Charles River laboratories (Québec, ON, Canada) at approximately 4-5 weeks of age (~200-250g). Rats were given ad libitum access to regular chow food and water and allowed to acclimatize upon arrival for one week. To avoid high endogenous levels of circulating ghrelin as a potentially confounding factor, food was reintroduced 2-3 h prior to experiments following an overnight fast, and it was visually confirmed that the food was consumed. Rodents were anesthetized with an intraperitoneal injection of sodium pentobarbital (6mg per 100g body mass) prior to all surgical procedures.

Materials and Reagents

Reagents, molecular weight markers and nitrocellulose membranes were purchased from BioRad (Mississauga, ON, Canada). Western lighting plus enhanced chemiluminescence (ECL) was purchased from Perkin-Elmer (NEL105001EA). The following antibodies were purchased from Cell Signalling: total-AMPK (Cat. No. 2532), phospho-AMPK (Cat. No. 2535), total-ACC (Cat. No. 3676), phospho-ACC (Cat. No. 11818), total-HSL (Cat. No. 4107), total-ERK (Cat. No. 4695), phospho-ERK (Cat. No. 4370), phospho-HSL 660, 565, 563 (Cat. Nos. 4126, 4137, 4139). The following antibodies were purchased from Abcam: total-ATGL (Cat. No. 109251), phospho-ATGL (Cat. No. 135093). NP40 cell lysis buffer (Cat. No. FNN0021) was obtained from Life Technologies and PMSF and protease inhibitor were obtained from Sigma (Cat. Nos. 78830 and 9599). Insulin (Humulin rDNA origin) was purchased from Eli Lilly (Toronto, ON,

Canada). Recombinant human growth hormone protein was purchased from Abcam (Cat. No. ab116162). Acylated (Cat. No. H-4862) and unacylated (Cat. No. H-6264) ghrelin were sourced from Bachem (Torrance, CA, USA). For adipose tissue incubations, fatty-acid free bovine serum albumin (Cat. No. 10775835001), Medium 199 (Cat. No. M7653), L-glutamine (Cat. No. G7513), dexamethasone 21-phosphate disodium salt (Cat. No. D1159) and CL 316,243 (Cat. No. C5976) were all sourced from Sigma. Antibiotic/antimycotic solution (Cat. No. 30004Cl) was purchased from Corning.

Ex vivo (Adipose Tissue Organ Culture - ATOC) lipolysis and signalling

Following anesthesia, surgeries were carefully performed to excise both subcutaneous (inguinal) and visceral (retroperitoneal) adipose tissue which was immediately placed into M199 media supplemented with 1% antibiotic/antimycotic, 50µU insulin and 2.5nM dexamethasone. 250mg portions were snipped, minced in petri dishes and covered with 3.5ml of ATOC media after which they remained incubated under warmed (37°C), humidified and gassed (95% O2, 5% CO2) conditions for 24 h. Following 24 h pre-treatment, media was swapped with fresh media to which respective treatments (sterile H_2O, 150ng/ml AG, 150ng/ml UnAG, 250ng/ml GH, or 1µM CL) were added. Previous work has found that these doses for AG, UnAG and CL elicit significant metabolic effects; the dose used for GH represents a concentration that is achieved in response to ghrelin administration in rats (125, 147, 154). Sampling of media for the quantification of glycerol and free fatty acids was done at t=2, 4 and 8 h. Aliquots were immediately frozen at -80°C for further analysis. CL significantly increased glycerol accumulation (subcutaneous depot shown, for simplicity) compared to control at each time point (t=2 h: Con, 1.0 ± 0.1 vs. CL, 2.3 ± 0.2; t=4 h: Con, 1.0 ± 0.3 vs. CL: 3.3 ± 1.0; t=8 h: Con, 1.0 ±

0.2 vs. CL, 2.9 ± 0.8 mM/g). Given that the accumulation was significant at 2 h and was not linear beyond this point, 2 h was chosen as the time point of collection for all future measurements of lipolysis. For assessing signalling proteins in adipose tissue samples from ATOC, tissue was rinsed in ice cold phosphate-buffered saline (PBS) following 2 h of incubation with treatment, snap frozen in liquid nitrogen, and stored at -80°C until further processing for western blotting.

In vivo lipolysis and signalling

Following anesthesia, rats were administered an intraperitoneal injection of either CL, CL+AG or CL+UnAG, or a body mass-adjusted bolus of saline (all 1mg/kg). Other *in vivo* work (10) has demonstrated significant metabolic and physiological effects using a similar injection dose of ghrelin. Also, previous work in our lab (147) has demonstrated a robust increase in lipolysis following CL administration by 30 min. As such, this time point was used for blood and tissue collection. Specifically, a cardiac puncture was performed to obtain terminal blood samples. Blood was allowed to rest on ice for an additional 30 min and then centrifuged for 10 min at 1500g. Serum was collected and immediately snap frozen in liquid nitrogen and stored at -80°C for further processing. For *in vivo* signalling, subcutaneous (inguinal) and visceral (retroperitoneal) adipose tissue were excised 30 min post-injection, rinsed in saline, blotted dry and frozen in liquid nitrogen to be stored at -80°C.

Glycerol quantification

Glycerol was quantified from ATOC media and serum samples via single-wavelength spectrophotometry using commercially available reagents from Sigma derived from their

glycerol assay kit. These reagents included glycerol standard (Cat. No. G7793) and glycerol reagent (Cat. No. F6428).

Non-Esterified Fatty Acid (NEFA) quantification

NEFAs were quantified from ATOC media and serum samples using commercially available reagents (Cat. Nos. 995-34791, 993-35191, 999-34691, 991-34891) and standards (Cat. No. 276-76491) from Wako Diagnostics (Richmond, VA), which are included in their free fatty acid assay kit.

Primary Fatty Acid Re-esterification

Primary fatty acid re-esterification was calculated as the difference between the theoretical fatty acid release (3 x glycerol release into media/serum) and the measured fatty acid release. This assumes negligible rates of fatty acid oxidation in adipose tissue and describes absolute rates of fatty acid re-esterification, as described previously by Van Harmelen *et al.* (94).

Western blotting

Adipose tissue (~250-300mg) was placed directly into homogenization tubes containing lysis beads prior to being frozen in liquid nitrogen. Protease inhibitor and PMSF were then added, after which samples were homogenized and centrifuged at 4°C. The supernatant was then carefully removed (to not disrupt the fat layer) and transferred to a new tube. BCA assays were performed to determine protein content from homogenized samples to facilitate subsequent sample preparation for western blots (200).

For western blots, equal amounts of sample protein (10μg) were loaded onto 10% gels (5% for ACC), as we have published previously (32). Samples were then transferred for 1 h at 100V onto nitrocellulose membranes and blocked in non-fat skim milk powder and TBST. Membranes were then incubated at 4°C overnight in primary antibody supplemented with 5% BSA/5% skim milk powder as per manufacturer guidelines. Following primary incubation, membranes were washed with TBST and exposed to secondary (anti-rabbit) antibody (1:2000) for 1 h at room temperature and then washed again (2x TBST, 1x TBS). Protein bands were visualized using ECL and quantified using densitometry on Alpha Innovate Software. All membranes were ponceau stained and quantified to assess equal loading and blots were normalized to their respective ponceau image.

Statistics

All data are expressed as mean ± standard error. A repeated measure (by both factors - with or without CL or ghrelin) 2-way analysis of variance (ANOVA) was performed for *ex vivo* ATOC experiments. Pending significance with the ANOVA, a Tukey's multiple comparisons post-hoc test was used to find any main (CL or AG/UnAG) or interaction (CL x AG/UnAG) effects of treatments. A repeated measure, non-parametric one-way ANOVA (Friedman's test) was performed for the GH-treated subsets of *ex vivo* ATOC experiments as well as all *in vivo* injection and western blotting experiments. If significance was detected with the ANOVA, multiple comparisons were assessed using a Dunn's post-hoc test. In all figures, letters were used to denote statistical significance, such that groups sharing a common letter are not significantly different from each other. Data was considered significant at $p < 0.05$. Data points greater than two standard deviations from the mean were considered outliers and removed.

Results

Ex vivo, ATOC experiments:

Ex vivo, AG and UnAG do not independently affect AT lipolysis or fatty acid re-esterification

During ATOC incubations with iWAT and RP, neither AG nor UnAG had a significant effect on indices of lipolysis i.e. glycerol (**Figures 1A and D**) and free fatty acid release (**Figures 1B and E**). Calculated fatty acid re-esterification (**Figures 1C and F**) was also unaffected. Given the lack of any independent ghrelin effect on these functional measurements, AG and UnAG treatments were not further pursued for signalling purposes.

Ex vivo, CL-stimulated glycerol release and fatty acid re-esterification are attenuated by AG and UnAG

CL treatment independently stimulated lipolysis (**Figures 1A, B, D and E**). This was evidenced in both fat depots by an increase in glycerol (iWAT, ~2.7-fold, $p<0.01$; RP, ~3.9-fold, $p<0.01$) and free fatty acid (iWAT, ~1.2-fold, $p<0.05$; RP, ~1.6-fold, $p<0.01$) release. CL's stimulatory effect on glycerol release was significantly attenuated ($p<0.05$) by AG in both (**iWAT: Figure 1A and RP: Figure 1D**) AT depots, whereas UnAG had a significant effect in RP (**Figure 1D**) and trended ($p=0.06$) towards a reduction in iWAT (**Figure 1A**). Interestingly, AG and UnAG did not suppress CL's ability to increase fatty acid release from either fat depot (**Figure 1B**) and RP (**Figure 1E**). A large proportion of hydrolyzed fatty acids are reesterified back into TAGs; accordingly, given CL's stimulatory effect on lipolysis, CL also significantly increased fatty acid re-esterification in both depots (**Figures 1C and F**). AG and UnAG significantly ($p<0.01$) blunted this re-esterification response to CL in iWAT (**Figure 1C**), whereas only CL+UnAG was significant in RP (**Figure 1F**).

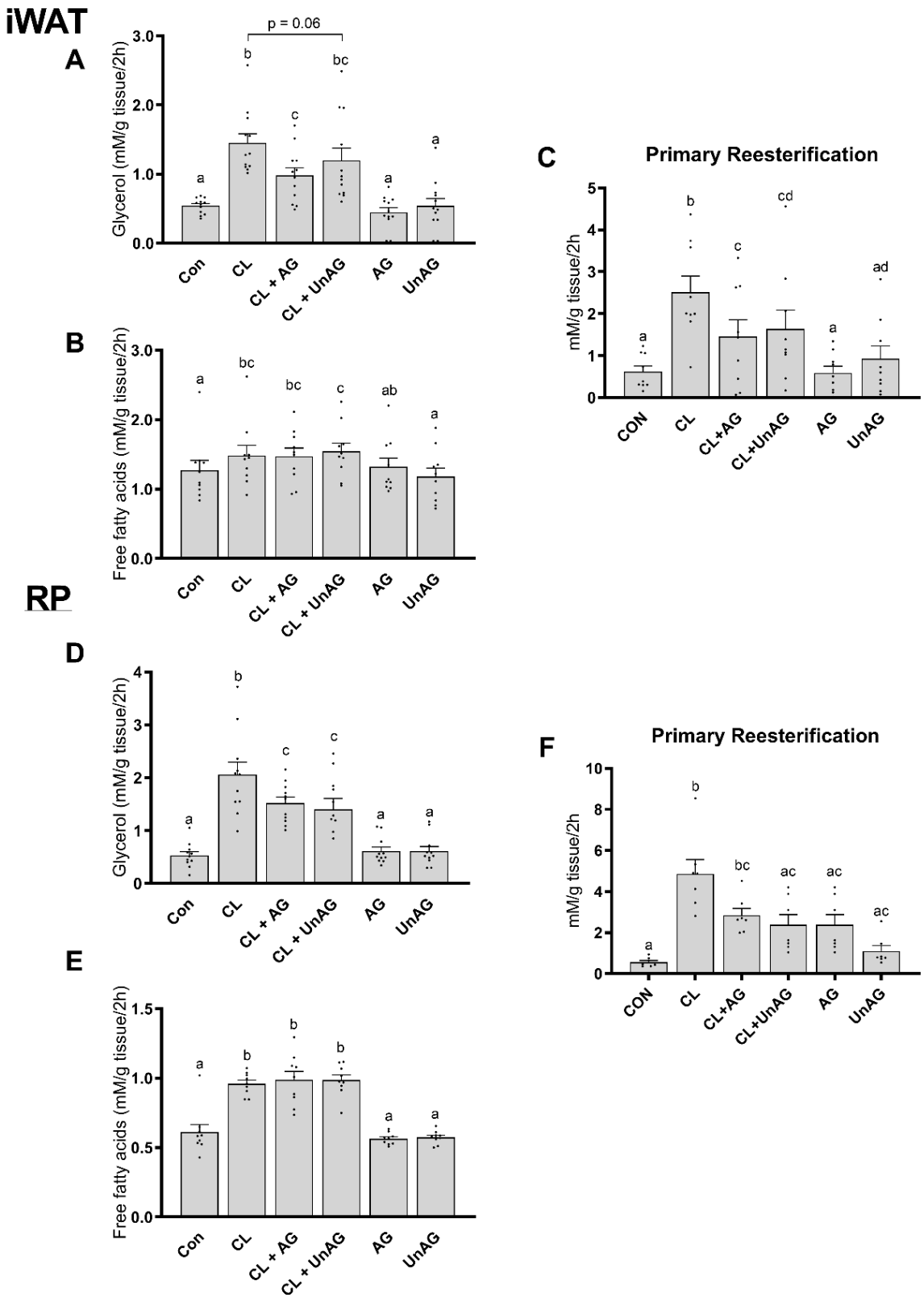
iWAT
A
p = 0.06
Glycerol (mM/g tissue/2h)
3.0
2.0
1.0
0.0
a
b
c
bc
a
a
Con
CL
CL + AG
CL + UnAG
AG
UnAG
B
Free fatty acids (mM/g tissue/2h)
3.0
2.0
1.0
0.0
a
bc
bc
c
ab
a
Con
CL
CL + AG
CL + UnAG
AG
UnAG
C
Primary Reesterification
mM/g tissue/2h
5
4
3
2
1
0
a
b
c
cd
a
ad
CON
CL
CL+AG
CL+UnAG
AG
UnAG
RP
D
Glycerol (mM/g tissue/2h)
4
3
2
1
0
a
b
c
c
a
a
Con
CL
CL + AG
CL + UnAG
AG
UnAG
E
Free fatty acids (mM/g tissue/2h)
1.5
1.0
0.5
0.0
a
b
b
b
a
a
Con
CL
CL + AG
CL + UnAG
AG
UnAG
F
Primary Reesterification
mM/g tissue/2h
10
8
6
4
2
0
a
b
bc
ac
ac
ac
CON
CL
CL+AG
CL+UnAG
AG
UnAG

Figure 1: *Ex vivo* Lipolysis and Fatty Acid Re-Esterification with CL and Ghrelin

The *ex vivo* effects of CL and CL + ghrelin combination treatments on lipolysis as measured by glycerol (A and D) and free fatty acid (B and E) release and fatty acid re-esterification (C and F) in subcutaneous inguinal white (top) and visceral retroperitoneal (bottom) adipose tissue. Data were analyzed using a repeated measures two-way ANOVA (n = 9–12) and expressed as mean ± standard error, in mM/g tissue/2h. Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

Ex vivo, GH alone or in combination with AG and UnAG, does not affect AT lipolysis

Given AG's physiological role in stimulating GH release, we determined whether ghrelin could modulate GH's purported role as a stimulator of AT lipolysis (165, 173). However, with our acute (2 h) treatment protocol, when compared to control, GH did not alter glycerol (**Figure 2A and D**) or free fatty acid (**Figures 2B and E**) release alone or in combination with CL in iWAT or RP. CL significantly increased ($p<0.001$) glycerol release in iWAT (**Figure 2A**) and RP (**Figure 2D**) as expected. CL also significantly increased ($p<0.001$) free fatty acid release in RP (**Figure 2E**) but not iWAT within this subset of incubations (**Figure 2B**). Furthermore, AG and UnAG did not elicit any effects when combined with GH, as lipolytic endpoints were not significantly different from control or GH alone (**Figures 2A, B, D and E**). Only the CL-treated AT, which showed an increase in lipolysis, demonstrated an increase in fatty acid re-esterification (**Figures 2C and F**). Given the lack of any direct, acute effect on the functional measurements of lipolysis and fatty acid re-esterification, further experiments with GH were not pursued for determination of protein signalling.

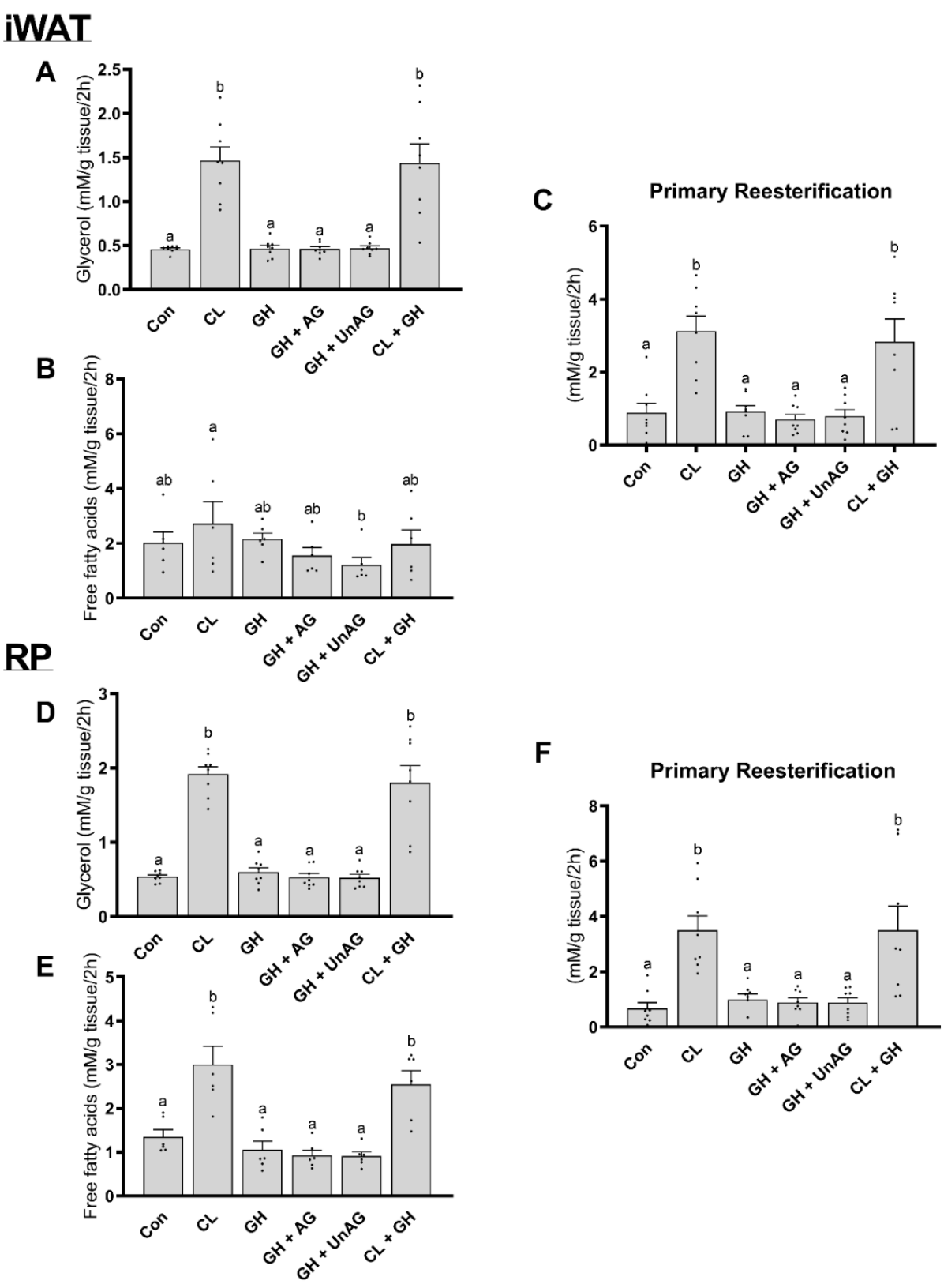
iWAT
A
Glycerol (mM/g tissue/2h)
B
Free fatty acids (mM/g tissue/2h)
C
Primary Reesterification
(mM/g tissue/2h)
RP
D
Glycerol (mM/g tissue/2h)
E
Free fatty acids (mM/g tissue/2h)
F
Primary Reesterification
(mM/g tissue/2h)
Con
CL
GH
GH + AG
GH + UnAG
CL + GH

Figure 2: *Ex vivo* Lipolysis and Fatty Acid Re-Esterification with GH and Ghrelin

The *ex vivo* effects of GH and GH + ghrelin or CL combination treatments on lipolysis as measured by glycerol (A and D) and free fatty acid (B and E) release and fatty acid re-esterification (C and F) in subcutaneous inguinal white (top) and visceral retroperitoneal (bottom) adipose tissue. Data were analyzed using a repeated measures one-way ANOVA (n = 6–8) and expressed as mean ± standard error, in mM/g tissue/2h. Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

Ex vivo, CL-stimulated HSL phosphorylation is attenuated with both AG and UnAG

CL significantly increased the phosphorylation of HSL at activating $Ser^{563/660}$ residues in both iWAT and RP (**Figures 3A and B**; $p<0.01$). CL treatment also significantly reduced the phosphorylation of HSL at its inhibitory Ser^{565} residue, but this effect was only observed in RP (**Figure 3B**). In iWAT, AG blunted the CL-mediated increase in HSL phosphorylation at both Ser^{563} and Ser^{660} ($p<0.05$), whereas UnAG only reduced HSL activation at Ser^{660} (**Figure 3A**). In RP, both AG ($p = 0.07$) and UnAG ($p < 0.05$) attenuated CL's activation of HSL at Ser^{563} but had no significant effect at Ser^{660} (**Figure 3B**). In the presence of CL, AG and UnAG did not further alter HSL phosphorylation at its inhibitory Ser^{565} site in either adipose tissue depot (**Figures 3A and B**). The activation of ATGL (a TAG hydrolase) at Ser^{406}, was unaltered by all treatments in both iWAT (**Figure 3A**) and RP (**Figure 3B**).

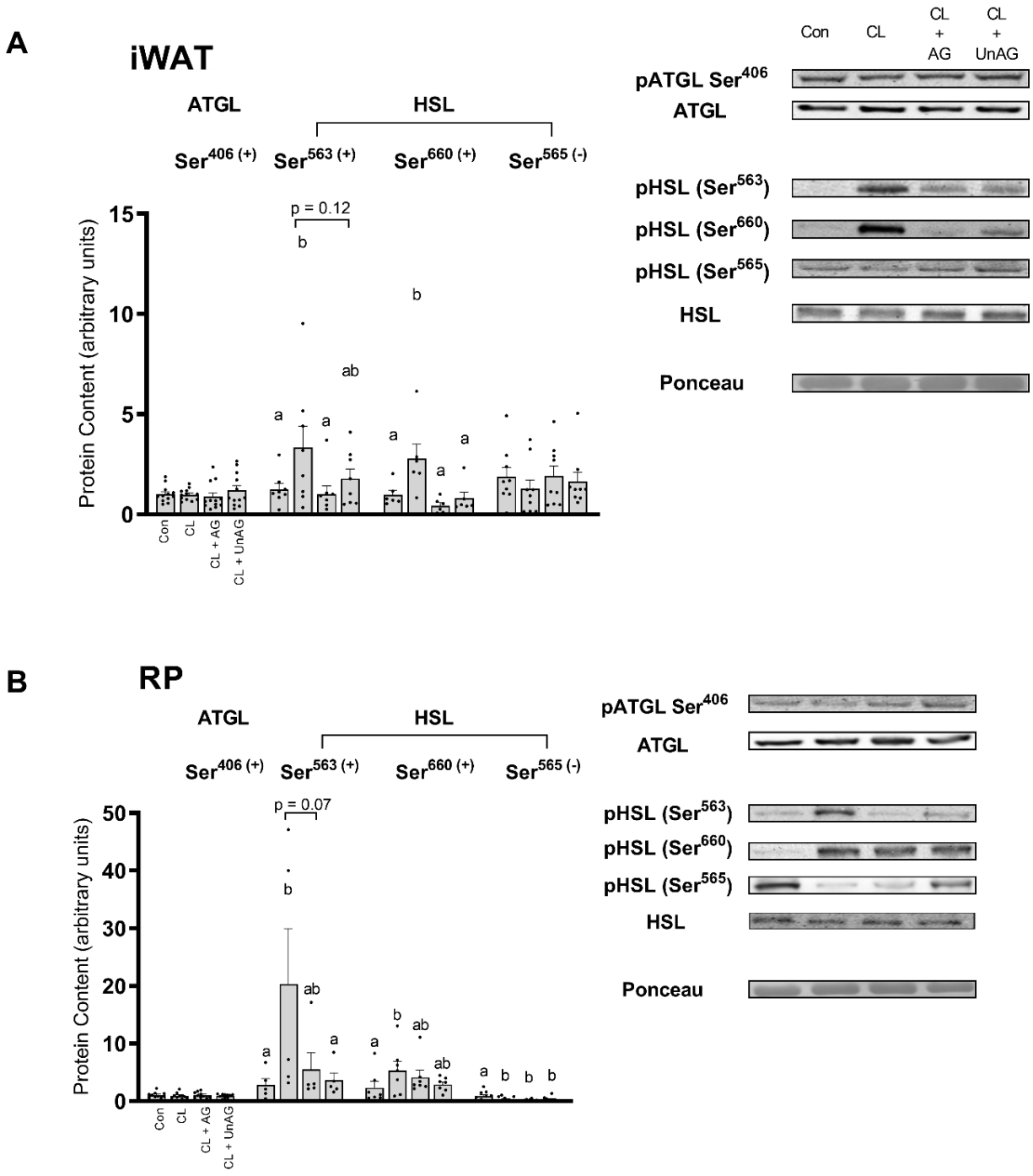

Figure 3: *Ex vivo* Lipolytic Signalling with CL and Ghrelin

The *ex vivo* effects of CL and CL + ghrelin combination treatments on the activation of lipolytic enzymes ATGL and HSL in subcutaneous inguinal white (a) and visceral retroperitoneal (b)

adipose tissue. Data were analyzed using a repeated measures one-way ANOVA (n = 6–12) and expressed as mean ± standard error, in arbitrary protein units (phospho/total). Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

Ex vivo, AMPK, ACC and ERK activation remained unaltered by CL and ghrelin treatments

AMPK Thr^{172} phosphorylation was significantly ($p<0.05$) elevated following all CL treatments, but only in RP (**Figure 4B**), but this did not translate to its downstream target ACC, as its Ser^{79} phosphorylation was unaffected. No consistent treatment effects on AMPK or ACC activation were apparent in iWAT (**Figure 4A**). ERK phosphorylation at its Thr^{202} and Tyr^{204} residues were not significantly different between any groups in iWAT or RP (**Figures 4A and B**).

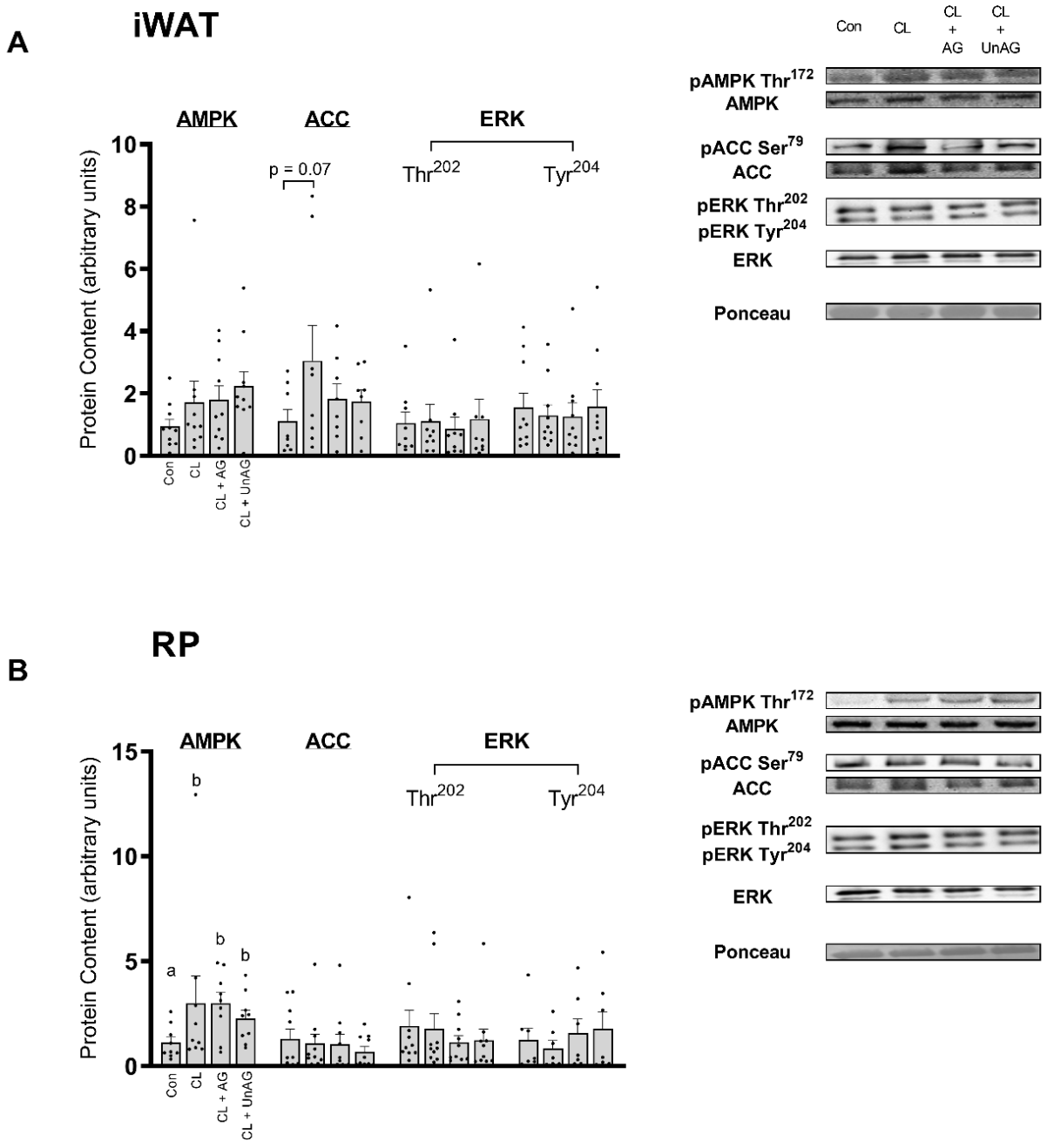

Figure 4: *Ex vivo* AMPK/ACC Signalling and ERK Activation with CL and Ghrelin

The *ex vivo* effects of CL and CL + ghrelin combination treatments on the activation of ERK, the cellular energy-sensing enzyme AMPK and its downstream target ACC in subcutaneous inguinal white (A) and visceral retroperitoneal (B) adipose tissue. Data were analyzed using a repeated

measures one-way ANOVA (n = 8–10) and expressed as mean ± standard error, in arbitrary protein units (phospho/total). Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

<u>*In vivo* injection experiments:</u>

Stimulation of lipolysis with CL is not blunted with AG and UnAG in vivo

CL-injection significantly ($p<0.01$) increased lipolysis *in vivo*, as shown by a 2-fold rise in both whole-body circulating glycerol (**Figure 5A**) and free fatty acids (**Figure 5B**), compared to saline control. Similarly, all *in vivo* CL-injections showed a significant ($p<0.05$) increase in fatty acid re-esterification (**Figure 5C**). Contrary to our direct, *ex vivo* (ATOC) findings, co-injection of AG or UnAG with CL did not alter circulating glycerol (**Figure 5A**) or fatty acid (**Figure 5B**) concentrations when compared to CL alone. AG trended ($p = 0.08$) towards reducing CL-stimulated free fatty acid release (**Figure 5B**).

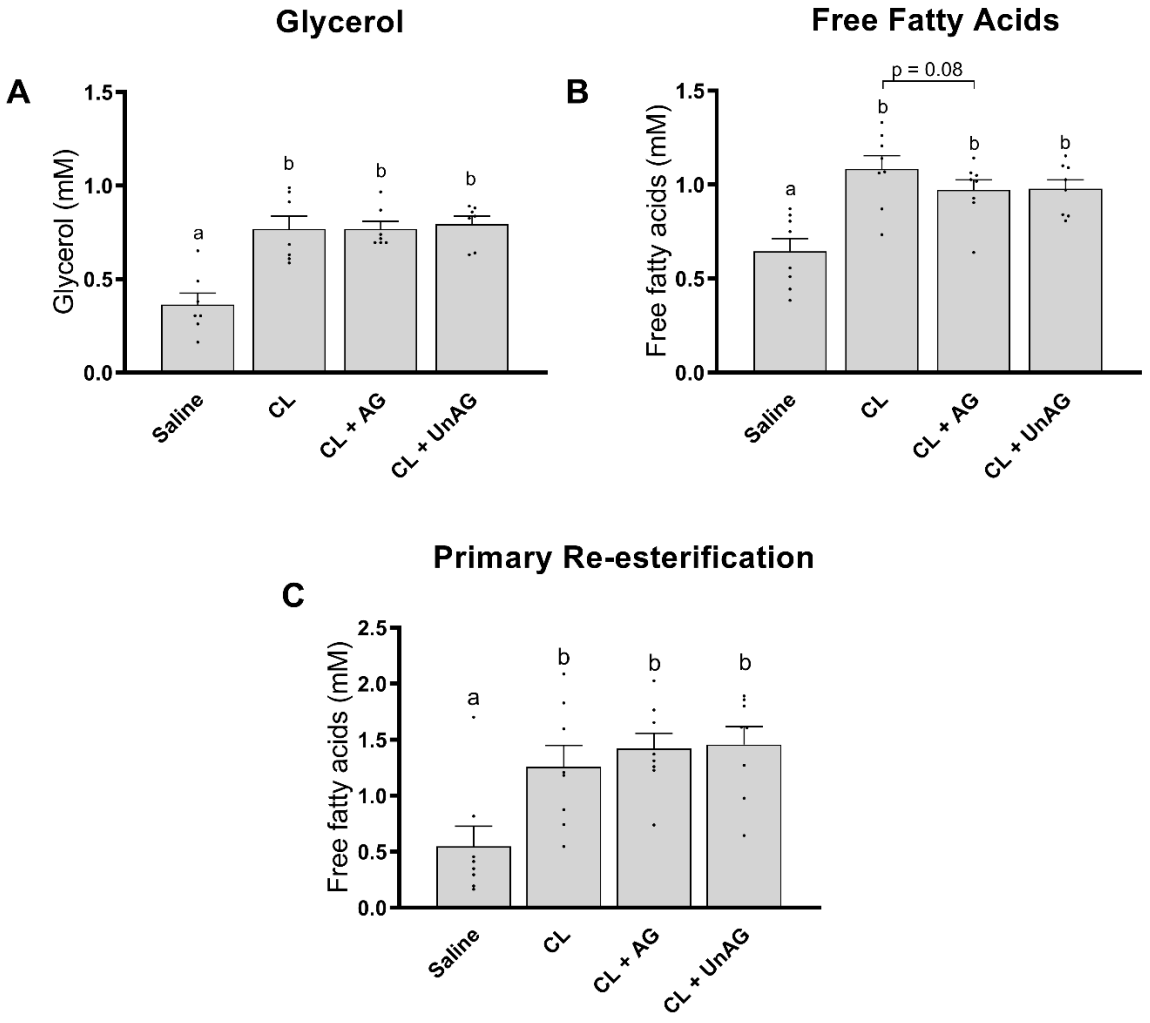

Figure 5: *In vivo* Lipolysis and Re-esterification Following CL and Ghrelin Injection

The *in vivo* effects of CL and CL + ghrelin co-injections on lipolysis as measured by circulating glycerol (A) and free fatty acid (B) release and fatty acid re-esterification (C). Data were analyzed using a repeated measures one-way ANOVA (n = 7–8) and expressed as mean ± standard error, in mM. Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

In vivo, neither AG nor UnAG blunt CL's activation of HSL

CL-injection significantly increased the phosphorylation of HSL at both Ser^{563} and Ser^{660} residues in iWAT (**Figure 6A**; $p<0.05$); in RP, only Ser^{563} phosphorylation was increased by *in vivo* CL-injection. The phosphorylation of HSL at activating $Ser^{563/660}$ residues following co-administration of CL with AG and UnAG were not significantly reduced from CL alone in either AT depot (**Figures 6A and B**). The phosphorylation of HSL at its inhibitory Ser^{565} residue was unchanged in both iWAT (**Figure 6A**) and RP (**Figure 6B**) following all injections. The activation of ATGL was unaffected by all treatments (**Figures 6A and B**).

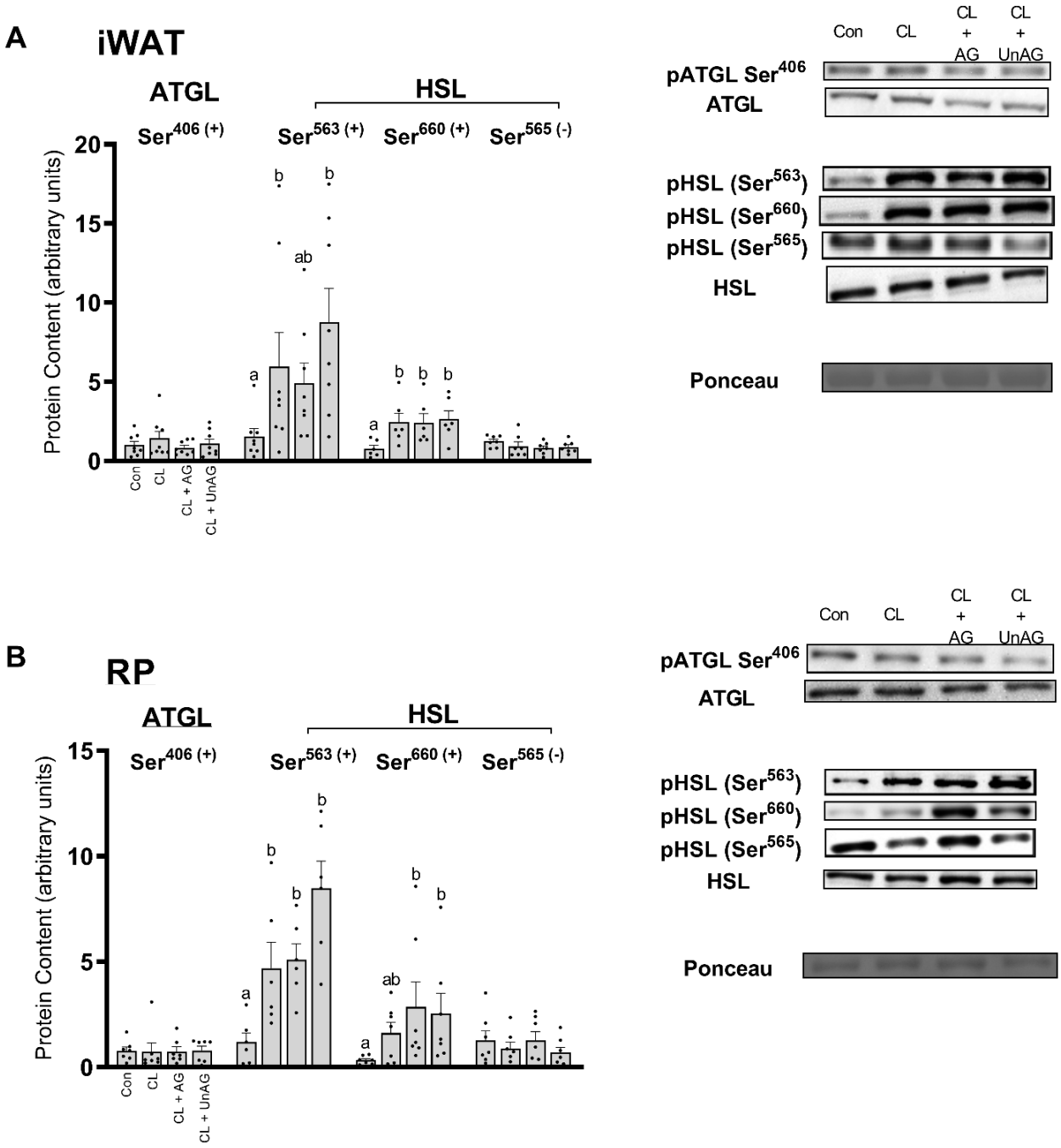

Figure 6: *In vivo* Lipolytic Signalling Following CL and Ghrelin Injection

The *in vivo* effects of CL and CL + ghrelin co-injections on the activation of lipolytic enzymes ATGL and HSL in subcutaneous inguinal white (A) and visceral retroperitoneal (B) adipose

tissue. Data were analyzed using a repeated measures one-way ANOVA (n = 6–8) and expressed as mean ± standard error, in arbitrary protein units (phospho/total). Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

In vivo, AMPK and ACC activation are unaltered by CL and ghrelin

AMPK (Thr^{172}) and its downstream target ACC (Ser^{79}) were not statistically different between any injection treatment in either adipose tissue depots (**Figures 7A and B**).

***In vivo,* ERK phosphorylation is variably increased with CL and ghrelin**

Injection of saline, CL and CL+UnAG had no effect on the activation of ERK in iWAT (**Figure 7A**). Interestingly, co-administration of CL with AG significantly ($p<0.05$) increased the activation of ERK at its Thr^{202} but not Tyr^{204} residue in iWAT (**Figure 7A**). All CL-stimulated treatments had a stimulatory effect on ERK activation at its Tyr^{204} residue in RP (**Figure 7B**). AG and UnAG further increased ERK phosphorylation at Thr^{202} beyond CL alone (**Figure 7B**).

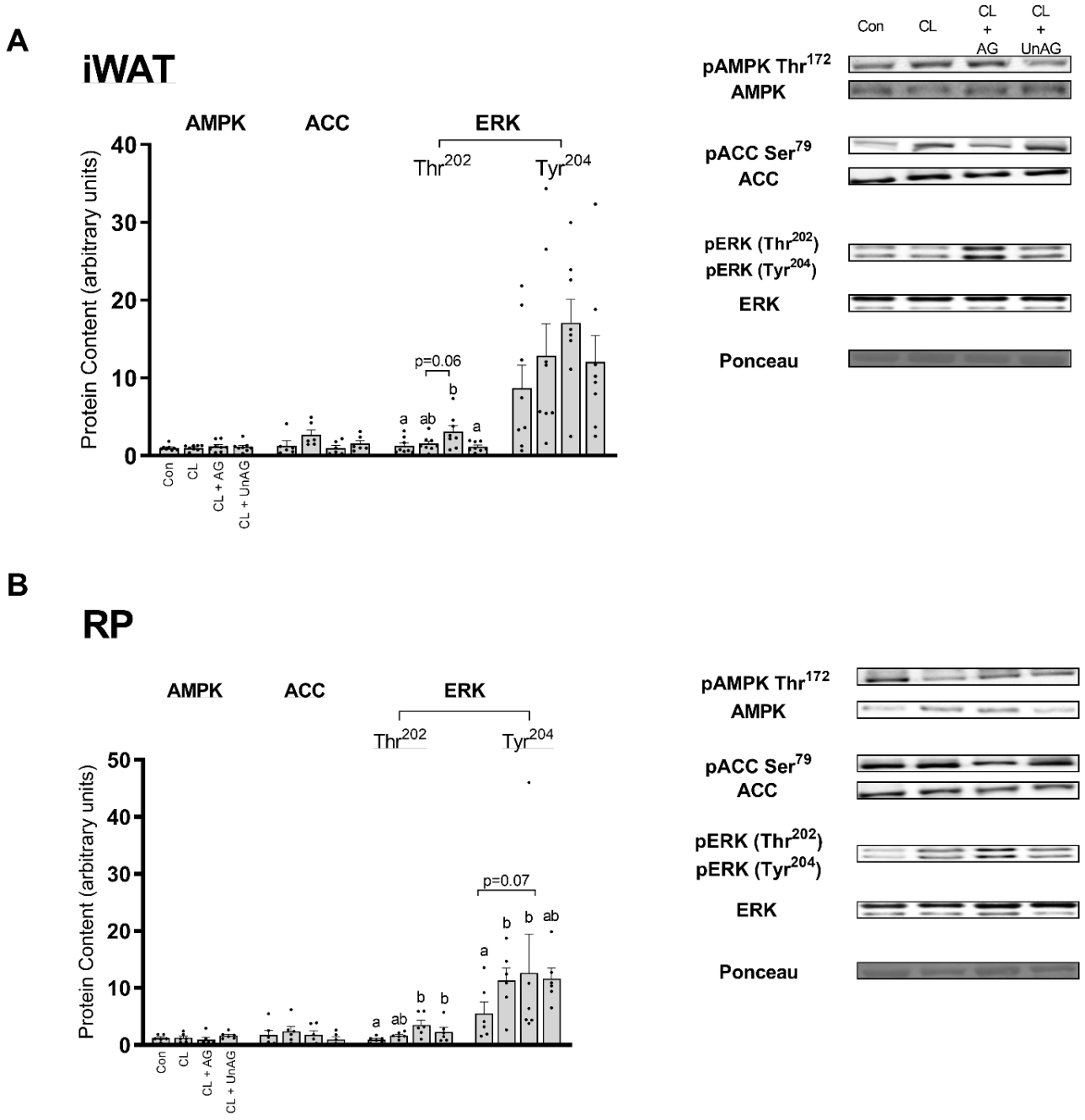

Figure 7: *In vivo* AMPK/ACC and ERK Signalling Following CL and Ghrelin Injection

The *in vivo* effects of CL and CL + ghrelin co-injections on the activation of ERK, the cellular energy-sensing enzyme AMPK and its downstream target ACC in subcutaneous inguinal white (A) and visceral retroperitoneal (B) adipose tissue. Data were analyzed using a repeated

measures one-way ANOVA (n = 6–8) and expressed as mean ± standard error, in arbitrary protein units (phospho/total). Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

In vivo injections of CL increase insulin, but do not affect AT Akt phosphorylation

CL injection has been reported to cause insulin secretion *in vivo* (86) therefore we measured insulin following injections. All CL-injections significantly increased circulating insulin concentrations compared to saline (Table 1). Interestingly, the co-injection of UnAG with CL yielded a significantly greater rise in insulin when compared to CL alone. However, these concentrations were not sufficient to blunt CL's effects in stimulating lipolysis. This is likely due to the fact that Akt phosphorylation, a mediator of insulin action in peripheral tissues and inhibitor of lipolysis, was unaltered (**Figure 8A and B**). Despite the CL-mediated increases in serum insulin, the phosphorylation of Akt at its Ser^{473} and Thr^{308} residues was unaffected by treatments in iWAT and RP (**Figure 8A and B**).

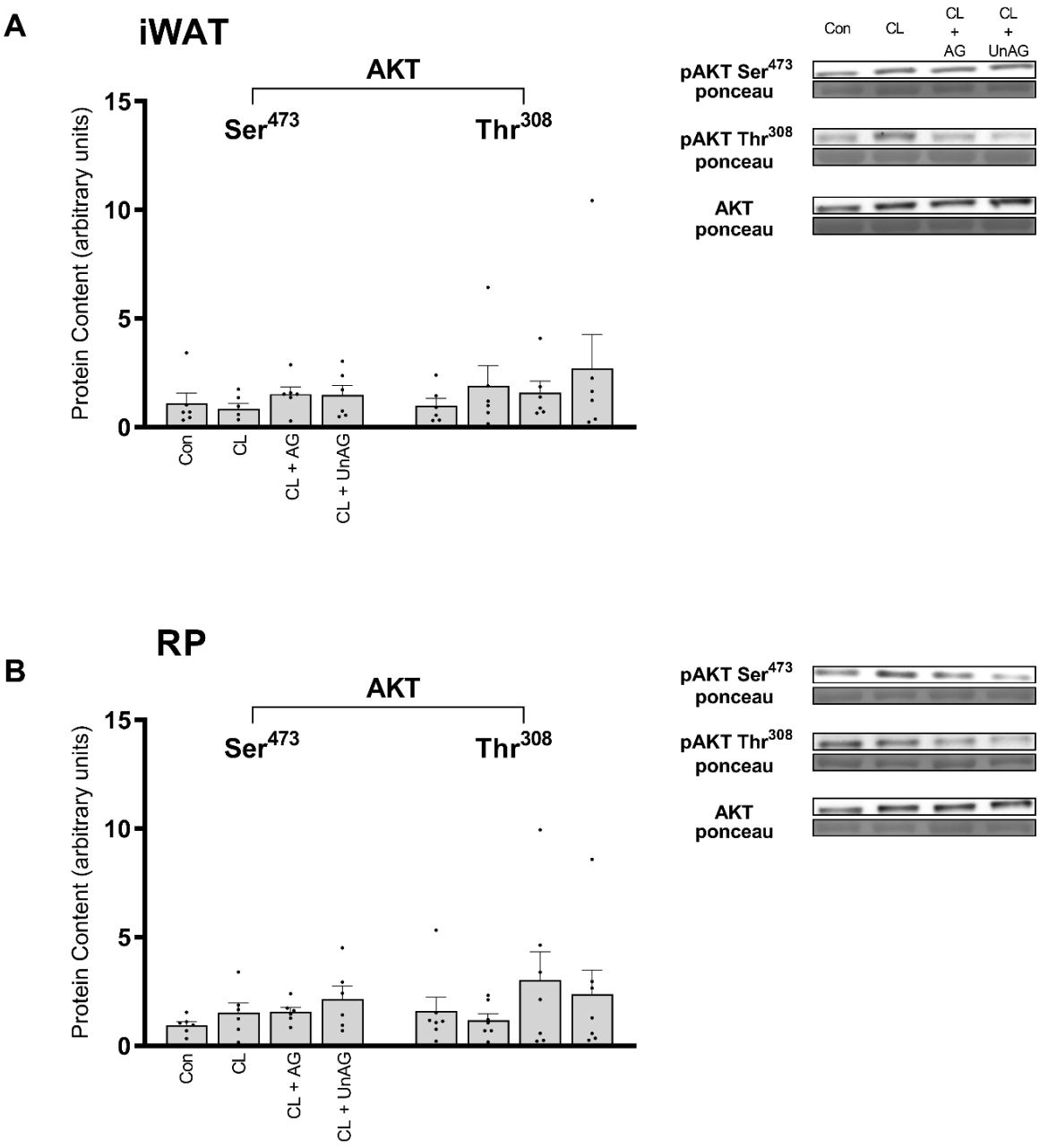

Figure 8: *In vivo* Akt Activation Following CL and Ghrelin Injection

The *in vivo* effect of CL and CL + ghrelin combination treatments on Akt phosphorylation (a marker of insulin signalling activation) in subcutaneous iWAT (A) and visceral RP (B) adipose tissue depots (arbitrary units, phospho/total, normalized for ponceau. Data are expressed as mean

± standard error. Data were analyzed using a repeated measures one-way ANOVA (n = 6–8). Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

	Treatment			
	Saline (control)	**CL 316, 243**	**CL + AG**	**CL + UnAG**
Serum Insulin (ng/ml)	4.6 ± 0.6^{a}	8.1 ± 0.6^{b}	9.2 ± 0.7^{bc}	11.3 ± 0.5^{c}

Table 1: Serum Insulin Concentrations Following CL and Ghrelin Injection

The *in vivo* effect of CL (1mg/kg) and CL + ghrelin combination treatments on serum insulin concentrations (ng/ml). Data are expressed as mean ± standard error. Data were analyzed using a repeated measures one-way ANOVA (n = 6–8). Data sharing a letter are not statistically different from each other. $p < 0.05$ was considered statistically significant.

Discussion

Overall finding

Ghrelin spikes immediately prior to entrained mealtimes. We hypothesized that ghrelin would exert an antilipolytic effect, which would facilitate the storage of lipid following an anticipated meal. Interestingly, ghrelin's usual entrained rise and fall are lost during times of prolonged energy deficit (143), which would presumably facilitate lipolysis to spare blood glucose. However, such a direct role for ghrelin in the regulation of AT lipolysis has not been thoroughly examined. In the current study we demonstrate that both forms of ghrelin may be important regulators of fatty acid turnover in mature rodent adipose tissue (AT). *Ex vivo*, high doses of AG and UnAG directly attenuated the ability of the ß3-agonist CL 316, 243 to release glycerol from subcutaneous and visceral AT depots. For the first time, lipolytic cellular signalling was extensively explored, which demonstrated that in the presence of CL, both AG and UnAG likely exerted their antilipolytic effects by reducing the phosphorylation of HSL at two of its stimulatory (Ser^{563} and Ser^{660}) residues. Interestingly, neither AG nor UnAG reduced media FA concentration with CL treatment, suggesting that ghrelin may also directly hinder the ability for AT to re-esterify fatty acids. To our knowledge, we are the first to demonstrate this. Finally, *in vivo,* we did not demonstrate any blunting effect of CL-induced increases in circulating FAs or glycerol concentrations subsequent to ghrelin injection. This may be due to confounding secondary factors, although our incubation experiments would suggest that this is not due to the acute action of GH, despite purported as being lipolytic. Our data supports a potential role for ghrelin as a regulator of lipolysis, at least in isolated AT. Further work needs to examine whether this effect is exhibited *in vivo*, and if not, the mechanisms underlying its disappearance.

Ghrelin directly regulates the ß3-induction of lipolysis

Our data indicate that ghrelin inhibits lipolysis, as evidenced by reduced glycerol appearance in both subcutaneous and visceral AT depots. Importantly, however, ghrelin does not serve this role independently, but only in the presence of adrenergic stimulation (ß-agonist, CL). These are important distinctions that have not been previously assessed (9, 154). This would be physiologically relevant as ghrelin's peak concentration would coincide with that of catecholamines stimulating AT mobilization during times of energy deficit. CL-stimulated glycerol release in the presence of ghrelin was still higher than control, suggesting that ghrelin may not be sufficient to completely abolish lipolysis in the absence of other potent antilipolytic stimuli that would increase with the consumption of a meal, such as insulin (72). This should be further examined. Ghrelin's direct effects on fatty acid transport and storage in AT also remain to be studied.

Ghrelin impairs fatty acid re-esterification

When TAG is completely hydrolyzed, three FAs are released for each glycerol, and either delivered to other tissues via circulation, or re-esterified. In our study, unlike glycerol, the increase in media FAs induced by CL was not attenuated by ghrelin. This suggests that ghrelin also inhibited the re-esterification of liberated fatty acids, allowing their concentration to remain elevated. While it is logical to speculate that ghrelin may reduce glucose-sparing AT lipolysis prior to mealtime (and the ingestion of glucose), the inhibition of re-esterification is perplexing. It could be that secondary to its role in reducing AT lipolysis, ghrelin maintains elevated fatty acids via a reduction in re-esterification to protect blood glucose in the event that a meal is missed (i.e. glucose is not consumed). Then, upon meal consumption, other hormones (e.g.

insulin) can govern lipid turnover in AT. Nevertheless, perhaps at its simplest, the reduction in re-esterification simply reflects the observed absolute reduction in lipolysis, given that upwards of ~30% of hydrolyzed fatty acids can be cycled back to storage in rats (230). Mechanisms by which ghrelin may regulate fatty acid re-esterification should be explored.

Ghrelin alters lipolytic signalling through HSL

Despite some purported effects for ghrelin's positive (233) and negative (9) regulation of AT lipolysis, the ability for ghrelin to directly regulate lipolytic enzyme activity has been sparsely examined. One previous report assessing ghrelin action demonstrated a general trend towards antilipolytic signalling at phosphodiesterase, upstream of HSL (9). To our knowledge, the only study examining HSL did not assess its state of phosphorylation, nor did it have any positive control for lipolysis (130). Overall, we did not observe independent ghrelin effects on lipolysis. In line with this, HSL phosphorylation was unaffected by independent AG and UnAG treatments (data not shown). Expectedly, HSL activation was significantly decreased with ghrelin's addition to CL treatment, coinciding with the observed reduction in glycerol release, *ex vivo*. Although the mechanism mediating the ghrelin-driven reduction in HSL phosphorylation has yet to be tested directly, HSL activity (and subsequent lipolysis) appears to have been reduced by AG and UnAG via its stimulatory (Ser^{563} and Ser^{660}), but not inhibitory (Ser^{565}) residues.

Ghrelin does not affect the AMPK/ACC and ERK axes

Until now, there has been no effort towards elucidating potential ghrelin action on other enzymes and pathways thought to contribute to lipolysis (e.g. ATGL, ERK). In the current

investigation there were no observed changes in ATGL phosphorylation with any treatment *in vivo* or *ex vivo*, although it has been speculated that this covalent modification may not entirely reflect ATGL activity within adipose tissue and that PKA is not likely to be the enzyme responsible for its alteration (256). Some evidence would suggest an AMPK requirement for ATGL phosphorylation (4). In line with this, we did not observe any alterations in AMPK activation. Extracellular signal-regulated kinases (ERKs), have been previously shown to respond to ß-adrenergic agonists in adipocytes (153, 199) and stimulate lipolysis and HSL activity (85). In ATOC experiments, we did not observe changes in ERK activation with CL or ghrelin treatments, although this could be due to the timing of our measurements. Tissue was frozen for assessment of signalling to match out determination of lipolysis, which occurred at 2h and may have missed acute changes in ERK, upstream of HSL modification. *In vivo*, ERK was activated in visceral, but not subcutaneous AT, following all CL treatments. It is likely that visceral AT responded to a greater degree to the adrenergic stimulus due to its increased ß3-receptor function (100, 145, 188). Interestingly, the CL+AG combined injection consistently demonstrated the highest increase in ERK activation, and this treatment significantly activated ERK in iWAT. This could potentially be due to a secondary rise in GH, which has been shown to signal through the ERK pathway in AT (17). Clearly, however, this was not sufficient to drive a further increase in lipolysis by 30min, *in vivo*.

Ghrelin's in vivo effects on lipolysis

In the presence of the ß3-agonist CL, ghrelin's ability to down-regulate AT lipolysis was not observed *in vivo*. The assessment of the metabolic effects of ghrelin administered *in vivo* is abundant in literature but has limitations. Acutely, AG has been shown to drive GH release in

both rodents (125) and humans (211). This is potentially confounding given that GH, although subject to large interindividual variability, typically elicits an increase in lipolysis in humans (93), as well as in cell culture (165). As such, we initially sought to determine whether ghrelin could modify the lipolytic response of our ATOC preparation to GH. Unexpectedly, we did not observe any increases in lipolysis with GH. There are other reports on the lack of GH effect on lipolysis in explant (63) and adipocyte (150) models. While the mechanistic nature and time-course for GH's ability to alter lipolysis is beyond the scope of our investigation, it is worthy of further consideration as some have pursued (150). Also, it is interesting that others whom have made efforts to control for GH (*in vivo*) through the use of somatostatin, still observed increases in lipolysis with AG (233, 234). It must be given deliberation that studies demonstrating a ghrelin-mediated increase in lipolysis *in vivo* have typically been compared to baseline, or in some cases an antilipolytic hyperinsulinemic clamp as a control (233). CL is very potent, and it may be difficult to observe changes in lipolytic endpoints in the context of this pharmacological agent, which could explain why AG did not elicit any increase in lipolysis *in vivo* in the current study. We also attempted to isolate whole-body lipolytic responses to AT with CL; however, the potential contribution of ghrelin's effects on lipid metabolism in other tissues cannot be discounted. Also, fatty acid-induced insulin release is another potential effect of CL administration (86), which could down-regulate AT lipolysis. However, we feel that this is unlikely to be a factor in the present study, as we found no change in Akt (a marker of insulin signalling) activation in AT across treatments.

Conclusion

In conclusion, our data would suggest that both forms of ghrelin can directly reduce ß3-driven lipolysis in mature inguinal white and retroperitoneal adipose tissue depots through a parallel reduction in HSL activation. The cellular nature of UnAG action on peripheral tissues remains elusive, particularly with numerous others suggesting that it may act independently of the GHS-R1a (56, 66). Nevertheless, these antilipolytic effects will require further testing to uncover any physiological relevance as well as extensive *in vivo* work to contextualize ghrelin's role as a regulator of fatty acid metabolism surrounding mealtime. Finally, mechanisms relating to ghrelin's ability to concurrently inhibit AT fatty acid re-esterification should be pursued.

CHAPTER 4: Acylated and unacylated ghrelin do not directly stimulate glucose transport in isolated rodent skeletal muscle

As published, with minor modifications: Cervone DT and Dyck DJ (2016).

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Abstract

Emerging evidence implicates ghrelin, a gut-derived, orexigenic hormone, as a potential mediator of insulin-responsive peripheral tissue metabolism. However, in vitro and *in vivo* studies assessing ghrelin's direct influence on metabolism have been controversial, particularly due to confounding factors such as the secondary rise in growth hormone (GH) after ghrelin injection. Skeletal muscle is important in the insulin-stimulated clearance of glucose, and ghrelin's exponential rise prior to a meal could potentially facilitate this. The present study was aimed at elucidating any direct stimulatory action that ghrelin may have on glucose transport and insulin signalling in isolated rat skeletal muscle, in the absence of confounding secondary factors. Oxidative soleus and glycolytic extensor digitorum longus skeletal muscles were isolated from male Sprague-Dawley rats in the fed state and incubated with various concentrations of acylated and unacylated ghrelin in the presence or absence of insulin. Ghrelin did not stimulate glucose transport in either muscle type, with or without insulin. Moreover, GH had no acute, direct stimulatory effect on either basal or insulin-stimulated muscle glucose transport. In agreement with the lack of observed effect on glucose transport, ghrelin and GH also had no stimulatory effect on Ser^{473} Akt or Thr^{172} AMPK phosphorylation, two key signalling proteins involved in glucose transport. Furthermore, to our knowledge, we are among the first to show that ghrelin can act independent of its receptor and cause an increase in calmodulin-dependent protein kinase 2 (CaMKII) phosphorylation in glycolytic muscle, although this was not associated with an increase in glucose transport. We conclude that both acylated and unacylated ghrelin have no direct, acute influence on skeletal muscle glucose transport. Furthermore, the immediate rise in GH in response to ghrelin also does not appear to directly stimulate glucose transport in muscle.

Introduction

Ghrelin is a gut-derived hormone with central effects controlling appetite. Its production and release increases exponentially prior to meals and returns to basal levels immediately postprandially (125, 143). Ghrelin can be post-translationally acylated by ghrelin-*O*-acyl-transferase (GOAT) and unacylated by serum esterases (88, 194, 249). It exists primarily unbound and unacylated, although many of ghrelin's effects can be attributed to its acylated form (143, 169). Acylated ghrelin (AG) acts through GHS-R1a in non-muscle peripheral tissues, and potentially the corticotropin-releasing factor (CRF-2) receptor in muscle (67, 79). Unacylated ghrelin (UnAG) is believed to act through an alternate receptor (79, 135) in peripheral tissues such as muscle. Ghrelin's effects on peripheral glucose utilization have not been extensively studied and are unclear. Several studies (203, 232–234) suggest that AG infusion acutely impairs the clearance of glucose from the blood. Furthermore, ghrelin KO and ghrelin receptor KO mice are protected from the negative effects of a high fat diet on glucose tolerance and insulin sensitivity (172). In contradiction to these findings, AG injection has recently been shown to improve blood glucose clearance during an oral glucose tolerance test in mice (74), which was linked to the ability of AG to stimulate the secretion of glucagon-like peptide-1 (GLP-1) (74) an intestinally released incretin which serves to potentiate insulin release (25, 29, 74). Taken together, there is controversy as to the *in vivo* effects of ghrelin on peripheral glucose metabolism.

A potentially confounding factor when examining the effects of *in vivo* ghrelin administration is the change in circulating concentrations of secondary hormones. For example, AG is well known to stimulate growth hormone release (125, 223, 246). Growth hormone has been shown to stimulate adipose lipolysis (63), which may in turn alter (i.e. reduce) peripheral

glucose utilization. However, AG has also been demonstrated to acutely increase GLP-1 and subsequently insulin (74), which conversely could lead to increased peripheral glucose transport. The direct, isolated effects of ghrelin isoforms on peripheral insulin sensitive tissues such as skeletal muscle, in the absence of these secondary hormonal changes, have not been extensively examined. Such an examination is required to fully understand the metabolic role of ghrelin in tissues such as skeletal muscle.

The effects of ghrelin on skeletal muscle metabolism have not been extensively investigated and are inconsistent. One study reported increases in key insulin responsive proteins (Akt, glycogen synthase kinase, GLUT4) in oxidative muscle (11) in response to *in vivo* AG injections over a period of several days in rats, while others exhibit no alteration in Akt following injection or infusion, in both rats and humans (12, 234). There is also some, albeit limited evidence for a direct effect of ghrelin on isolated muscle cells. In C2C12 myocytes, direct exposure to AG for 24h increases GLUT4 translocation to the cell surface and glucose transport (79). However, to date there has been no examination of ghrelin's direct and isolated effect on glucose transport in mature, intact skeletal muscle. Given ghrelin's rise just prior to the consumption of food, it is tempting to consider whether ghrelin could prime tissues such as skeletal muscle in the preparation for the delivery of glucose, making them more responsive to insulin. To our knowledge this potential role for ghrelin has largely been ignored. Therefore, the overall aim of this study was to determine the direct effects of ghrelin's two main isoforms on the transport of glucose in isolated rat skeletal muscle, in the presence and absence of insulin. More specifically, we hypothesized that ghrelin would directly stimulate glucose transport in skeletal muscle in the presence, but not the absence of insulin.

Methods

Animals

All procedures were approved by the Animal Care Committee at the University of Guelph and followed Canadian Council of Animal Care guidelines. Male Sprague-Dawley rats were purchased from Charles River Laboratories (Québec, ON, Canada) at approximately 6 weeks of age (~200 g). Rats were given free access to water and a regular chow diet, *ad libitum*. Food was provided until approximately 3h prior to experiments to ensure that animals were in the fed state for terminal surgeries. Rodents were anesthetized with an intraperitoneal injection of pentobarbital sodium (6mg per 100g body mass) prior to all surgical procedures.

Materials and Reagents

Reagents, molecular weight markers, and nitrocellulose membrane were purchased from BioRad (Mississauga, ON, Canada). Western Lightning Plus enhanced chemiluminescence (ECL) was purchased from Perkin-Elmer (NEL105001EA). The following primary antibodies were purchased from Cell Signalling: phospho-Akt Ser^{473} (Cat. # 4060), phospho-Akt Thr^{308} (Cat. # 9275S), phospho-CaMKII Thr^{287} (Cat. # 12716), phospho-STAT5b Tyr^{694} (Cat. # 4322), total Akt (Cat. # 9272S), total AMPK (Cat. # 2603S), total CAMKII (Cat. # 4436S) and total STAT5 (Cat. # 9363T). NP40 cell lysis buffer was acquired from Life Technologies and PMSF and protease inhibitor cocktail were obtained from Sigma (Cat. # 78830 and 9599). Insulin (Humulin rDNA origin) was purchased from Eli Lilly (Toronto, ON, Canada).

Incubation buffer (Krebs-henseleit base) constituents were purchased from Sigma-Aldrich and include: D-glucose (Cat. #G-8270), D-mannitol (Cat. #M-9546), sodium pyruvate (Cat. #P8574) and 3-methyl-*O*-glucopyranose (Cat. #M-4879). Growth hormone was obtained from Abcam (Cat. #ab68388). Acylated (Cat. #H-4862) and unacylated (Cat. #H-6264) ghrelin were sourced from Bachem (Torrance, CA, USA). Radioactive ^{14}C-mannitol (Cat. #CFA-238 - 250µCi/1250µl) and 3-O-[^{3}H]methyl-D-glucose (Cat. #ART-126 - 1mCi/1000µl) tracers were purchased from American Radiolabeled Chemicals (St. Louis, MO, USA).

Ghrelin Stability Verification

To our knowledge, the stability of either ghrelin isoform in an incubation medium has not been reported. To test this, incubation buffer samples were taken at t = 0, 30, 60 and 120 min and analyzed for ghrelin concentration using a commercially available kit (EMD Millipore Cat. EZRGRT-91K) according to manufacturer instructions. Accuracy and intra-assay comparisons were validated using two quality control standards (run in triplicate) provided with each kit. Buffer samples were assayed in duplicate.

Glucose Uptake Assays

Glucose transport assays in isolated skeletal muscle were carried out as previously described (26, 156, 183, 216). Briefly, media were pre-gassed with 95% O_2 and 5% CO_2 and heated in a shaking water bath at 30˚C. One muscle strip for each condition within a set of experiments was obtained from a single animal, as each muscle can be stripped into 2-3 viable 20-30 mg sections. Muscles were allowed to float in medium and did not have tension applied.

Soleus (oxidative) and extensor digitorum longus (glycolytic) muscles were stripped lengthwise, excised with tendons intact and pre-incubated in medium (M1) containing 8 mM D-glucose and 32 mM D-mannitol for 1h. Next, muscles were carefully transferred to vials containing a medium (M2) of 36 mM D-mannitol and 4 mM pyruvate to undergo two separate 15 min washes. Lastly, muscles were transferred to vials which contained a medium (M3) of 8mM 3-methyl-O-glucopyranose, 28mM D-mannitol, pyruvate 4mM, 0.5µl/ml 3-*O*-[^{3}H]methyl-D-glucose, 1.0µl/ml ^{14}C-mannitol for 1 h. Acylated or UnAG was added to all media (M1, 2 and 3) for non-insulin stimulated glucose transport determinations. To determine whether AG or UnAG had a priming effect on insulin-stimulated glucose transport, AG or UnAG were added to pre-incubation and wash incubations only (M1 and 2), but not the final transport medium (M3) in an attempt to mimic the physiological state of high insulin and low/declining ghrelin following meal consumption. To determine the effects of GH on glucose transport, GH was added to the wash and final transport incubation (M2 and M3).

For insulin-stimulated glucose transport assays, insulin was added to both wash media (M2) as well as the final transport incubation (M3) at either 0.5mU/ml (moderate) or 10mU/ml (high) concentrations (183, 217). Following incubation, muscles were trimmed of their tendons, blotted and weighed. Muscles were then boiled and solubilized for ~10 min in 1ml of 1M NaOH and vortexed periodically throughout. A 200µl sample of muscle digest was added to scintillation vials with 5ml of Cytoscint scintillation fluorescent cocktail (MP Biomedicals - via Cedarlane - Burlington, ON, Canada) and swirled to ensure proper mixing. Samples were left to quench overnight in darkness and then counted for 5 min per sample using a Beckman-Coulter LS5600 liquid scintillation counter and were considered acceptable when the detected Lumex

percentage (random counts per minute) was less than 5%. Glucose transport was calculated as the accumulation of intracellular labelled glucose as we have previously reported (183).

Ghrelin, growth hormone and insulin signalling

Incubations for the purpose of determining hormonal signalling was carried out so as to closely mimic our glucose transport protocol. In short, muscle strips from soleus and EDL were removed from one leg and incubated for 1 h in pre-incubation buffer, then transferred to a second (untreated) buffer for 15 min, as described above. Strips from the contralateral leg were also excised, pre-incubated for 1 h, and then transferred to a second experimental buffer containing various treatments (i.e. 150ng/ml AG or UnAG, 250ng/ml GH, 10mU/ml insulin) for 15 min. This brief window was chosen to detect any rapid transient changes in signalling proteins (e.g. phosphorylation of Akt, AMPK, etc.). All tissues were immediately frozen in liquid nitrogen and then stored at -80˚C until further processing for western blotting.

Western blotting

Muscles were chipped under liquid nitrogen into 20-30mg pieces, and then placed directly into homogenization tubes containing lysis beads and stored in liquid nitrogen. Cell lysis buffer supplemented with protease inhibitor and PMSF were then added, and samples were homogenized and centrifuged at 4°C. Supernatant was then removed and transferred to a new tube. BCA assays were performed to determine protein content from homogenized samples for subsequent sample preparation and western blots (200).

For western blots, equal amounts of sample protein (Akt: 20µg; CaMKII: 25µg; STAT5: 30µg) were loaded onto 10% gels, as we have published previously (156, 183). Samples were then transferred for 1 h at 0.2A onto nitrocellulose membranes and blocked in non-fat skim milk

powder and TBST. Membranes were then incubated with primary antibody (1:1000 Akt and CaMKII; 1:500 STAT5) diluted in 5% BSA and incubated overnight at 4°C. Afterwards, membranes were washed with TBST and exposed to secondary (anti-rabbit) antibody (1:2000) for 1 h at room temperature and then washed again (2x TBST, 1x TBS). Bands were visualized using ECL and quantified using densitometry on Alpha Innovate Software. Ponceau staining was used as a loading control.

Statistics

All data are expressed as mean ± standard error. A repeated measure one-way analysis of variance (ANOVA) was performed for all experiments examining the effects of ghrelin and growth hormone, and their interaction with insulin, on glucose transport and signalling proteins. If significance was detected with the ANOVA, multiple comparisons were assessed using Tukey's post-hoc test. A paired student's t-test was performed for experiments examining growth hormone effects (independent of insulin) on glucose transport and signalling proteins. In all glucose transport figures, letters are used to denote statistical significance, such that groups sharing a letter are not significantly different. Data was considered significant at $p < 0.05$. Data points greater than two standard deviations from the mean were considered outliers and removed.

Results

AG and UnAG remain stable in incubation buffer for 2 h

Despite the prevalence of studies which use cell (e.g. adipocyte, myocyte) incubations to assess ghrelin's effects, there has not been any previously reported validation of ghrelin's stability in such a preparation. Thus, prior to commencing glucose uptake incubations, it was first confirmed that AG and UnAG, added to produce a final buffer concentration of 4 and 12 ng/ml respectively, remained stable at 30˚C for a 2 h period. Sampling was done at t = 0, 30, 60 and 120 min and there were no indications to suggest that ghrelin was declining in the incubation solution. Acylated and unacylated ghrelin concentrations at 0, 30, 60 and 120 min were: AG; 3.6, 3.4, 3.1 and 3.4 ng/ml and UnAG; 9.3,11.2, 12.5 and 13.1 ng/ml.

AG and UnAG do not impact muscle's ability to uptake glucose ex vivo, either in the presence or absence of insulin

Over a wide range of concentrations (1-150ng/ml), neither AG nor UnAG had any significant effect on the rate of 3-*O*-[^{3}H]-methyl-D-glucose uptake independent of insulin, within oxidative soleus **(Figure 9A, B)** or glycolytic EDL skeletal muscle **(Figure 9C, D)**.

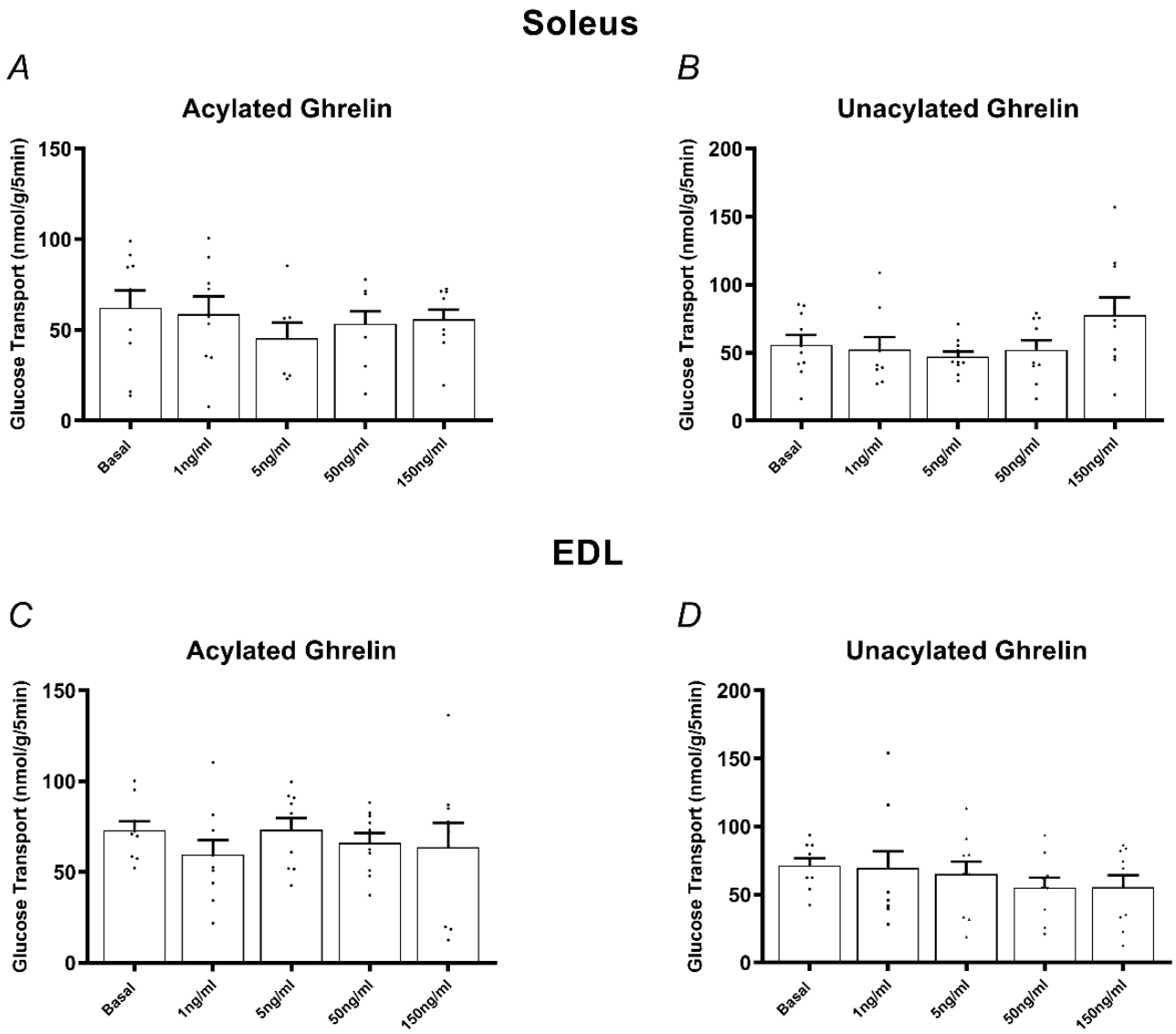

Figure 9: Basal Glucose Uptake in Isolated Skeletal Muscle

Effect of acylated and unacylated ghrelin on glucose transport in the absence of insulin. 3-O-[^{3}H]-methyl-D-glucose transport (nmol/g/5 min) in incubated oxidative soleus (A and B) and glycolytic EDL (C and D) muscles with ghrelin alone. Data were analyzed using a repeated measures one-way ANOVA (n = 10). There were no statistically significant differences among groups.

In both soleus and EDL, a moderate dose of insulin (0.5mU/ml) significantly increased glucose uptake, on average, by 44% and 52% ($p<0.05$), respectively, compared to control **(Figure 10A-D)**. However, insulin-stimulated glucose uptake was not altered by the presence of physiological and supraphysiological concentrations of AG **(Figure 10A, C)** or UnAG **(Figure 10B, D)** in either oxidative or glycolytic muscle. It should also be noted that in soleus, neither of the insulin conditions with ghrelin (**Figure 10A, B**) was statistically different from the basal condition. Thus, it might be argued that ghrelin prevented insulin from stimulating glucose transport, although this is complicated by the fact that none of the insulin conditions (insulin, insulin + AG, insulin + UnAG) were statistically different from each other. This is also true in EDL with the highest concentration of AG and UnAG (**Figure 10C, D**). Taken together, while it is somewhat tenuous to state that ghrelin decreased insulin-stimulated glucose transport, our data are very clear that the ghrelin did not increase glucose transport, either in the presence or absence of insulin, which was the specific hypothesis that we were testing.

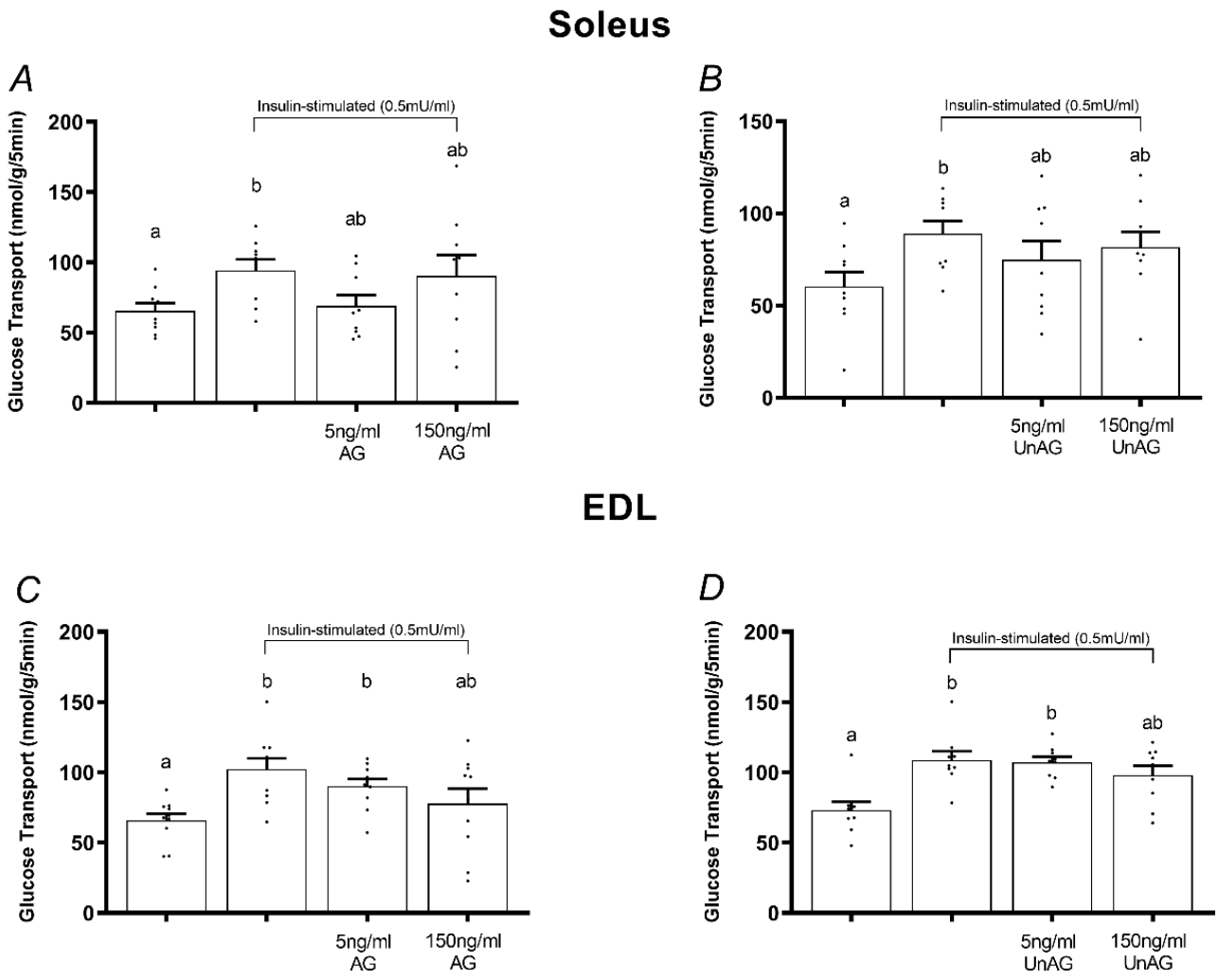

Figure 10: Submaximal Insulin-Stimulated Glucose Uptake in Isolated Skeletal Muscle

Effect of acylated and unacylated ghrelin on glucose transport with a moderate dose of insulin. 3-O-[^{3}H]-methyl-D-glucose transport in oxidative soleus (A and B) and glycolytic EDL (C and D) muscles following preincubation with either moderate insulin (0.5mU/mL) alone or moderate insulin in conjunction with ghrelin. Groups sharing a letter are not statistically different. AG, acylated ghrelin; UnAG, unacylated ghrelin; Ins, insulin; EDL, extensor digitorum longus. Data were analyzed using a repeated measures one-way ANOVA (n = 9–10).

High dose insulin-stimulation resulted in a 2-fold increase ($p<0.05$) in the rate of glucose uptake in soleus **(Figure 11A)** and a 65% increase ($p<0.05$) in EDL **(Figure 11B)**. As previously observed, neither AG nor UnAG significantly increased glucose transport in the presence of insulin in either muscle. Again, it is perhaps worthwhile noting that glucose transport in the presence of insulin was not statistically different from the basal condition with a high dose of AG in soleus, and UnAG in EDL.

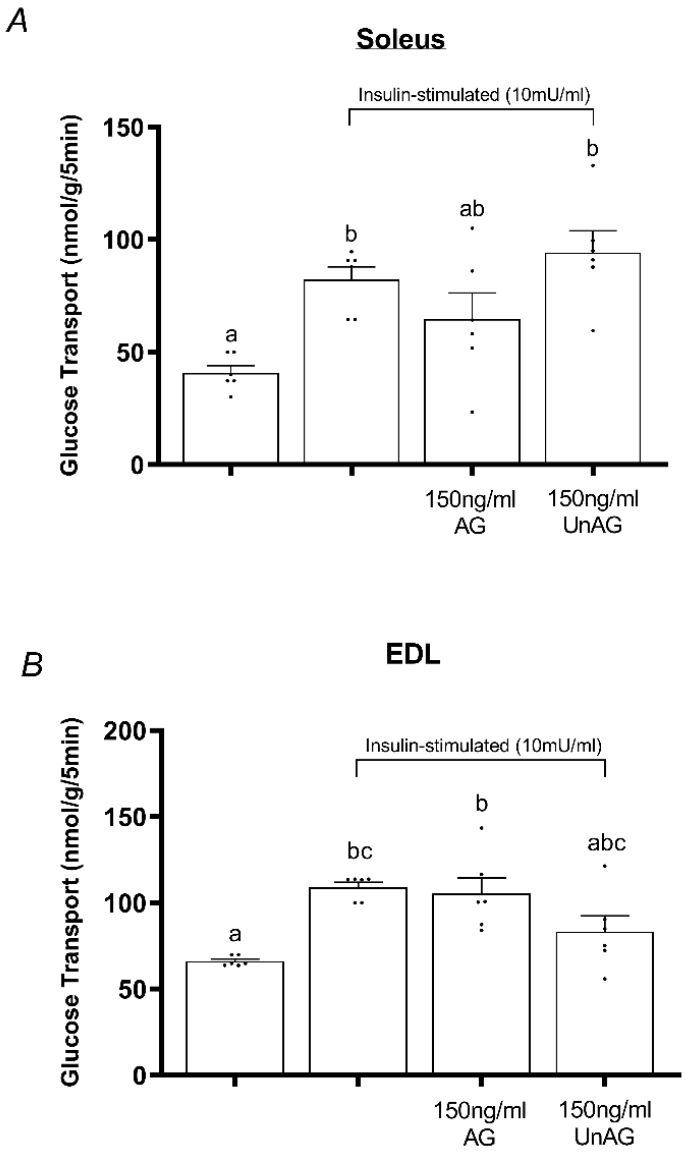

Figure 11: Maximal Insulin-Stimulated Glucose Uptake in Isolated Skeletal Muscle

Effect of acylated and unacylated ghrelin with a high dose of insulin on glucose transport. 3-O-[^{3}H]-methyl-D-glucose transport in oxidative soleus (A) and glycolytic EDL (B) muscles following preincubation with either high insulin (10 mU/mL) alone or high insulin in conjunction with ghrelin. Groups sharing a letter are not statistically different. AG, acylated ghrelin; UnAG, unacylated ghrelin; Ins, insulin; EDL, extensor digitorum longus. Data were analyzed using a repeated measures one-way ANOVA (n = 6).

Akt Ser^{473} phosphorylation is increased following insulin, but not AG or UnAG incubation

There was a robust increase in the phosphorylation of the insulin signalling protein Akt at one of its activating sites (Ser^{473}) in both soleus (7.1 ± 0.5 arbitrary units; $p<0.05$) and EDL (5.4 ± 0.24; $p<0.05$), when subjected to a high dose (10mU/ml) of insulin **(Figure 12A, B)**. However, AG and UnAG had no acute effect on the activation of Akt, which is in agreement with the lack of stimulatory effect of ghrelin on glucose transport.

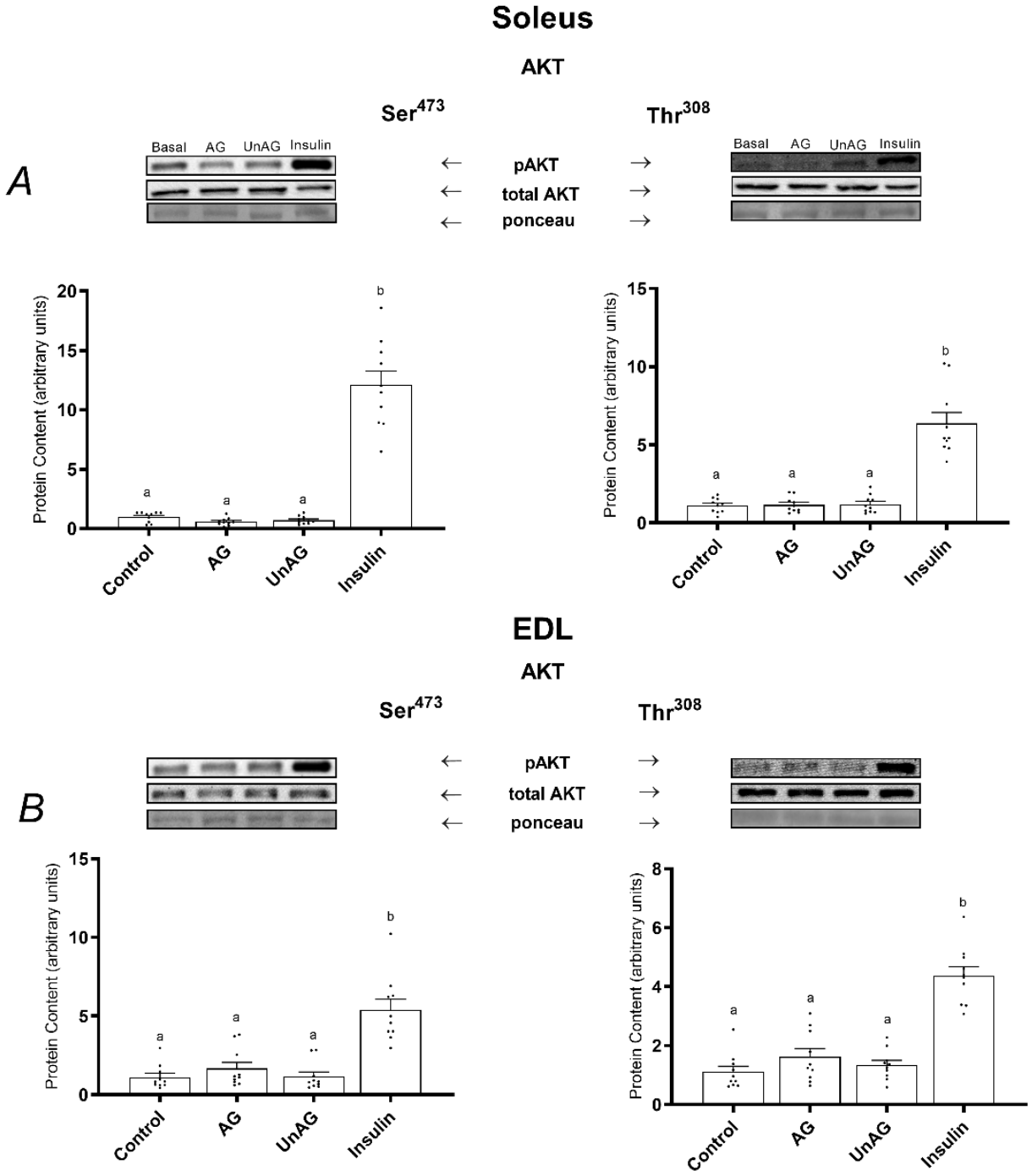

Figure 12: Effects of Ghrelin on Insulin Signalling

Effect of acylated and unacylated ghrelin, and insulin on the phosphorylation of Akt Ser^{473} and Thr^{308} protein content following incubation with ghrelin (150ng/mL) or insulin (10mU/mL) in

(A) oxidative soleus and (B) glycolytic EDL muscles. Groups sharing a letter are not statistically different. AG, acylated ghrelin; UnAG, unacylated ghrelin; EDL, extensor digitorum longus. Data were analyzed using a repeated measures one-way ANOVA (n = 10).

CaMKII Thr^{287} phosphorylation is significantly increased following AG and UnAG incubation, but only in glycolytic skeletal muscle

Acylated ghrelin signals via GHS-R1a in several peripheral tissues, although its levels in skeletal muscle have been considered to be negligible (82). Rather, AG is thought to potentially interact with the corticotropin-releasing factor (CRF-2) receptor in muscle (67, 79), while UnAG does not have an identified receptor, although it is not a ligand for GHS-R1a and has been shown to have metabolic effects in peripheral tissues distinct from AG (67, 135). Although UnAG signalling/receptor interactions remain largely unknown, classical ghrelin signalling via GHS-R1a results in the transient release of calcium (104, 125). However, whether ghrelin activates similar signalling pathways in isolated mature skeletal muscle has not been examined to our knowledge. To this end, we examined CaMKII phosphorylation, the major multifunctional calmodulin-kinase in skeletal muscle (187), as an initial attempt to detect ghrelin signalling in skeletal muscle. CaMKII (δ: ~57kDa, γ: ~62kDa and β_M: ~72kDa) phosphorylation was detected as three bands, consistent with what has been previously reported in skeletal muscle (187). During pilot analysis, the lower two (δ and γ) bands followed a loading dose-response relationship and were therefore quantified for study purposes. Since we did not have antibody for total CaMKIIδ, only pCaMKIIγ is shown expressed relative to total gamma protein. Interestingly, CaMKII phosphorylation was not increased in oxidative soleus muscle (**Figure 13A**). However, the content of pCaMKIIδ (not shown) and pCaMKIIγ was increased in EDL, but

only following incubation with AG (**Figure 13B**), serving as a positive control for ghrelin signalling in this muscle.

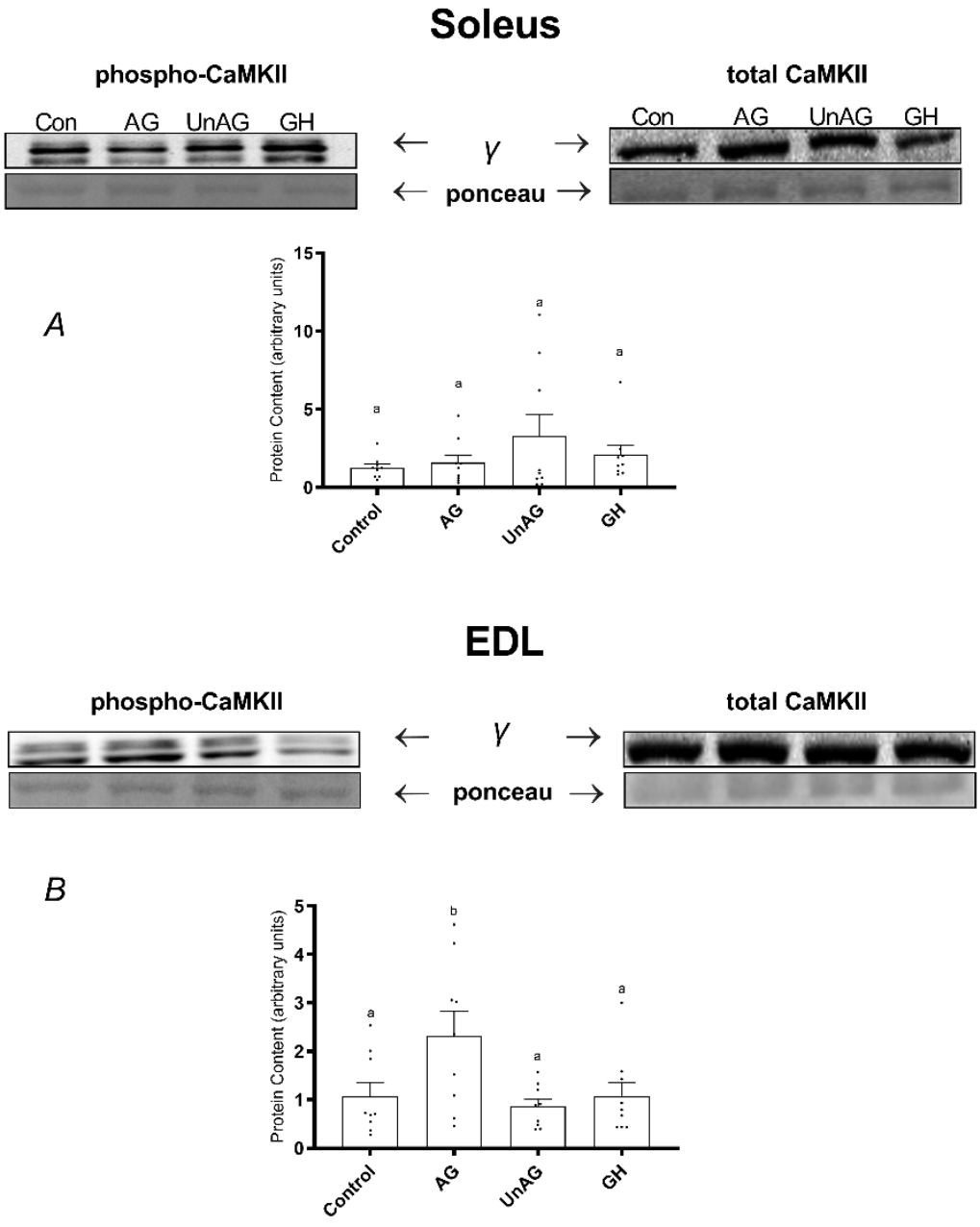

Figure 13: Effects of Ghrelin on CaMKII Signalling

Effect of acylated and unacylated ghrelin, and growth hormone on CaMKIIγ (Thr^{287}) protein content following incubation with ghrelin (150ng/mL) or GH (250ng/mL) in (A) oxidative soleus

and (B) glycolytic EDL muscles. Groups sharing a letter are not statistically different. AG, acylated ghrelin; UnAG, unacylated ghrelin; GH, growth hormone; EDL, extensor digitorum longus. Data were analyzed using a repeated measures one-way ANOVA (n = 10).

AMPK phosphorylation (Thr^{172}) is unchanged following incubation with AG, UnAG and GH

Consistent with previous findings with ghrelin in rodent skeletal muscle (127), there was no observed changes in the state of AMPK activation in soleus or EDL following ghrelin or GH treatment **(Figure 14A, B)**.

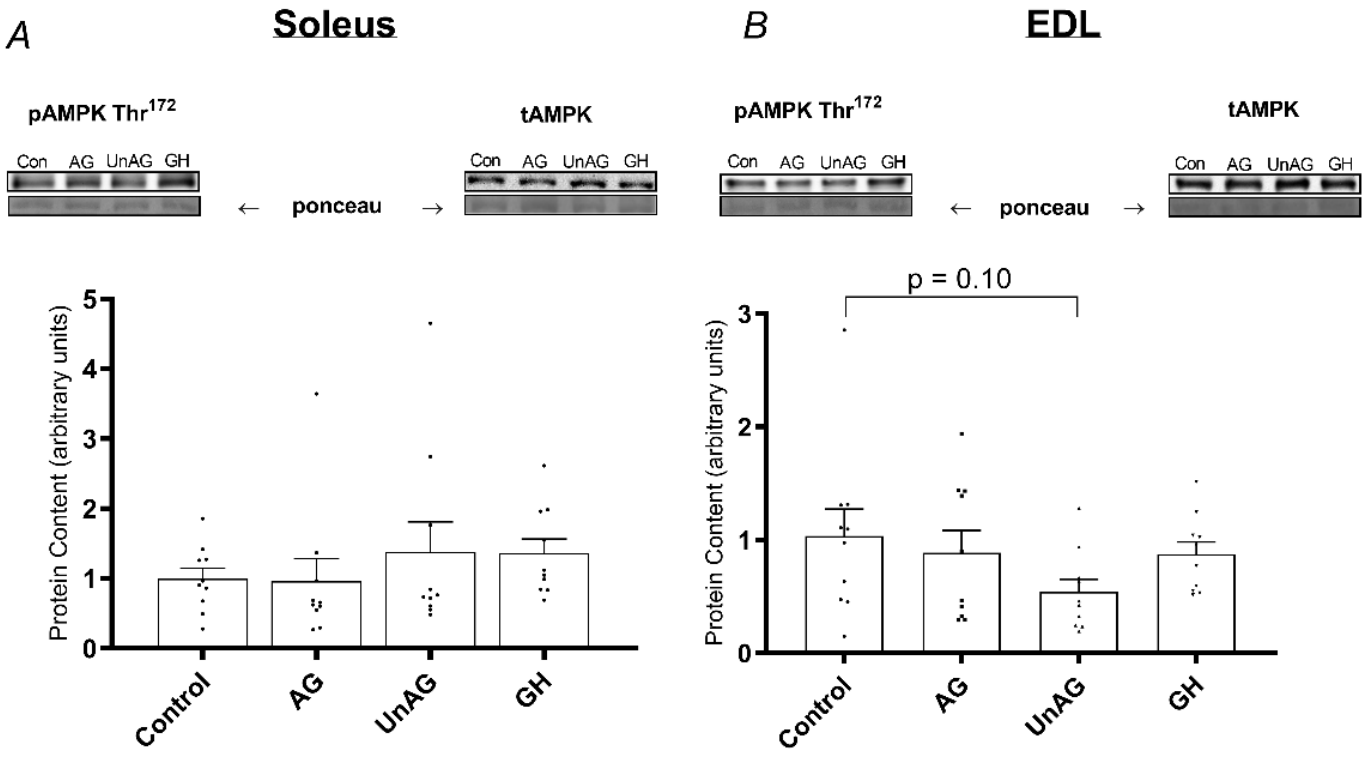

Figure 14: Effects of Ghrelin on AMPK Activation

Effect of acylated and unacylated ghrelin, and growth hormone on the phosphorylation of AMPK Thr^{172} protein content following incubation with ghrelin (150ng/mL) or GH (250ng/mL) in (A) oxidative soleus and (B) glycolytic EDL muscles. There were no statistically significant differences among groups. AG, acylated ghrelin; UnAG, unacylated ghrelin; GH, growth hormone; EDL, extensor digitorum longus. Data were analyzed using a repeated measures one-way ANOVA (n = 10).

Incubation with GH has no effect on basal or insulin-stimulated glucose transport, or activation of insulin signalling protein, Akt (Ser^{473} and Thr^{308})

Because GH is robustly increased *in vivo* subsequent to ghrelin treatment, we also determined whether GH might directly influence glucose transport in muscle. GH did not directly affect glucose transport in the absence of insulin (**Figure 15A and B**). Also, GH did not alter glucose transport in the presence of insulin ($p = 0.36$, compared to insulin alone), although there was an apparent decrease such that this was no longer different from the basal condition (**Figure 15C and D**). Akt phosphorylation was not significantly affected by GH in either oxidative or glycolytic muscles (**Figure 15E and F**).

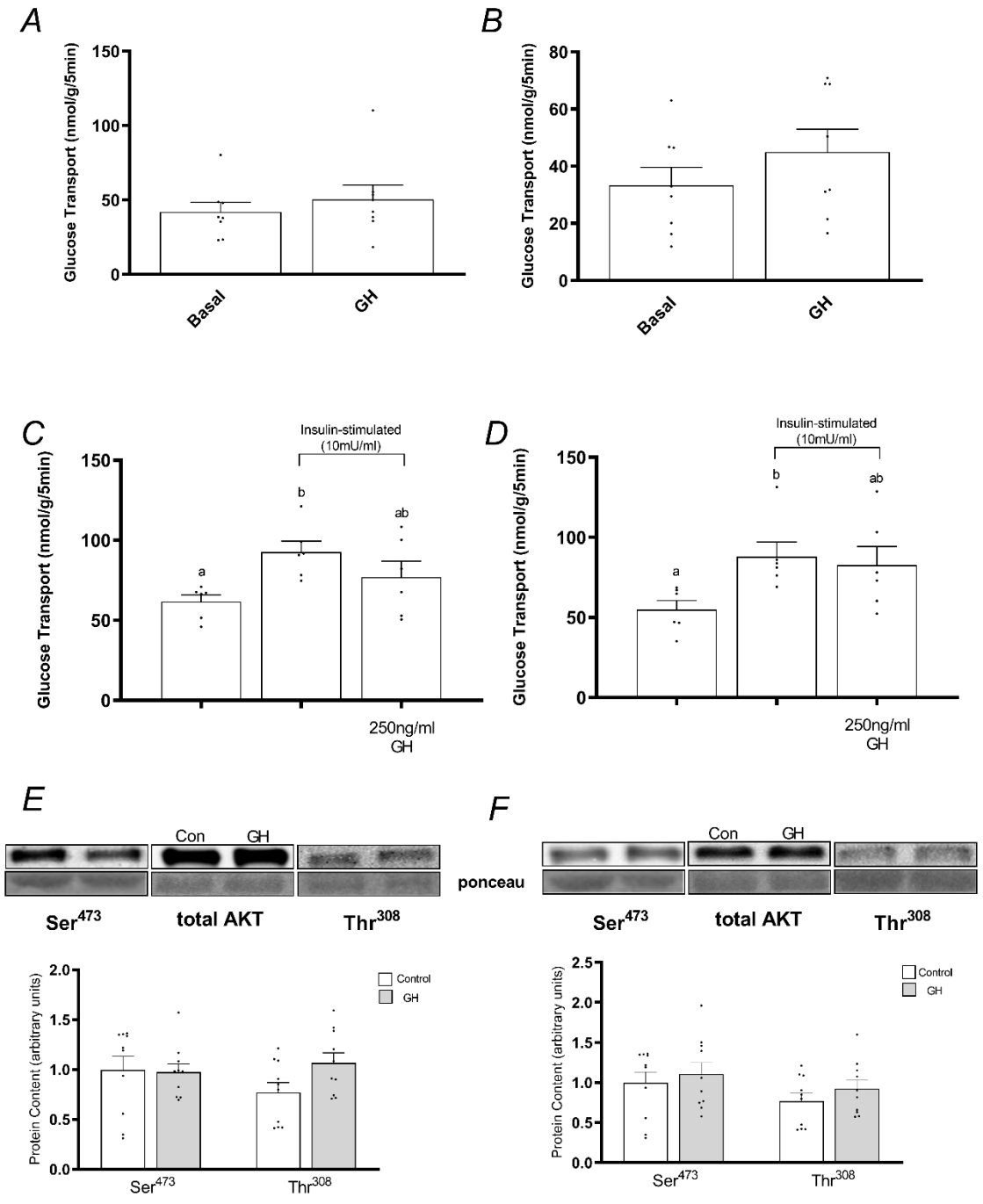

Figure 15: Effects of Growth Hormone on Glucose Uptake and Insulin Signalling

Effect of growth hormone on basal (A, B) and insulin-stimulated (C, D) 3-O-[^{3}H]-methyl-D-glucose uptake and phosphorylation of Akt Ser^{473} and Thr^{308} (E, F) in muscles treated with GH

(250ng/mL). Groups sharing a letter are not statistically different. AG, acylated ghrelin; UnAG, unacylated ghrelin; GH, growth hormone. Basal glucose uptake and insulin signalling data were analyed using an unpaired t-test (n= 8 and n = 10, respectively). Insulin-stimulated glucose uptake data were analyzed using a repeated measures one-way ANOVA (n = 6).

GH, but not AG or UnAG, potently increases the phosphorylation of STAT5 (Tyr^{694})

Since some of the effects of ghrelin on peripheral tissues may be due to secondary rise in GH, and we measured glucose uptake in the presence of GH, we wanted to verify that GH signalling was present in both muscle fiber types. We examined the phosphorylation of Tyr^{694} of STAT5, which is a common downstream target of GH action and JAK2/STAT signalling (7). Phosphorylation of STAT5 Tyr^{694} was significantly elevated in soleus in response to GH ($p<0.05$) and trended towards an increase in EDL ($p= 0.08$; **Figure 16A and B**). Acylated and unacylated ghrelin had no effect on STAT5 phosphorylation in either muscle (**Figure 16A and B**).

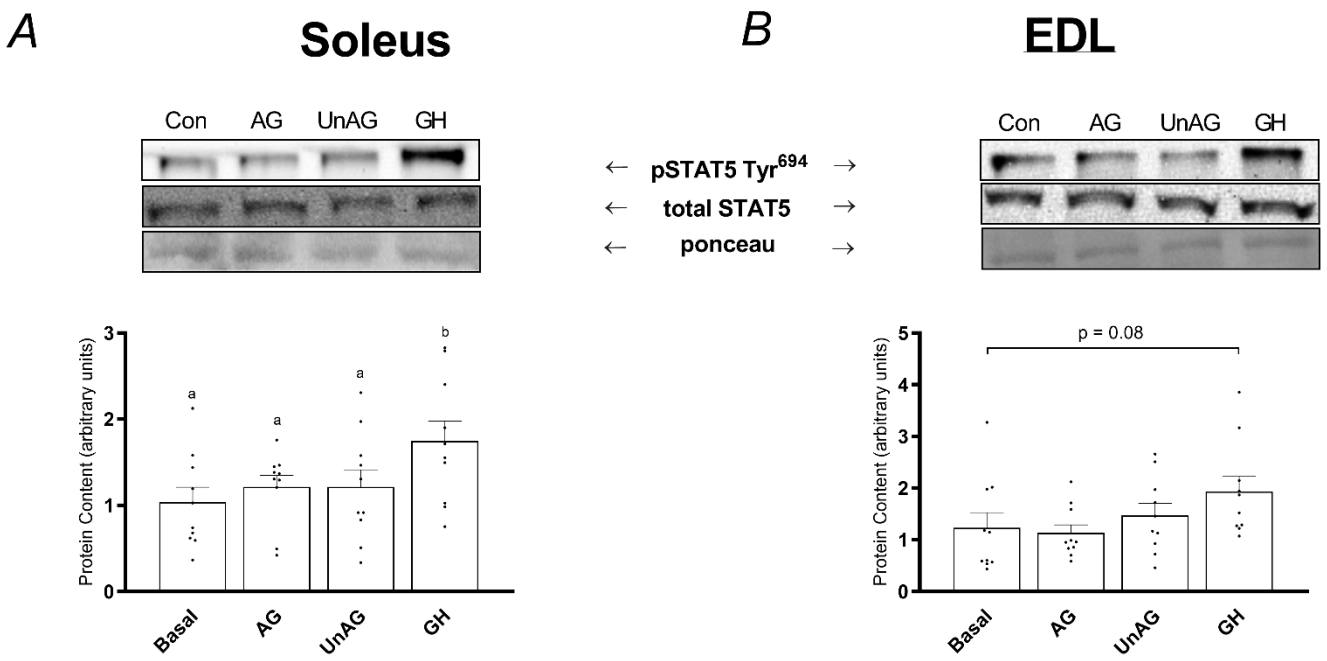

Figure 16: Effects of Ghrelin and Growth Hormone on STAT5 activation

Effect of ghrelin isoforms and growth hormone on phosphorylation of STAT5 Tyr^{694}. pSTAT5 protein content following incubation with GH (250ng/mL) in oxidative soleus (A) and glycolytic EDL (B) muscles. AG, acylated ghrelin; UnAG, unacylated ghrelin; GH, growth hormone; EDL, extensor digitorum longus. Data were analyzed using a repeated measures one-way ANOVA (n = 10).

Discussion

Given the rapid rise of ghrelin prior to habitual mealtime, it is plausible that ghrelin is involved in the regulation of insulin-mediated glucose transport in skeletal muscle. We therefore hypothesized that ghrelin would further stimulate insulin-mediated glucose transport in skeletal muscle but would not alter glucose transport in the absence of insulin. Our rationale for this was that in the absence of insulin, such an effect of ghrelin *in vivo* could cause a decrease in blood glucose in the absence of consuming food. To our knowledge, the direct effect of ghrelin isoforms on glucose transport in isolated mature skeletal muscle, in the absence of secondary increases in GH, GLP-1, FAs, etc., has not been examined. The results of our study demonstrate that neither AG nor UnAG over a wide range of concentrations significantly stimulate glucose trasnport in either oxidative or glycolytic skeletal muscle, regardless of the presence or absence of insulin. Given that ghrelin administration potently stimulates GH release, we also determined whether GH might have any direct stimulatory effect on muscle glucose uptake that would help to explain the reported *in vivo* effects of ghrelin. Direct incubation with GH did not significantly increase glucose transport. Although not statistically significant, there was a trend toward a reduction in glucose transport in the presence of insulin following GH incubation, which could potentially explain previous reports of impaired glucose tolerance following ghrelin administration in humans. In agreement with the lack of observed stimulation on muscle glucose transport, AG, UnAG and GH did not affect Akt Ser^{473} or Thr^{172} AMPK. To our knowledge, we are the first to demonstrate an AG-mediated increase in Thr^{287} CaMKII phosphorylation in muscle, although this clearly was not associated with an increase in glucose transport. Altogether, our findings suggest that any effects of acute ghrelin injection on glucose homeostasis, and in particular postprandial glucose clearance, are not due to the direct effects of

ghrelin on skeletal muscle. However, our findings in no way should be taken to discount the potential metabolic effects of chronic ghrelin administration, as this has been shown to produce significant effects on the regulation of glucose metabolism in muscle (11, 30).

Skeletal muscle is an important insulin-responsive tissue involved in the clearance of plasma glucose (55) and management of glucose homeostasis. To date there has been sparse data available to suggest ghrelin as a mediator of insulin signalling in muscle. Gershon et al. reported an increase in GLUT4 translocation and glucose uptake in C2C12 muscle cells treated for 24 h with AG (79). Other groups have reported no change in the phosphorylation of insulin signalling markers, such as Akt, in acute response to ghrelin administration (233). However, the response to chronic ghrelin injections appears to be quite different. Barazzoni and colleagues were among the first to demonstrate that ghrelin can increase key insulin-responsive proteins, including Akt, GLUT4, and GSK3 (11), subsequent to the chronic (4 days) subcutaneous administration of AG. There were no functional assessments of glucose metabolism to indicate whether glucose uptake was also increased in response to ghrelin (11). In addition, 4 days of UnAG ghrelin injection has recently been demonstrated to prevent high fat diet-induced muscle inflammation and preserve normal muscle glucose uptake (30). Our own data indicates that over a time course of ~1h (and therefore relevant to the time course of the rapid rise and fall in ghrelin surrounding entrained mealtimes), high physiological concentrations of AG and UnAG ghrelin do not result in enhanced insulin signalling or glucose transport in isolated oxidative or glycolytic skeletal muscle.

Growth hormone's direct effects on muscle glucose transport and Akt phosphorylation were also examined given that AG's effects on glucose metabolism *in vivo* could potentially be mediated or confounded through the secondary induction of GH. Treatment with GH in

differentiated adipocytes *ex vivo*, as well as in rat skeletal muscle *in vivo*, suggest that chronically GH can induce insulin resistance as indicated by reductions in IRS-1/2 tyrosine and Akt phosphorylation and deoxyglucose transport (201, 210, 214), which may in part be mediated by an increase in adipose lipolysis and circulating FAs (164). Initial studies with ghrelin in humans attempted to control for ghrelin's secondary GH release, using either infusion of somatostatin to inhibit GH release, or GH-deficient participants (77, 233). However, somatostatin is unable to fully blunt significant elevations in serum GH (233), and GH-deficient individuals often exhibit impaired insulin sensitivity (99). Our results show that in isolated muscle, GH acutely stimulates STAT5, but does not increase the phosphorylation of Akt, which is consistent with previous findings, some of which indicate GH-induced reductions in Akt activity (210). In line with this, there was no observed functional increase in glucose transport following muscle incubation with a maximal GH dose. Next, we considered whether GH could influence glucose transport in the presence of insulin, as both hormones would be elevated immediately following a meal. Similarly, GH did not alter glucose transport in the presence of insulin; rather, there was a trend toward a reduction in glucose transport although this was not statistically different from our insulin control. Thus, it would seem that any potential stimulatory effect of ghrelin on glucose clearance *in vivo* is not due to the direct effect of GH on skeletal muscle glucose transport.

Data from human studies suggest that AG administration causes hyperglycemia and insulin resistance, whereas UnAG may be insulin-sensitizing (77, 233). In rodents, animals lacking ghrelin, or the ghrelin receptor are resistant from the induction of impaired glucose tolerance and insulin sensitivity induced by a high fat diet (172). In contradiction to these findings, Gagnon et al. (74) demonstrated that AG injection in mice improves blood glucose

clearance during an oral glucose tolerance test, an effect which appears to be dependent on the release of the incretin GLP-1 (29). Thus, the role of ghrelin *in vivo* in the regulation of blood glucose homeostasis is unclear. However, we find the results from Gagnon et al. (74) intriguing as it suggests a link between ghrelin release and the subsequent metabolism of ingested glucose. Studies directly assessing ghrelin's influence on glucose transport have produced mixed results. In isolated 3T3-L1 adipocytes and C2C12 myocytes, supramaximal concentrations of both isoforms of ghrelin have been shown to stimulate glucose transport in the absence of insulin (79, 168). However, Patel et al. found that only AG, but not UnAG, was able to potentiate insulin's stimulatory effect on glucose uptake in adipocytes (168). Unfortunately, there was no assessment of insulin signalling. Although the known ghrelin receptor GHS-R1a does not appear to be detectable in muscle, ghrelin may act through the CRF-R2 receptor to stimulate GLUT4 translocation in C2C12 cells, an effect which is blocked in the presence of the selective CRF-R2 antagonist, anti-sauvagine (79).

Glucose transport in skeletal muscle is mediated through distinct insulin and contraction mediated pathways, and their maximal effects can be additive (75). Signals such as Ca^{2+}/CaMKII and AMPK appear to be involved in contraction mediated GLUT4 translocation and glucose transport (95, 247). Ghrelin's effects on these stimulatory processes do not appear to be consistent. For example, neither AG nor UnAG affects the phosphorylation of AMPK in skeletal muscle (127); however, in non-skeletal muscle peripheral tissues such as adipose and liver, AG has been shown to inhibit AMPK (127). AG has been shown to increase Ca^{2+} release in cardiomyocytes (208) and CaMKII activation in glial cells (39). To our knowledge, we are the first to report AG-induced phosphorylation of CaMKII in skeletal muscle. However, the observed increase in CaMKII activation with AG was not accompanied by an increase in glucose

transport. Although initial reports by Holloszy indicated that increasing cytosolic Ca^{2+} alone could increase glucose transport (251), CaMKII's role in skeletal muscle glucose transport has been recently disputed (113, 243). Thus, we speculate that the ghrelin induced increase in CaMKII activation observed in this study was not sufficient to increase glucose transport. We have no explanation for the lack of effect of AG on CaMKII phosphorylation in the oxidative soleus. Interestingly, it was the UnAG that tended to increase CaMKII phosphorylation in this muscle type, although the response was not statistically significant. In this study, we did not observe any significant change in AMPK phosphorylation with either AG or UnAG, although there was a trend toward a reduction in the glycolytic EDL with UnAG ($P = 0.10$).

Conclusion

In conclusion, our results indicate that at least acutely, ghrelin does not appear to have any direct stimulatory effects on muscle glucose transport or relevant signalling mechanisms. Further, the lack of a direct effect of GH on glucose transport and signalling (Akt, CaMKII and AMPK) questions the secondary role of GH in directly mediating any acute effects on muscle glucose transport. Further investigation examining any potential direct, acute ghrelin action in other insulin-sensitive peripheral tissues such as the liver may be important in revealing any short-term effects of ghrelin responsible for the maintenance of blood glucose homeostasis following the consumption of carbohydrate.

CHAPTER 5: Unacylated Ghrelin Stimulates Fatty Acid Oxidation to Protect Skeletal Muscle Against Palmitate-Induced Defects to Insulin Action in Lean but not High-Fat Fed Rats

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Abstract

Given that ghrelin spikes in circulation before mealtime and recent *ex vivo* evidence suggests that it may stimulate skeletal muscle fatty acid oxidation, it was hypothesized that during an exposure to elevated fatty acids, ghrelin may promote their oxidation to preserve muscle insulin response. Rat soleus muscle was isolated for the measurement of palmitate oxidation, glucose uptake and insulin signalling. During high palmitate exposure, unacylated ghrelin (UnAG) was a more potent stimulator of palmitate oxidation. Both ghrelin isoforms generally protected insulin-mediated phosphorylation of Akt Ser^{473} and Thr^{308}, as well as downstream AS160 Ser^{588}. However, only UnAG preserved insulin-stimulated glucose uptake. Etomoxir, an inhibitor of carnitine palmitoyltransferase (CPT-1) abolished this protection, strongly suggesting that the stimulation of fatty acid oxidation by UnAG may be essential for protection. Following 6-weeks of high-fat diet, UnAG was unable to preserve insulin-stimulated signalling or glucose transport during acute high palmitate exposure. UnAG was also unable to further stimulate AMPK or fatty acid oxidation. Corticotropin-releasing factor receptor-2 (CRF-2R) content was significantly decreased in muscle from high-fat fed animals, which may partially account for the loss of UnAG's effects. UnAG can protect muscle from acute lipid exposure, likely due to its stimulation of fatty acid oxidation. This effect is lost in high-fat fed animals, implying a muscle resistance to ghrelin. The underlying mechanisms accounting for ghrelin resistance remain to be discovered.

Introduction

By virtue of its mass, skeletal muscle represents a major tissue for substrate clearance, and its ability to take up glucose and fatty acids following a meal relies heavily on its responsiveness to hormones such as insulin, a signal which becomes impaired in diabetes. Dysfunctional lipid metabolism is a major contributor towards the development of insulin resistance. The accumulation of reactive lipid intermediates is widely regarded as a causal factor in the impairment of insulin action in tissues like skeletal muscle (2, 5). As little as 4 hours exposure of isolated human or rodent skeletal muscle to high concentrations of fatty acids can impair insulin signalling and insulin-stimulated glucose uptake likely in part, due to their uptake and deposition as DAGs or ceramides (58, 252). Therefore, in the context of chronically elevated circulating fatty acids e.g. obesity or exposure to an acute, high-fat meal, it would appear beneficial to have physiological strategies in place that preserve muscle insulin sensitivity and attenuate the negative outcomes associated with persistent hyperglycemia.

Ghrelin is an appetite-stimulating peptide that has recently garnered attention through its ability to influence peripheral tissue metabolism. Both acylated (AG) and unacylated (UnAG) ghrelin isoforms exhibit a large pre-prandial rise in the plasma which precedes anticipated meal ingestion (143). Since skeletal muscle is a large sink for postprandial substrates (glucose, fatty acids) and a key site for insulin action, recent research has examined whether ghrelin is able to regulate skeletal muscle's uptake and utilization of glucose and fatty acids. *In vivo* studies suggest contrasting roles for ghrelin's effects on glucose metabolism, in that AG worsens (77, 233, 234) but UnAG improves (30, 77) insulin-stimulated glucose disposal. However, with regards to AG infusion, confounding increases in circulating growth hormone (GH) may indirectly impair muscle's ability to uptake glucose (164). Isolating ghrelin's direct effects on

substrate metabolism has been met with obstacles *in vivo*. Therefore, recent approaches have utilized isolated tissue to directly assess and compare the two bioactive forms of ghrelin. Data from our own laboratory has shown that ghrelin may not directly regulate glucose uptake, either independently or with insulin, in rat skeletal muscle (32). Notably, in all cases and as with previous work examining ghrelin's direct effects (32, 79), glucose uptake has been assessed in the absence of increased fatty acid availability, which can alter the fuel partitioning in resting skeletal muscle (176). We and others have also recently demonstrated that both ghrelin isoforms can stimulate fatty acid oxidation in both myocytes and mature skeletal muscle (91, 131). Given that saturated fatty acids such as palmitate can acutely impair muscle insulin signalling and insulin-stimulated glucose uptake (216), we hypothesized that any potential effects of ghrelin on glucose transport would manifest during scenarios of increased fatty acid availability. Specifically, we postulated that ghrelin would promote the oxidation of saturated fatty acids and thereby mitigate their detrimental effects on skeletal muscle insulin action.

Further adding to the complexity of ghrelin's metabolic effects are changes in its circulating concentrations in obesity. More specifically, fasting total ghrelin levels are lower in obese individuals, while the acute postprandial decrease that is evident in lean individuals appears to be absent in the obese (224). Moreover, both AG and total ghrelin concentrations appear to continue to rise following a missed, anticipated meal in obese humans (40). These findings stress the importance for elucidating ghrelin's effects on glucose and lipid metabolism in skeletal muscle, and whether it plays a role in the dysregulation that occurs in obesity. Finally, whether skeletal muscle can become resistant to ghrelin's effects is unknown. Therefore, we aimed to address this knowledge gap by investigating ghrelin's effects in the context of a high-fat diet. The objectives of the present study were to determine whether i) ghrelin isoforms can

further stimulate fatty acid oxidation when skeletal muscle is exposed to high saturated fatty acid (palmitate) concentrations (2mM); ii) ghrelin isoforms can prevent acute high palmitate-induced impairment in skeletal muscle insulin signalling (Akt and AS160) and glucose uptake; iii) ghrelin's beneficial effects in preserving insulin action, if present, are due to its ability to stimulate the oxidation of fatty acids; and iv) whether skeletal muscle from high-fat fed rats becomes resistant to ghrelin's effects on glucose and fatty acid metabolism. We hypothesized that ghrelin would preserve insulin action in healthy skeletal muscle through its ability to stimulate palmitate oxidation, and that muscle derived from high-fat fed animals would become resistant to ghrelin.

Methods

Animals

All procedures were approved by the Animal Care Committee at the University of Guelph and followed Canadian Council of Animal Care Guidelines. For experiments in healthy animals, male Sprague-Dawley rats were ordered from Charles River Laboratories (Quebec, ON, Canada) at approximately 5 weeks of age (~150-200g). Rats were given ad libitum access to chow diet (Teklad 2018 laboratory diet, Envigo) and water and acclimatized for one week prior to experiments. To avoid high levels of endogenous circulating ghrelin that normally occur in the morning, overnight fasted animals were allowed food access (re-fed) for a short duration at the start of their dark cycle, approximately 2-3 h prior to tissue collection in the post-absorptive state, as confirmed previously (131). Rats were anesthetized with an intraperitoneal bolus of pentobarbital sodium (6mg per 100g body mass) prior to all surgical procedures. A low-fat (10% kcals fat), sucrose-matched diet was used (Cat No. D12450J, Research Diets) as the control diet

in a subset of animals during dietary intervention experiments. The other subset of rats (approximately 3 weeks of age; ~50-75g) were provided a 60% kcal fat diet (Cat No. D12492, Research Diets) ad libitum for 6 weeks. This timeline consistently increases weight gain and impairs glucose tolerance.

Materials and reagents

Reagents, molecular weight markers and nitrocellulose membranes for western blots were purchased from BioRad (Mississauga, ON, Canada). Western lighting plus enhanced chemiluminescence (ECL) was purchased from Perkin-Elmer (NEL105001EA). The following antibodies were purchased from Cell Signalling: phospho-Akt Ser^{473} (Cat. No. 9271), phospho-Akt Thr^{308} (Cat. No. 9275), Akt (Cat. No. 9272), phospho-AS160 Ser^{588} (Cat. No. 8730), phospho-AMPK Thr^{172} (Cat. No. 2531S), AMPK (Cat. No. 2603S), phospho-ACC Ser^{79} (Cat. No. 3661) and ACC (Cat. No. 3662). CRF-2 receptor (Cat. No. Ab104368) and GHS-R1 (Cat. No. Sc-374515) antibodies were sourced from Abcam and Santa Cruz Biotechnology, respectively. AS160 antibody (Cat. No. 07-741) was purchased from Millipore Sigma. NP40 cell lysis buffer (Cat. No. FNN0021) was obtained from Life Technologies and PMSF (Cat. No. 78830) and protease inhibitor (Cat. No. 9599) were obtained from Millipore Sigma. Insulin (Humulin rDNA origin) was purchased from Eli Lilly (Toronto, ON, Canada). Synthetic acylated (Cat. No. H-4862) and unacylated (Cat. No. H-6264) ghrelin were ordered from Bachem (Torrance, CA, USA). For skeletal muscle incubations, fatty-acid free bovine serum albumin (Cat. No. 10775835001), palmitic acid (Cat. No. P0500), Dulbecco's modified eagle's media (DMEM; Cat. No. D5030), D-glucose (Cat. No. G-8270), D-mannitol (Cat. No. M-9546), sodium pyruvate (Cat. No. P8574) and 2-deoxyglucose (Cat. No. D8375) were all obtained from

Millipore Sigma. Radiolabeled palmitic acid (Cat. No. 0172A), D-mannitol (Cat. No. 0127) and deoxy-D-glucose (Cat. No. 0103) were all purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA).

Fasting blood glucose and glucose tolerance tests

At the end of 6 weeks on the specified dietary intervention, intraperitoneal glucose tolerance tests (IPGTT) were performed following an overnight fast. Animals were injected with a bolus of glucose (2.0g/kg body weight) and blood glucose measurements were taken at intervals over a 2 h period (t = 0, 15, 30, 45, 60, 120 min) using tail vein blood and a handheld FreeStyle Lite glucometer. The incremental area under the curve (AUC) for the duration of the blood glucose response was calculated. T = 0 min was used as a measure of fasting blood glucose in low and high fat-fed animals.

Blood collection and plasma fatty acid analysis

Following surgeries for the measurement of glucose uptake and fatty acid oxidation, terminal blood samples were obtained from low and high-fat fed animals by cardiac puncture. Blood was placed on ice for an additional 30 min and was then centrifuged for 10 min at 1500 g. Serum was collected and immediately snap frozen in liquid N_2 to be stored at -80℃ for further analysis. Plasma non-esterified free fatty acids (NEFA) were determined using commercially available reagents (Cat. No. 995-34791, 993-35191, 999-34691, 991-34891) and standards (Cat. No. 276-76491) from Wako Diagnostics (Richmond, VA).

^{14}C Fatty Acid (Palmitate) Oxidation

All incubations were carried out in a heated (30°C), gassed (95% O_2, 5% CO_2) shaking water bath as previously described (131) and the experimental design was identical for experiments with low and high-fat fed animals. Briefly, soleus skeletal muscle strips were excised tendon to tendon and pre-incubated for 30 min in DMEM supplemented with 8mM glucose prior to being transferred into a flask containing either low (0.2mM) or high (2mM) palmitate. Following 2 h in buffer containing non-radiolabeled palmitate, muscles were transferred into vials containing media with labelled 0.5mCi/mL ^{14}C-palmitic acid for the measurement of palmitate oxidation (2 h). Following the 2 h incubation, the muscle was carefully removed from the flask, trimmed of its tendons and weighed. The muscle strip was then re-added to the flask with which 250µL of benezethonium hydroxide was added to an eppendorf tube inside and 1mL of 1M sulfuric acid was added (via a syringe) to both incubation media and muscle. The flask with acidified media and muscle was left at room temperature for 2 h to allow for the radiolabeled $^{14}CO_2$ gas to be liberated and captured in the benzethonium hydroxide solution. The entire eppendorf containing trapped $^{14}CO_2$ and benzethonium hydroxide was placed in a vial containing scintillation cocktail to quench overnight prior to scintillation counting using a PerkinElmer Tri-Carb LSC 4910TR liquid scintillation counter. Samples were counted for 5 min.

^{3}H Deoxyglucose uptake

Glucose uptake assays were carried out as previously described (32) and the experimental design was identical for experiments with healthy and obese animals. Briefly, incubation media were pre-gassed (95% O_2, 5% CO_2) in a heated (30°C), shaking water bath. Given that soleus

muscle can be reliably stripped lengthwise into 3 viable, 20-30mg sections, one strip (from a leg of one animal) was used for each treatment (i.e. each full set of treatments could be obtained from the muscles of one animal. Muscles were left to freely float in incubation media for the duration of the experiment. A total molarity of 40mM was maintained for each media, as previously described (92, 179). Muscle strips were first pre-incubated in medium containing 8mM D-glucose and 32mM D-mannitol for 30 min. Following pre-incubation, muscles were carefully transferred to a 4 h palmitate exposure containing either low (0.2mM) or high (2mM) palmitate concentrations. Two hours into the 4 h palmitate exposure, all vials containing muscle strips were re-gassed with the same humidified, 95% O_2, 5% CO_2 gas. Following palmitate exposure, muscles were washed in media containing 36mM D-mannitol and 4mM pyruvate for 30 min prior to being transferred into the final buffer containing 28mM D-mannitol, 8mM 2-deoxyglucose, radiolabeled 1mCi/mL [1,2]-^{3}H-2-deoxy-D-glucose and 0.1mCi/mL [1]-^{14}C-mannitol for 30 min. Acylated and unacylated ghrelin (150ng/ml) were added to the appropriate vials for the 4 h palmitate exposure onwards, whereas insulin (10mU/ml) was only added during the wash (following 4 h palmitate) and the final radioactive glucose uptake buffer. Following the final uptake buffer, muscle strips were trimmed of their tendons and blotted dry on gauze. Muscle strips were then weighed and subsequently boiled and digested in 1M sodium hydroxide solution (~10 min, swirled periodically). Muscle homogenate was then sampled (200µL) to which 5mL of Cytoscint scintillation fluorescent cocktail (MP Biomedicals (via Cedarlane) Burlington, ON, Canada) was added. Samples were shaken vigorously and left to quench overnight in complete darkness. The next day, samples were counted (5 min/sample) using a PerkinElmer Tri-Carb LSC 4910TR liquid scintillation counter and were considered acceptable when the detected Lumex percentage (random counts per minute) was < 5%. Glucose uptake was

calculated as the accumulation of intracellularly labelled glucose as we have previously reported (32).

^{3}H Deoxyglucose uptake with Etomoxir (CPT-1 inhibitor)

Glucose transport was also assessed in the presence or absence of the CPT-1 inhibitor etomoxir (100µM; dissolved in dimethyl sulfoxide (DMSO)), to determine whether any beneficial effect of ghrelin on preserving insulin-stimulated glucose transport was dependent on stimulation of fatty acid oxidation. Etomoxir was added to the palmitate exposure buffers (4 h) which contained ghrelin isoforms but was removed in the subsequent muscle washes and radiolabeled glucose uptake buffer.

Total ghrelin ELISA

Although we have previously demonstrated that ghrelin remains stable in our incubation preparation over the course of 2 h, the nature of experiments that acutely induce insulin resistance via palmitate exposure required approximately 4 h. As such, we again tested the stability of ghrelin within the heated, oxygenated incubation buffer at t = 0, 120, 240 min. Ghrelin concentrations were measured using a commercially available kit (Millipore Sigma – Cat. No. EZRGRT-91K) according to manufacturer instructions. Accuracy and intra-assay comparisons were validated using two quality control standards (in triplicate) that were provided with the kit. Incubation buffer samples were assayed in triplicate. Both AG (2.34, 1.46, 1.40 ng/ml) and UnAG (2.89, 2.22, 3.43 ng/ml) remained stable at each timepoint, over the 4 h duration.

Western Blotting

Incubations to assess protein signalling following muscle palmitate exposures were carried out similarly to the functional measures of glucose uptake and palmitate oxidation. Briefly, for incubations assessing insulin-signalling, muscles were pre-incubated in DMEM containing 8mM D-glucose and 32mM D-mannitol for 30 min. Muscles were then transferred to their respective palmitate vials (0.2mM vs. 2mM) for 4 h prior to being washed for 30 min (36mM D-mannitol, 4mM pyruvate) before undergoing insulin treatment (30 min). For incubations assessing fatty acid oxidation signalling, muscles were pre-incubated in DMEM containing 8mM D-glucose for 30 min. Muscles were subsequently transferred to palmitate exposure vials (with or without ghrelin) for 1 h. Muscles were then blotted on gauze, weighed, snap frozen in liquid N_2 and frozen at -80°C until further analyses. ~20-30mg muscle strips were homogenized (Qiagen TissueLyser LT) in 500µL of ice-cold cell lysis buffer (containing Na_3VO_4 and NaF) that was supplemented with PMSF and P_i to minimize the action of both serine and threonine proteases and phosphatases. Homogenization was 3x3 min, and samples were left on ice at each interval. Samples were then centrifuged at 1500 *g* for 15 min. The supernatant was removed, and protein concentrations were assessed using the bicinchoninic acid assay method (200). Equal amounts of proteins were separated by molecular weight via electrophoresis on 10% acrylamide gels (5% for ACC, 7.5% for AS160) and then wet transferred at 4°C onto nitrocellulose membranes for 1 h at 100V. Membranes were subsequently blocked in (5%) skim milk powder + TBST for 1 h before being washed in TBST for 10 min. Primary antibody (1:1000) was then added to the membrane and left overnight in a dark, 4°C cold-room. The following day, membranes were washed in TBST (2x15 min) and incubated in secondary antibody (1:2000), shaking at room temperature for 1 h. Next, membranes were washed in TBST

(2x15 min) and then once in TBS (10 min). Bands were visualized using ECL and quantified using Alpha Innotech Software. Vinculin was used as a loading control. Western blots are shown as the quotient of phosphorylated to total protein where appropriate.

Statistics

All data are expressed as mean ± standard error. For insulin signalling experiments in lean animals, basal levels (i.e. without insulin) of insulin signalling activation (Akt and AS160) were not different between LP and HP conditions. Therefore, these groups were excluded from analysis such that only insulin-stimulated conditions were analyzed. To compare western blots and fasted plasma substrates from 6-week low vs. high fat-fed animals, an unpaired t test was used. Food intake, energy intake and body weights between dietary groups were analyzed using a two-way (by both factors – diet and time) analysis of variance (ANOVA). If statistical significance was observed with the ANOVA, a Fisher's multiple comparisons post-hoc test was used to determine whether any interaction (diet x time) of treatments was present. All other experimental data (i.e. glucose uptake, palmitate oxidation, protein signalling) were analyzed using a repeated measure, one-way analysis of variance (ANOVA). In all figures, letters were used to denote statistical significance, such that groups sharing a common letter are not significantly different from each other. Data was considered significant at $p < 0.05$. Data points greater than two standard deviations from the mean were considered outliers and removed.

Results

Ghrelin's effects on glucose and fatty acid metabolism in lean skeletal muscle

Palmitate impairs insulin activation of Akt at phosphorylation sites Ser^{473} and Thr^{308} and downstream AS160 activation at phosphorylation site Ser^{588}

Similar to what has been shown previously in obese human rectus abdominus muscle strips (216), the present work using isolated rodent soleus muscle demonstrated that 4 h exposure to 2mM of saturated fatty acids (high palmitate; HP) acutely induced defects in insulin signalling. Specifically, palmitate significantly impaired insulin's (10mU/ml) ability to increase phosphorylation of Akt at both Ser^{473} ($p < 0.05$; **Figure 17**) and Thr^{308} ($p < 0.05$; **Figure 17**) residues as well as AS160 phosphorylation at its Ser^{588} residue ($p < 0.05$; **Figure 17**), when compared to insulin action in the presence of low palmitate (LP; 0.2mM) control.

Ghrelin preserves skeletal muscle insulin action following high palmitate exposure

Insulin-mediated phosphorylation of Akt at both Ser^{473} ($p < 0.05$; **Figure 17**) and Thr^{308} ($p < 0.05$; **Figure 17**) as well as AS160 phosphorylation at Ser^{588} ($p < 0.05$; **Figure 17**) following the acute (4 h) exposure to HP were preserved when AG was present, such that HP+ins+AG activation of Akt and AS160 were not different ($p > 0.05$) from the insulin-stimulated control group, LP+ins. UnAG also preserved insulin activation of AS160 ($p < 0.05$; **Figure 17**) and Akt at its Thr^{308} ($p < 0.05$; **Figure 17**), but not Ser^{473} ($p > 0.05$; **Figure 17**) residue.

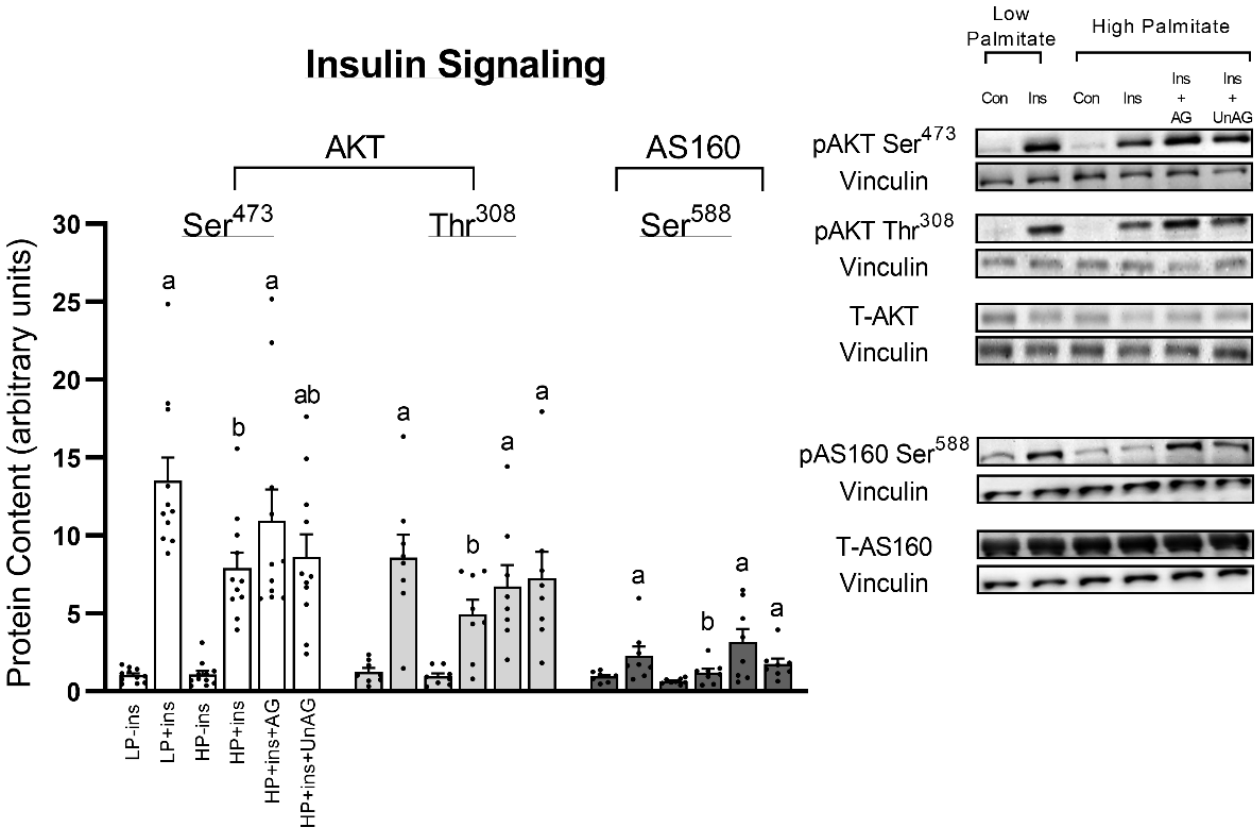

Figure 17: Effects of Ghrelin on Insulin Signalling During Palmitate Exposures

The activation of insulin signalling protein Akt at phosphorylation sites Ser^{473} and Thr^{308} as well as AS160 at site Ser^{588} in isolated soleus muscle following muscle exposure to low (LP; 0.2mM) or high (HP; 2mM) palmitate concentrations with or without AG/UnAG (150ng/ml) and insulin (10mU/ml). Data were analyzed using a repeated measure one-way ANOVA (n = 8-11) and expressed as individual data points and the mean ± standard error, in arbitrary protein units (phospho/total). Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

Unacylated, but not acylated ghrelin, preserves insulin-stimulated glucose uptake in the presence of high palmitate

Insulin significantly increased the rate of glucose uptake in muscle following a 4 h exposure to LP control ($p < 0.001$; **Figure 18A**). In agreement with insulin signalling data, 4 h exposure to HP impaired insulin-stimulated glucose uptake ($p < 0.001$; **Figure 18A**). UnAG led to a significantly higher rate of insulin-stimulated glucose transport following HP exposure ($p < 0.01$) when compared to HP alone (**Figure 18A**), indicating a protective effect. However, despite its ability to generally preserve insulin signalling, AG was unable to protect muscle's functional response to insulin i.e. insulin-stimulated glucose uptake ($p > 0.05$; **Figure 18A**) following the acute HP exposure compared to HP alone.

UnAG is more potent than AG in stimulating palmitate oxidation in soleus muscle

Rates of fatty acid oxidation were significantly increased ($p < 0.0001$; **Figure 18B**) in the presence of high palmitate (i.e. 0.2mM vs. 2mM). We replicated and built upon previous work from our laboratory which used 1mM palmitate (131), in demonstrating that ghrelin maintains its ability to further stimulate fatty acid oxidation during a 4 h incubation with a higher (2mM) concentration of palmitate (AG: $p < 0.005$; UnAG: $p < 0.0001$; **Figure 18B**) when compared to HP conditions alone. Also, following the addition of ghrelin isoforms to incubation media, their stimulatory effects on oxidation were greater in the latter half (i.e. t = 2-4 h) of the 4 h exposure to palmitate (**Figure 18B**). Specifically, when compared to HP alone, AG only significantly increased palmitate oxidation during t = 2-4 h ($p < 0.01$) but not t = 0-2 h ($p > 0.05$; **Figure 18B**). UnAG appeared to be a more effective stimulus for fatty acid oxidation compared to AG ($p < 0.005$; **Figure 18B**). UnAG significantly increased rates of palmitate oxidation by 40% at t

= 0-2 h ($p < 0.05$) and by 78% at t = 2-4 h ($p < 0.01$) versus HP alone, compared to the ~28% increase in rates of oxidation achieved by AG during t = 0-2 h and the ~40% increase during t = 2-4 h (**Figure 18B**).

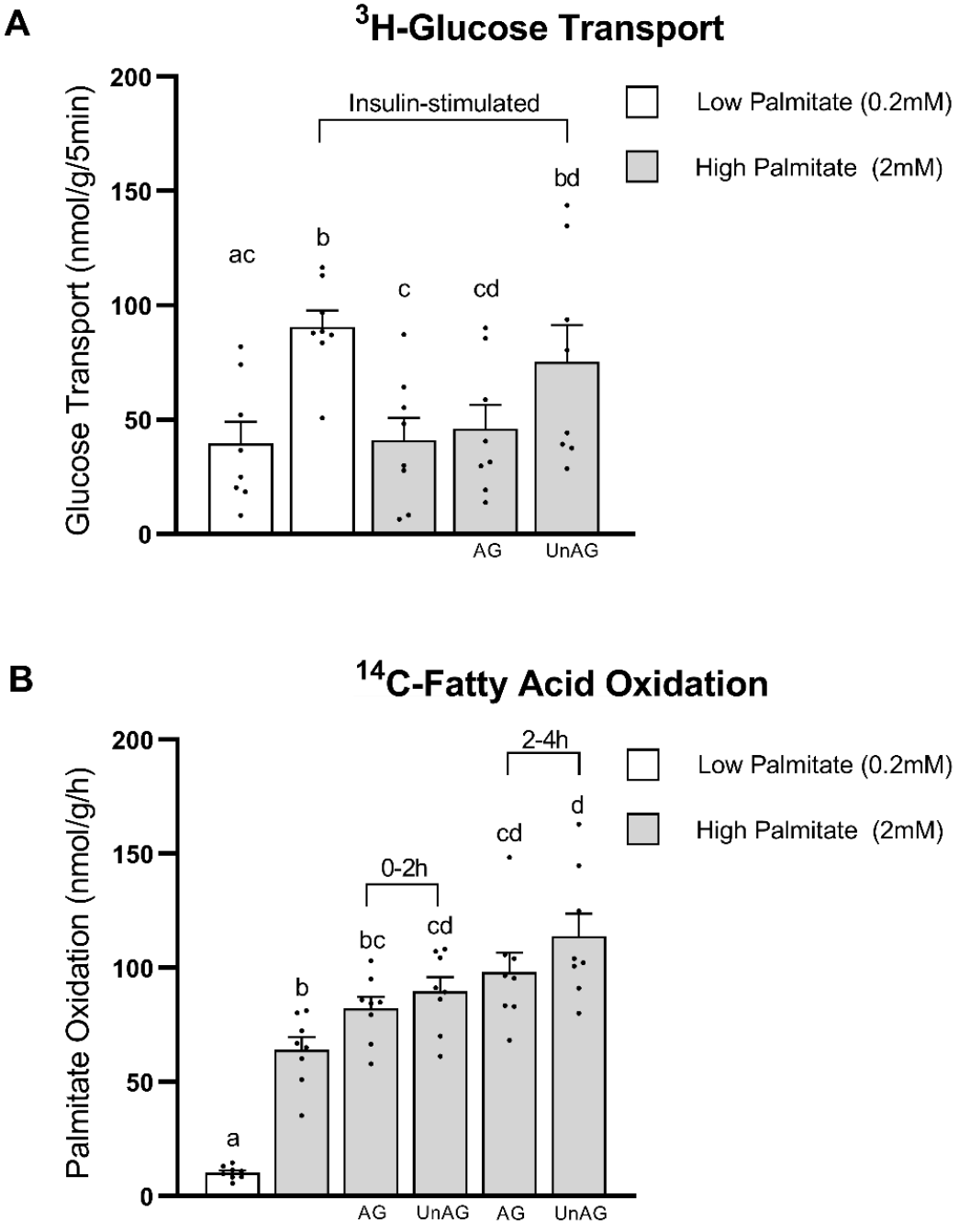

Figure 18: Glucose Uptake and Palmitate Oxidation

Rates of basal and insulin-stimulated (10mU/ml) glucose uptake **(A)** and palmitate oxidation **(B)** following the exposure of isolated soleus muscle to either low (LP) or high (HP) palmitate concentrations with or without AG/UnAG (150ng/ml). Data were analyzed using a repeated measure one-way ANOVA (n=8 for each) and expressed as individual data points and the mean ± standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

The ability of UnAG to preserve insulin-stimulated glucose uptake in the presence of HP is likely dependent on its stimulation of fatty acid oxidation

Etomoxir is an irreversible inhibitor of CPT-1, the rate-limiting enzyme for fatty acid transport into the mitochondria, and significantly impairs rates of mitochondrial β-oxidation in various metabolic tissues (54, 229). Pilot work with etomoxir demonstrated that, in our isolated skeletal muscle incubation preparation, 100μM was an effective dose to reduce rates of palmitate oxidation by ~50% compared to vehicle ($p < 0.05$; **Figure 19A**). Furthermore, etomoxir inhibited fatty acid oxidation by ~55% ($p < 0.01$) during the latter 2 h of the (4 h total) muscle exposure to palmitate, while the initial 2 h exposure (i.e. 0-2 h) of muscle to etomoxir was relatively ineffective ($p > 0.05$) at reducing rates ($p > 0.05$; **Figure 19B**). We sought to determine whether ghrelin's beneficial effects on insulin-stimulated glucose uptake were dependent on its ability to stimulate fatty acid oxidation. Consistent with initial findings, HP exposure significantly reduced insulin-stimulated glucose uptake versus LP ($p < 0.01$; **Figure 19C**). During HP exposure, soleus muscle concurrently exposed to UnAG ($p < 0.005$; **Figure 19C**), but not AG ($p > 0.05$; **Figure**

19C) demonstrated significantly higher rates of insulin-stimulated glucose transport versus HP alone. Etomoxir's presence had no effect on rates of insulin-stimulated glucose transport during HP exposure with AG ($p > 0.05$; **Figure 19C**). However, UnAG was unable to preserve insulin's ability to stimulate glucose transport in soleus muscle exposed to HP when co-treated with etomoxir, when compared to HP alone ($p > 0.05$; **Figure 19C**). This was evident due to significantly lower rates of insulin-stimulated glucose transport achieved by UnAG during HP and etomoxir exposure when compared to UnAG's effects during HP exposure in the absence of etomoxir ($p < 0.05$; **Figure 19C**).

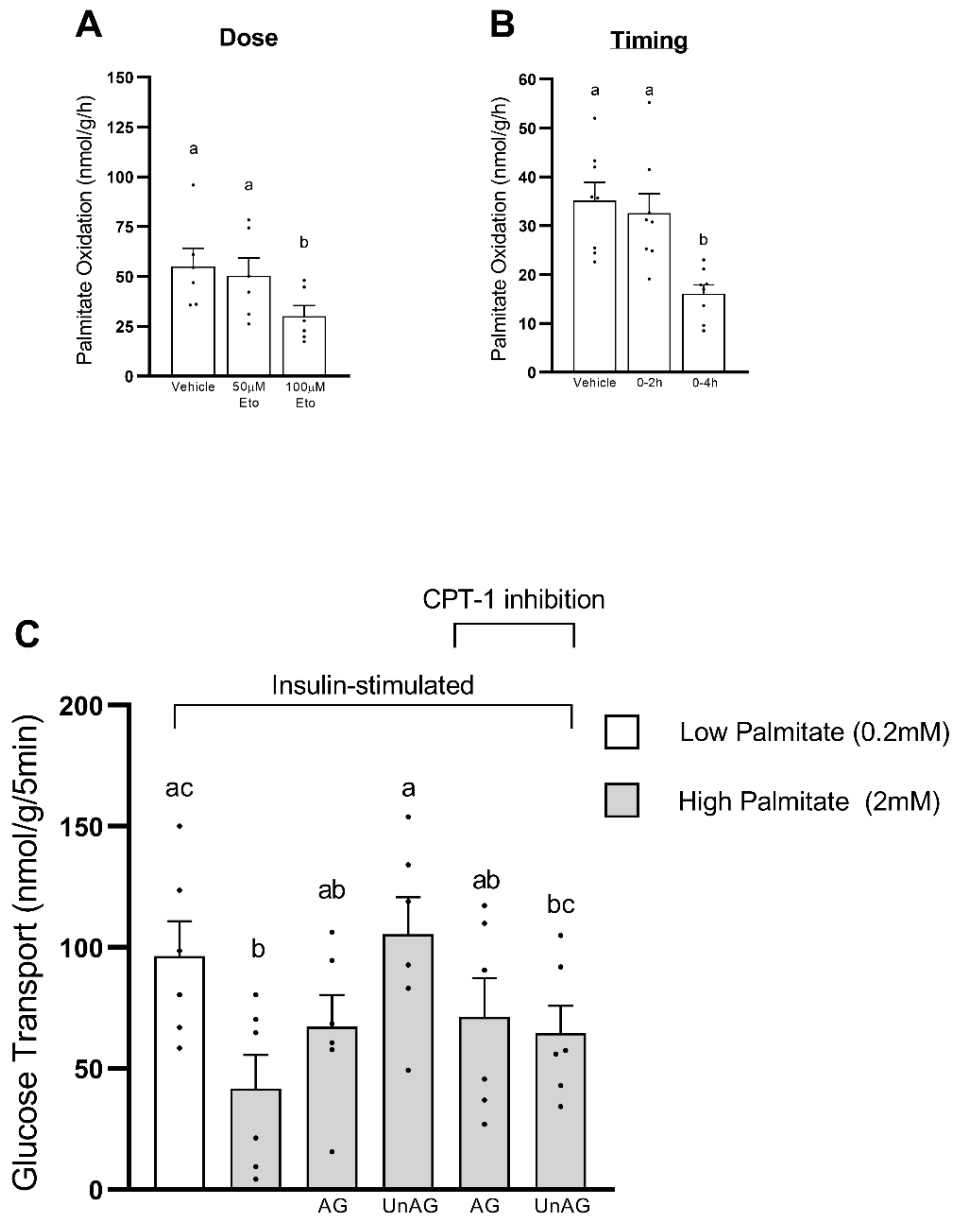

Figure 19: Inhibition of Fatty Acid Oxidation and Glucose Transport

The effects of etomoxir (CPT-1 inhibitor) dose **(A)** and exposure time **(B)** or vehicle (DMSO) on rates of palmitate oxidation in isolated soleus muscle during an exposure to 2mM palmitate. Rates of insulin-stimulated (10mU/ml) glucose transport **(C)** were also assessed following 4 h low (LP) or high (HP) palmitate exposure with or without AG/UnAG (150ng/ml) in the presence

or absence of 100μM etomoxir (4 h). Data were analyzed using a repeated measure one-way ANOVA (n = 6-8) and expressed as individual data points and the mean ± standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

Ghrelin's effects on glucose and fatty acid metabolism in skeletal muscle from high-fat fed rats

6-weeks of high-fat diet causes weight gain and glucose intolerance

There were significant main effects of both diet ($p < 0.0001$) and time ($p < 0.0001$) on changes to both food and energy intake over the 6-week diet intervention (**Figure 20A, B**). Low-fat diet (LFD) animals consumed more food in grams than high-fat diet (HFD) animals ($p < 0.05$; **Figure 20A**), at all timepoints. However, apart from week 1 ($p < 0.05$), HFD animals had significantly greater energy intake (kcals) during each week of the dietary intervention ($p < 0.001$; **Figure 20B**). Animals on the HFD also significantly ($p < 0.05$) increased their caloric intake with each successive week, except for weeks 4-5 ($p > 0.05$; **Figure 20B**). Over the 6-week dietary protocol, there was a statistically significant interaction between the effects of diet and time on changes to body weight ($p < 0.0001$; **Figure 20C**). There was a significant main effect of both time ($p < 0.0001$; **Figure 20C**) and diet ($p < 0.0001$; **Figure 20C**) on changes to animal body weights. There were no differences in body weights between groups of animals at baseline ($p > 0.05$; **Figure 20C**). By week 2, rats consuming the high-fat diet were significantly heavier than low-fat control animals ($p < 0.05$; **Figure 20C**). This difference persisted until the

conclusion of the study (i.e. week 6), with high-fat animals weighing ~75g more than low-fat controls, on average.

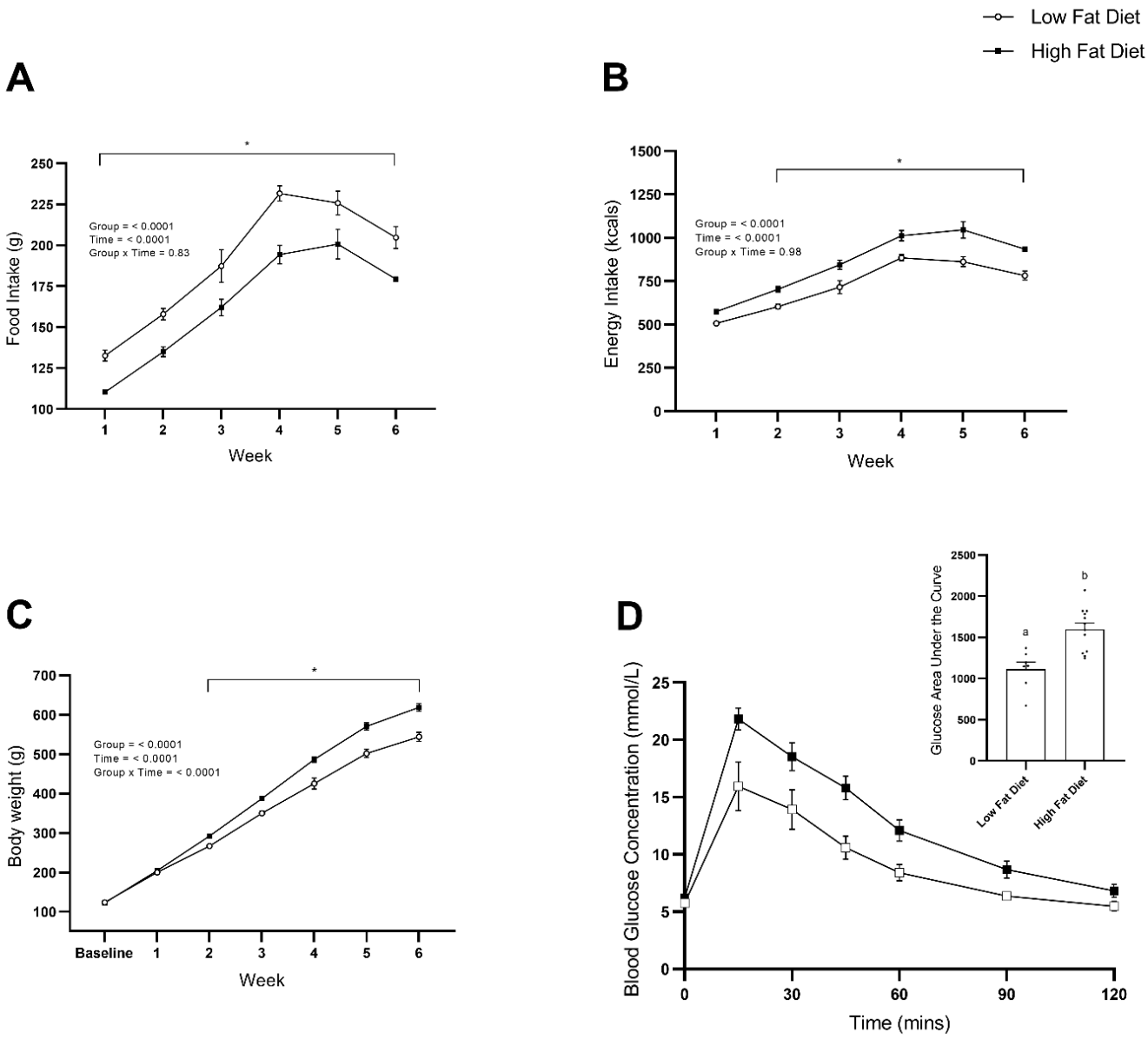

Figure 20: Whole-Body Measurements During Dietary Intervention

Food **(A)** and energy **(B)** intake as well as body weights **(C)** during a 6-week dietary intervention providing rats with diet either low (10%) or high (60%) in kcals from fat, *ad libitum*. Following an overnight fast, blood glucose response was tracked following an IPGTT **(D)** at the end of 6-

weeks on specified diet. Food, energy intake and body weight data were analyzed using a two-way (main effects and interaction displayed in text) ANOVA (n=8-12). Glucose AUC data were analyzed using an unpaired t test (LFD: n=7; HFD: n=12). All data were expressed as individual data points and the mean ± standard error. An asterisk (*) depicts a significant difference at a given week between dietary groups (main effect; HFD vs. LFD). $P < 0.05$ was considered statistically significant.

Chronic (6-week) high-fat diet impairs blood glucose homeostasis

At the end of the 6-weeks, animals on the HFD had impaired glucose tolerance as evidenced by a significantly greater glucose AUC following an IPGTT compared to healthy, lean controls ($p < 0.0001$; **Figure 20D**). High-fat fed animals also had significantly higher fasting blood glucose levels compared to low-fat fed animals ($p < 0.05$; **Table 2**).

	Diet	
	Low-fat	**High-fat**
Fasting Blood Glucose (mM)	5.8 ± 0.16^{a}	6.3 ± 0.20^{b}

Table 2: Fasting Blood Glucose in 6-Week High-Fat vs. Low-Fat Fed Animals

Overnight fasted blood glucose (mM) in rats having consumed 6-weeks of diet either low (10%) or high (60%) in kcals from fat. Data were analyzed using an unpaired t test (LFD: n=7; HFD: n=11). Data is expressed as mean ± standard error. Groups not sharing a letter were considered statistically different from each other. $P < 0.05$ was considered statistically significant.

Skeletal muscle from high-fat fed rats is resistant to ghrelin's ability to stimulate fatty acid oxidation and preserve insulin-stimulated glucose uptake during palmitate exposure

Data from the current study, as well as previous work from our laboratory (131), and others (91), has demonstrated a role for ghrelin as a potent stimulator of fatty acid oxidation in skeletal muscle. However, ghrelin's metabolic effects in the context of obesity or high-fat feeding are relatively unknown. Therefore, we examined ghrelin's capacity to stimulate palmitate oxidation in soleus skeletal muscle from high-fat fed rats. As expected, exposure of muscle to HP significantly increased rates of fatty acid oxidation compared to LP ($p < 0.0001$; **Figure 21A**). In contrast to ghrelin's effects in healthy muscle, neither isoform was able to further stimulate fatty acid oxidation in soleus muscle derived from high-fat fed animals ($p > 0.05$; **Figure 21A**) beyond the increased rates of oxidation that were observed with exposure to HP. Compared to basal (control) muscles, a maximal insulin concentration was still able to significantly increase glucose uptake (~46%) in muscle exposed to LP from high-fat rats ($p < 0.05$; **Figure 21B**). However, insulin no longer stimulated glucose uptake in the presence of HP ($p > 0.05$; **Figure 21B**); neither AG nor UnAG ($p > 0.05$; **Figure 21B**) had any effect in mitigating the HP-induced impairment (~55% reduction) in insulin-stimulated glucose uptake.

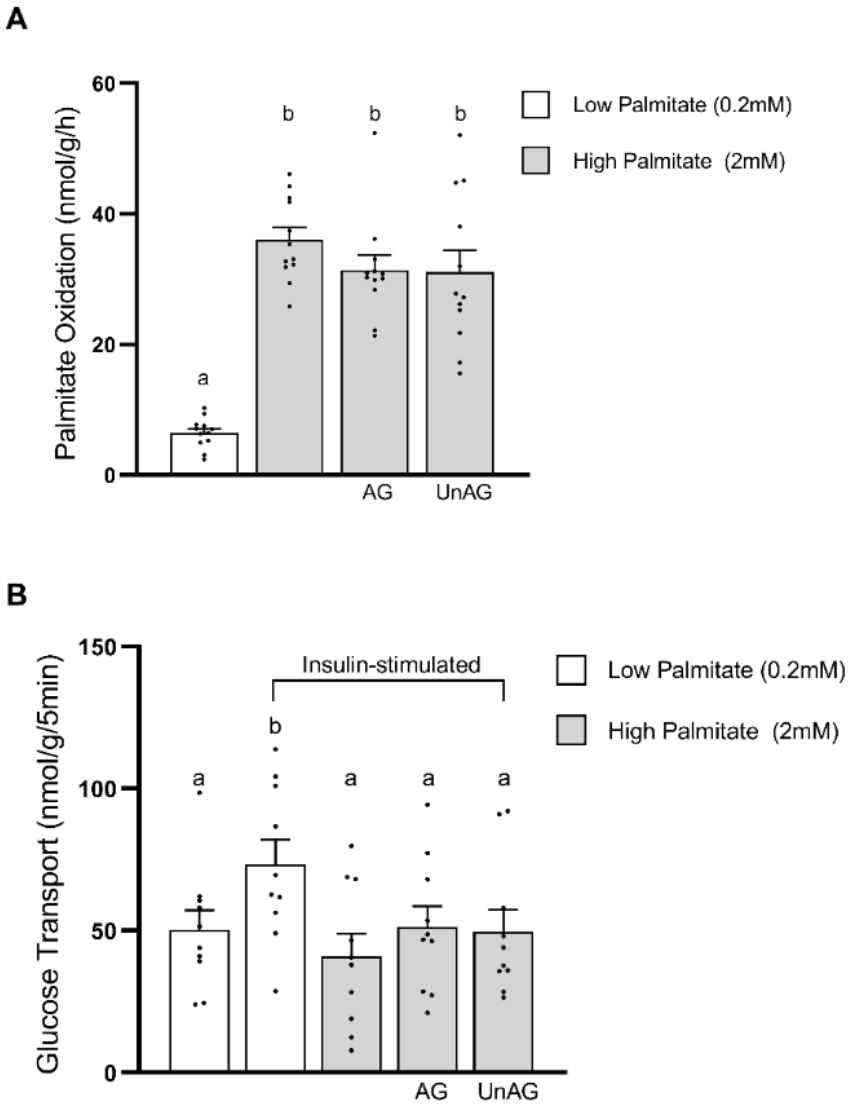

Figure 21: Glucose Uptake and Palmitate Oxidation in High-Fat Fed Animals

Rates of palmitate oxidation **(A)** and basal or insulin-stimulated (10mU/ml) glucose transport **(B)** following either low (LP) or high (HP) palmitate exposure either with or without AG/UnAG, in isolated soleus muscle from 6-week high-fat fed animals. Data were analyzed using a repeated measure one-way ANOVA (n=10-12) and were expressed as individual data points and the mean ± standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

Following 6-weeks of high-fat diet, ghrelin is unable to activate muscle AMPK/ACC axis

AMPK and its downstream target ACC are important regulators of fat oxidation in skeletal muscle and their phosphorylation at key residues can significantly alter their activity (1). Our group has previously demonstrated a role for activating the AMPK axis in carrying out some of the effects of ghrelin isoforms on stimulating fatty acid oxidation (131). Exposure of soleus to HP resulted in increased levels of AMPK phosphorylation/activation compared to LP ($p < 0.05$; **Figure 22A**), although this did not translate to an increase in ACC phosphorylation ($p > 0.05$; **Figure 22A**). Neither AG nor UnAG had any effect on the phosphorylation of AMPK or ACC in soleus muscle from high-fat fed rats following HP exposure ($p > 0.05$; **Figure 22A**).

6 weeks of high-fat diet significantly reduces CRF-2R protein content in soleus muscle

Studies indicate that ghrelin may exert some of its metabolic effects by acting through the corticotropin-releasing hormone receptor-2 (CRF-2R) in skeletal muscle (79). Interestingly, CRF-2R protein content was significantly lower in soleus skeletal muscle from animals having undergone 6-weeks of high-fat diet feeding ($p < 0.05$; **Figure 22B**). Also of interest was the GHS-R1 subtype (GHS-R1a) through which AG exerts many of its classical effects, i.e., GH-release and appetite-stimulation (82, 228). GHS-R1 content was unchanged in soleus muscle from high-fat fed animals compared to lean, healthy controls ($p > 0.05$; **Figure 22B**). The fatty acid transporter FABPpm was similarly unchanged in muscle from obese animals, compared to lean controls ($p > 0.05$; **Figure 22B**).

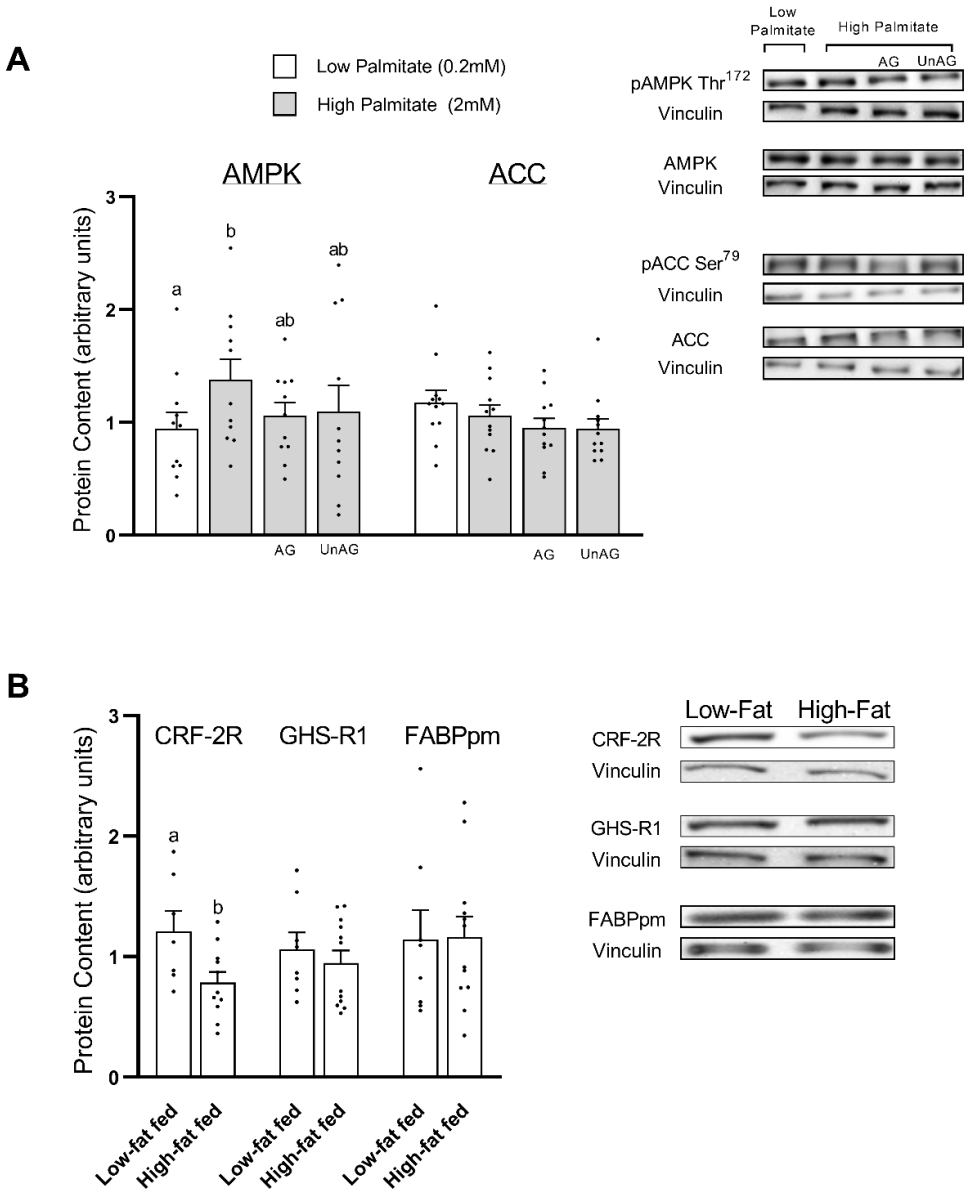

Figure 22: Muscle Signalling in High-Fat Fed Animals

The phosphorylation (activation) of the cellular energy-sensing protein AMPK (Thr^{172}) and its downstream target ACC (Ser^{79}) following low (LP) or high (HP) palmitate exposure either with or without AG/UnAG treatment, in isolated soleus muscle from 6-week high fat-fed, overweight rats. Data were analyzed using a repeated measure one-way ANOVA (n=11-12) and were expressed as individual data points and the mean ± standard error. Also shown, are CRF-2R and

GHS-R1 receptors, as well as the fatty acid transporter $FABP_{pm}$ from soleus muscle of the same 6-week high-fat fed rats. Data were analyzed using an unpaired t test (n=7-12) and were expressed as individual data points and the mean ± standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

Discussion

Current research interest in ghrelin extends beyond its known orexigenic effects and has begun to explore its' regulatory role on peripheral tissue glucose and fatty acid metabolism. Both acylated and unacylated ghrelin peak in the circulation immediately prior to anticipated mealtime, suggesting that ghrelin may affect substrate uptake and metabolism in tissues such as skeletal muscle, which is a major sink for glucose and fatty acids. However, ghrelin's direct metabolic effects on muscle remain poorly understood. We have shown previously that ghrelin isoforms do not independently affect muscle glucose uptake (32) in the absence of fatty acids. However, ghrelin's direct action on muscle metabolism have yet to be examined in the context of simultaneous glucose and lipid availability i.e. similar to a typical mixed meal. Recent work has demonstrated that ghrelin may alter skeletal muscle substrate preference through the stimulation of fatty acid oxidation (91, 131). Therefore, in the present study, we postulated that ghrelin's stimulation of fatty acid oxidation could protect muscle from fatty acid-induced impairment of insulin action, and ultimately glucose uptake. The results of our work in isolated rat soleus muscle demonstrates that both AG and UnAG further stimulate fatty acid oxidation in the presence of elevated palmitate and preserve muscle insulin signalling. While both ghrelin isoforms largely preserved insulin's activation of insulin signalling proteins (Akt, AS160),

UnAG was exclusively responsible for maintaining a functional increase in insulin-stimulated glucose uptake during the acute exposure of muscle to high palmitate concentrations. The protective effects of UnAG on muscle glucose uptake during high palmitate exposure appear to be mediated through its greater ability to stimulate fat oxidation, as this protection was no longer evident with the CPT-1 inhibitor, etomoxir. Interestingly, ghrelin's ability to preserve insulin action and glucose uptake in the presence of high palmitate was no longer evident in skeletal muscle derived from 6-week high-fat fed rats, implying an impaired response (resistance) to ghrelin. This coincided with a decrease in skeletal muscle expression of CRF-2R protein content, a receptor that is thought to contribute to ghrelin's metabolic effects in the periphery (79).

Ghrelin, Lipids and their Regulation of Skeletal Muscle Insulin Action

Skeletal muscle is important for blood glucose homeostasis, largely due to its overall mass and contribution to overall energy expenditure. Acute lipid oversupply can impair insulin-stimulated glucose uptake in muscle (18, 59, 110), which can last for several hours (18). Ghrelin may be a factor influencing muscle's response to an increased supply of lipid, as it peaks in the circulation immediately prior to the consumption of a meal (143). In the current study, 4 h of exposure to high palmitate impaired insulin-stimulated glucose transport in muscle concurrent with reduced activation of the signalling proteins Akt and its downstream target AS160. AG consistently preserved insulin stimulated Akt activation during the acute high palmitate exposure, whereas UnAG's effects were more variable on this marker. However, in terms of preserving a functional increase in insulin-stimulated glucose uptake, only UnAG was protective. Thus, it appears that AG and UnAG may act through distinct mechanisms to influence skeletal muscle insulin action, as observed in other tissues (66). This difference may potentially be

explained by UnAG's ability to more effectively stimulate fatty acid oxidation i.e. to a higher rate. Numerous contributors have been proposed to mediate muscle insulin resistance following lipid oversupply, but the exact mechanisms and their relative contribution remain elusive. Furthermore, lipid accumulation and defects in insulin signalling do not consistently accompany reductions in insulin-stimulated glucose clearance, *in vivo* (97, 106). LCFA-CoA intermediates may directly inhibit hexokinase, increase PKC activity (a negative regulator of insulin signalling) as well as provide substrate for DAG and ceramide production, which cumulatively lead to a net decrease in muscle glucose uptake and utilization (44, 241). It is plausible that UnAG maintains an increase in insulin-stimulated glucose uptake during high palmitate exposure through its ability to stimulate the oxidation of palmitate and reduce intracellular accumulation of certain lipid intermediates which would otherwise impair insulin action. However, in a previous study from our laboratory, UnAG did not divert labelled palmitate away from TAG or DAG (131). The effect of ghrelin on muscle ceramide synthesis has yet to be examined during exposure to high fatty acids. Increases in ceramide synthesis, accumulation and subsequent impairments to insulin-stimulated increases in Akt phosphorylation/membrane localization are often observed following lipid oversupply (36). Overall, UnAG appears to influence muscle insulin signalling through its stimulation of fatty acid oxidation, although it is worth noting that there are previous reports suggesting that UnAG is capable of directly modulating insulin action in the absence of lipid availability (30). The effects of AG are more difficult to interpret. Insulin signalling, but not glucose uptake, was preserved in the presence of high palmitate. AG was also able to stimulate fatty acid oxidation, albeit less so than UnAG. This implies that ultimately, the ability to stimulate fatty acid oxidation is critical to preserve glucose uptake, and that there is a disconnect

between the degree of phosphorylation of key insulin signalling proteins and functional glucose uptake during muscle's acute exposure to high palmitate concentrations.

Ghrelin's Regulation of Substrate Oxidation

There are multiple points of regulation that modulate skeletal muscle's reliance on fatty acids for fuel (120, 202). The literature assessing ghrelin's effects on muscle substrate oxidation and underlying mechanisms is relatively sparse. Following a standardized meal, the *in vivo* infusion of AG reduces insulin sensitivity in humans, whereas the co-administration of both AG and UnAG may improve it (77). Interestingly, the improvement in whole-body insulin sensitivity was paralleled by a reduction in circulating free fatty acid levels (77), suggesting a ghrelin-mediated reduction in lipid mobilization, which has been observed previously (34). Alternatively, ghrelin may be stimulating fatty acid uptake/clearance into peripheral tissues like skeletal muscle (77). Since the effects of AG *in vivo* are often assessed secondary to subsequent rises in GH, and possibly the incretin GLP-1, the ability to draw conclusions on the direct action of ghrelin in substrate mobilization/utilization is partly confounded (74, 77). A single study has demonstrated that as little as 4d of AG injection can reduce gastrocnemius TAG content and increase the expression of genes associated with skeletal muscle lipid utilization e.g. ACC, UCP2 (10). However, the underlying mechanisms for this reduction in tissue triglyceride content were not pursued (10). Recently, research has aimed to uncover the direct effects of ghrelin in the regulation of lipid metabolism. Han *et al.* (91) and work from our own lab (131) demonstrate that ghrelin can stimulate fatty acid oxidation. Han *et al.* observed increases in AMPK activation in myoblasts treated with AG (91), which contributed to the increase in fatty acid oxidation. Acute AG infusion in humans does not appear to alter AMPK activation (234) and initially, we did not

observe any changes to muscle AMPK activation when ghrelin's effects were assessed in the absence of fatty acid availability (32). However, in the presence of elevated fatty acids, ghrelin isoforms stimulated the AMPK/ACC axis (131). Ghrelin's activation of AMPK also appears highly tissue-dependent (127, 142). More work will be required to determine whether AMPK is an essential signal for the positive effects of ghrelin on fatty acid oxidation. Finally, it is unknown whether ghrelin isoforms influence the translocation of fatty acid transporters (e.g. FAT/CD36, $FABP_{pm}$) to the sarcolemmal membrane and whether this accounts for any of its stimulatory effects on fatty acid oxidation.

Ghrelin Signalling in Skeletal Muscle and Changes with High-Fat Feeding

The ability of ghrelin to stimulate fatty acid oxidation and preserve insulin-stimulated glucose uptake was no longer observed in muscle isolated from high-fat fed animals. This suggests that chronic muscle adaptations to the high-fat diet impacted either receptor density or sensitivity to ghrelin isoform stimulation. AG signals through GHS-R1a in the central nervous system, anterior pituitary gland and other tissues (125, 126). However, there has yet to be any definitive receptor attributed to the widespread effects of UnAG (130, 135, 215). Skeletal muscle exhibits relatively low expression of GHS-R1a, and although there are studies to suggest that GHS-R1a content may be altered in other peripheral tissues e.g. adipose tissue during aging (141), data depicting whether changes to receptor content also occurs as a consequence to different dietary interventions is lacking. Results from the current study suggest that GHS-R1 content is unaltered in skeletal muscle following 6-weeks of high-fat feeding. However, our GHS-R1 analysis does not distinguish between *a* and *b* isoforms of the receptor. Since AG only binds and signals through the GHS-R1a receptor isoform, more specific GHS-R1 antibodies are

required to draw definitive conclusions. More recently, some of ghrelin's direct metabolic effects have been attributed to the CRF-2R (79). Interestingly, the total CRF-2R content was significantly lower in muscle from high-fat fed rats. Future investigations examining the contribution of this receptor to AG and UnAG's metabolic effects in peripheral tissue's like skeletal muscle are merited.

Limitations and Considerations

In the current study, high-palmitate exposure was utilized to acutely induce defects in insulin action in skeletal muscle, as done previously (216). However, the relative contribution of different mechanisms towards the induction of acute insulin resistance is still unclear. Specifically, the roles of LCFA-CoAs and ceramides, and potentially reactive oxygen species, were not assessed in the current study. Future investigations may aim to target the relative contribution of each of these insulin-desensitizing components and whether they are affected by ghrelin. Next, the exact molecular transducers for ghrelin's effects in skeletal muscle remain uncertain. As such, no cellular measurement was made to directly confirm the presence of ghrelin resistance which was observed in the functional outcomes of fatty acid oxidation and glucose transport. Lastly, while the aim of isolated incubations is to attempt to replicate *in vivo* scenarios of substrate and hormonal concentrations, due to tissue limitations, there was no treatment assessing the effects of simultaneous AG and UnAG muscle exposure, as would be observed prior to a meal, *in vivo*.

Conclusion

Taken together, these findings provide further evidence for ghrelin isoforms as regulators muscle glucose and fatty acid metabolism. UnAG is a stimulator of muscle fatty acid oxidation which likely contributes to its protective effects on insulin-stimulated glucose uptake and Akt/AS160 activation. Skeletal muscle from high-fat fed rats is resistant to ghrelin's beneficial effects on activating the muscle AMPK/ACC axis, fatty acid oxidation and insulin-stimulated glucose transport, which requires further exploration.

Chapter 6: Integrative Discussion

6.1 Ghrelin and adipose tissue lipid metabolism

The first study of this thesis aimed to explore a potential role for ghrelin isoforms in regulating TAG breakdown in AT, which had also been previously identified by Muccioli et al. in isolated adipocytes (154). Specifically, we investigated whether ghrelin was antilipolytic during the adrenergic stimulation of lipolysis by the β_3-agonist, CL 316, 243. Our study is the first to elucidate cellular signalling mechanisms related to the antilipolytic effects of ghrelin. Also, no prior study had assessed ghrelin's effects on lipolysis in rats, *in vivo*, and since β_3-ARs are expressed in rodent AT but not skeletal muscle, this allowed us to interpret ghrelin's effects *in vivo* while isolating the observed lipolytic response to AT. Previous work with ghrelin in humans is lacking this element of control, which complicates the interpretation of ghrelin's effects on different tissues. Ghrelin had no effect on lipolysis or pertinent signalling proteins when injected *in vivo*. Also, AG and UnAG did not independently affect lipolysis in either AT depot, *ex vivo*. It was only in the presence of the agonist, CL, that each ghrelin isoform attenuated lipolysis, primarily by inhibiting the activation of HSL at stimulatory phospho-sites ($Ser^{563/660}$).

Ghrelin isoforms inhibit the adrenergic-stimulation of lipolysis in isolated AT, but the findings *in vivo* are highly variable and confounded by the lipolytic effects of GH (9, 34, 77, 154, 233). In our study, there was no effect of either ghrelin isoform on stimulated lipolysis *in vivo*. It is possible that this was a result of the high dose of CL used to stimulate lipolysis. On the one hand, the dose of CL administered *in vivo* may have already stimulated lipolysis to a maximal rate that AG, through its induction of GH release, could not further stimulate. Conversely, the

ability for UnAG to inhibit glycerol/FA efflux from AT may have been overcome by CL. Additional work is warranted to establish a dose-response for CL to more accurately assess the regulation of AT lipolysis. A single study has observed reductions to FA appearance in humans during the simultaneous infusion of AG and UnAG 2 h following a standardized meal in GH-deficient patients (77). The concurrent regulatory effects of ghrelin isoforms have yet to be directly tested in isolated AT, but this may be an important consideration to properly understand ghrelin's role *in vivo*. Finally, it remains unclear why insulin, a strong antilipolytic signal, was significantly elevated following CL+UnAG compared to other CL treatments. CL administration stimulates insulin secretion, *in vivo* (86), although it is unlikely that UnAG acutely impacts insulin secretion in healthy individuals (220). More importantly, none of the treatments translated to an increase in insulin signalling activation within AT depots which corroborates the view that CL was primarily driving the observed lipolytic response, *in vivo*.

CL injection significantly increased blood FAs to ~1mM and this lipolytic response was isolated to AT, due to the location of β_3-ARs. Thus, it seems that AG (and GH) did not stimulate the breakdown of TAG stores from other tissues like skeletal muscle since higher FA concentrations can be achieved *in vivo* e.g. via lipid infusion. In fact, since the publication of these data, our lab has demonstrated that ghrelin isoforms directly attenuate adrenergic-induced lipolysis in skeletal muscle, *ex vivo* (131). Also, GH may actually facilitate the uptake of FAs and suppress HSL activity in skeletal muscle (236). Nevertheless, ghrelin isoforms directly attenuate the adrenergic induction of lipolysis in isolated tissue while their effects *in vivo* remain elusive, particularly in different nutritional states. It will be interesting to see whether ghrelin's effects persist in AT isolated from obese animals. In fact, very recent unpublished data from our lab indicates the antilipolytic effects of ghrelin are lost in AT isolated from high-fat fed rats.

Though speculative, AT resistance to ghrelin's antilipolytic effects could potentially contribute to increased postprandial levels of circulating FAs exhibited in obesity and T2D.

In our study, ghrelin isoforms significantly blunted the augmented re-esterification of FAs that normally accompanies increased (CL-induced) lipolysis. However, FA re-esterification was crudely assessed i.e. without radiolabeled FA tracer. It is uncertain whether ghrelin diverted FAs away from re-esterification by affecting glucose or FA transport, the former being essential in the provision of dihydroxyacetone phosphate for glyceroneogenesis (180). To that end, changes to FA re-esterification through ghrelin's regulation of glucose uptake in the AT organ culture cannot be excluded since extracellular AT culture medium contained 5mM glucose. It could be that ghrelin maintains levels of circulating FAs in scenarios of reduced lipolysis. This could confer a protective effect if a meal is missed, and it would be beneficial to have FAs persist in circulation for other tissues in order to spare blood glucose. The ability for ghrelin to reduce the supply of glycerol to the liver and presumably gluconeogenesis should also be deliberated.

It is difficult to speculate on the cellular action of ghrelin when intracellular secondary messengers or signalling markers have not been fully elucidated in peripheral tissues such as AT. It is possible that ghrelin acts similarly to insulin in AT i.e. either by stimulating glucose uptake to encourage TAG synthesis, or by promoting the degradation of intracellular cAMP to reduce PKA-mediated HSL activation (50, 60). The latter possibility is supported by recent findings in rat visceral adipocytes (9). Finally, evidence exists to suggest that inhibiting CaMKII signalling in 3T3-L1 adipocytes blunts lipolysis by downregulating ERK-1/2 phosphorylation (178). However, based on our own data, ERK-1/2 activation was unchanged following CL and ghrelin co-administration, making it unlikely that ghrelin is working through this pathway. More work is

warranted to determine the importance of CaMKII in mediating ghrelin's effects in peripheral tissues.

6.2 Ghrelin and skeletal muscle metabolism

Initial findings supporting a role for AG as a stimulator of glucose uptake in C2C12 myocytes left several questions unanswered (79). Firstly, the ability for UnAG to regulate glucose uptake was not examined. Next, although measurements were made in the presence of insulin, C2C12 cell lines are a questionable model for insulin-stimulated GLUT4 translocation and glucose uptake, partly because they display high basal rates of glucose uptake and may even lack GLUT4 compartmentalization (160, 221). Since cells were treated with ghrelin for approximately 24 h, it is also possible that the stimulatory role of AG on glucose uptake involved protein translation mechanisms i.e. increases to GLUT4 content. Accordingly, two studies have observed increases in GLUT4 mRNA following prolonged AG exposure (11, 79). The appearance of AG in circulation is transient, which renders the physiological relevance of this mechanism (i.e. prolonged elevation) uncertain. Therefore, the second study of this thesis aimed to investigate the acute effects of both ghrelin isoforms on glucose uptake in mature skeletal muscle. Glucose uptake and insulin signalling were assessed in the absence of FA availability to isolate ghrelin's effects on glucose metabolism. Mature oxidative (soleus) and glycolytic (EDL) skeletal muscles were procured to determine whether ghrelin had any fibre-specific effects independently and during insulin treatment. Varying concentrations of both insulin and ghrelin were used, and it was concluded that neither ghrelin isoform acutely affected glucose uptake nor insulin signalling.

Our data did not support the notion that ghrelin isoforms may independently regulate glucose uptake. To that end, it seemed more reasonable to suggest that both isoforms may work

synergistically with insulin, particularly since ghrelin only peaks in circulation with habitual eating. However, insulin-stimulated glucose uptake and signalling were similarly unaffected by ghrelin isoforms, *ex vivo*. Ghrelin consistently affects glucose metabolism in the literature when chronically administered whereas its acute effects, which are arguably more relevant to physiology, remain difficult to interpret (16, 79). Future research may consider tracing the metabolic fate of radiolabeled glucose in peripheral tissues *in vivo*, following acute ghrelin administration to more definitively determine its effects on glucose handling in peripheral tissues.

In the third and final study of this thesis, we returned to consider whether ghrelin would be more impactful in the acute regulation of skeletal muscle glucose uptake through its modulation of lipid metabolism. Thus, insulin-stimulated glucose uptake was assessed during simultaneous glucose and FA availability, which is also more physiologically relevant. Prior, but limited studies, have demonstrated that ghrelin stimulates FA oxidation in muscle (91, 131). We replicated these findings, and importantly, further demonstrated that ghrelin, specifically UnAG, preserved insulin action in muscle during high FA exposure (91). These beneficial effects on protecting insulin-stimulated glucose uptake were dependent on the stimulation of FA oxidation, as blocking FA oxidation nullified ghrelin's protective effect. Additionally, we analyzed the metabolic effects of ghrelin in skeletal muscle from high-fat fed animals, which had only been sparsely examined previously. Our novel observation was that neither ghrelin isoform was able to stimulate FA oxidation in muscle from these animals i.e. implying ghrelin resistance.

Our results for insulin signalling were partly at odds with the outcome of glucose uptake. Specifically, AG more consistently preserved insulin signalling but this did not translate to insulin-stimulated glucose uptake. In contrast, UnAG was less consistent in maintaining insulin

signalling activation but ultimately preserved glucose uptake. In part, this may be explained by evidence that suggests Akt phosphorylation at site Thr^{308} may occur prior to Ser^{473} (218). Perhaps a more robust increase to Ser^{473} and downstream AS160 phosphorylation would have been observed following UnAG treatment had the muscle exposure to insulin been slightly prolonged. Also, a single timepoint was chosen for the measurement of insulin signalling cascade protein phosphorylation, which may not necessarily reflect the peak response to insulin.

Human and rodent skeletal muscle is comprised of multiple fibre-types that can be characterized by their contractile and metabolic phenotypes (254). Relevant to this thesis are rodent soleus and EDL muscles, which are predominately composed of type I (oxidative) and type II (glycolytic) fibers, respectively. Divergent fibre-type responses to several hormones have been reported in the literature while ghrelin's effects on different fibre-types remain relatively unexplored (46, 148). In the second study of this thesis, muscle incubation with AG increased the phosphorylation of CaMKII (a regulator of glucose uptake and FA oxidation) at its γ-subunit in EDL. UnAG treatment trended ($p = 0.1$) towards increasing CaMKII activation, but only in soleus, which may allude to ghrelin fibre-specific effects that were later unmasked, since UnAG was more effective in preserving insulin action in soleus in the third study. However, it is not possible to conclude any potential ghrelin fibre-type differences in the third study since we did not perform experiments in EDL. Moreover, if the beneficial effects of UnAG on insulin action require the stimulation of FA oxidation, it may be worthwhile considering its effects through CaMKII. CaMKII-dependent signalling may be especially important for FA uptake and oxidation in rodent skeletal muscle (177) and ghrelin signalling in the CNS has been shown to transiently increase intracellular Ca^{2+} (104). Future work may necessitate concomitant single

fibre analysis for fibre-type composition, metabolic measures of glucose uptake and cellular signalling to confidently conclude whether ghrelin isoforms exert fibre-specific effects.

In the second study of this thesis, we did not observe ghrelin-mediated activation of AMPK. However, downstream ACC phosphorylation was not assessed, which is likely to account for this discrepancy with other work from our lab which demonstrated increased ACC phosphorylation in acute response to ghrelin (131). The mechanism attributable to ghrelin's potential activation of the AMPK/ACC axis remains inconclusive. CaMKII is again a potential candidate since it may lie upstream of AMPK, and may increase FA uptake and oxidation during contraction (177). However, a first step would be to monitor intracellular messenger (e.g. Ca^{2+}) responses in peripheral tissues following exposure to ghrelin isoforms, similar to what has been performed in vascular smooth muscle cells (65).

6.3 Obesity

In obesity, or following obesogenic high-fat diets, skeletal muscle becomes resistant to many hormones, which can influence other aspects of muscle metabolism that eventually impinge on insulin action (183, 189, 204). The third study of this thesis provided evidence for a resistance to ghrelin at the skeletal muscle level. The effects of ghrelin on FA oxidation and glucose uptake in isolated muscle were compared between animals placed on a chronic, 6-week low versus high-fat diet.

Since it had only recently been determined in lean animals that ghrelin could preserve muscle insulin action during acute (4 h) palmitate exposure, this model was kept consistent for the analysis of ghrelin's metabolic effects in muscle from high-fat fed animals. Unfortunately, due to this element of control, it remains somewhat unclear whether the muscle had impaired insulin action prior to isolation and exposure to high palmitate concentrations. As little as one

week of HFD can impair muscle insulin action, although type I fibres exhibit the greatest resistance to these impairments (167). We used predominately type I fibre-expressing soleus muscle in the third thesis study. Insulin significantly stimulated glucose uptake in the low palmitate treatment (compared to control), although it is difficult to know whether the isolated muscle had some level of impaired insulin action prior incubation and measurement of glucose uptake. Future work could directly assess ghrelin's effects in insulin resistant muscle following chronic dietary intervention, as opposed to the combined effects of HFD and acute, saturated FA exposure on ghrelin action.

6.4 Other considerations

The accurate recapitulation of the *in vivo* nutritional and hormonal milieu should be a primary goal of isolated experiments that assess metabolic function. However, this is difficult to fully accomplish, particularly since ghrelin trends *in vivo* may precede the effects of several other hormones e.g. incretins, GH. Effort was made in this thesis to expose isolated muscle to ghrelin and relevant hormones in a manner similar to what would occur physiologically. For example, in the third study, muscle was incubated with ghrelin prior to insulin treatment and the measurement of glucose uptake. Although, several other factors were not accounted for across our experiments. For example, glucagon is another crucial hormone responsible for the mobilization of FAs from AT in which ghrelin's effects may be further contextualized. The first study in AT also warrants further investigation into ghrelin's overlapping role with insulin, the predominate negative regulator of AT lipolysis. Unlike lipolysis experiments, measurements of FA oxidation did not include catecholamines or other stimulants when assessing ghrelin's protective effects on insulin action. In the future, this could provide insight into the magnitude of ghrelin effect compared to other endogenous regulators of FA oxidation. Another consideration

is that, in a postprandial scenario, skeletal muscle is not provisioned glucose and FAs at the same rate, nor would the FA load be predominately one type e.g. palmitate.

6.5 Concluding remarks

Numerous questions remain unanswered regarding ghrelin's effects on peripheral tissue metabolism. While the literature continually supports a beneficial metabolic role for UnAG, extensive work is still required, particularly since research examining UnAG lags behind that of AG. Perhaps the plethora of emerging work highlighting the positive effects of UnAG will also lead to a greater emphasis on translational work in the coming years. It is intriguing to consider future directions addressing whether UnAG presents a therapeutic strategy to combat obesity or T2D. In addition to a possible resistance of skeletal muscle to ghrelin, circulating UnAG concentrations decline in obesity. Despite this, there are very few registered clinical trials applying UnAG as a therapy in the obese. Moving forward, it will be imperative that researchers confirm the presence of ghrelin resistance in skeletal muscle from obese or diabetic humans, and it will be important to characterize whether effective interventions such as exercise or specific nutritional strategies, can reverse the impaired ghrelin response in these conditions (183).

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Appendix I: Western Blotting

Reagents:

1. Laemmeli Buffer 4.0x
   - 50mL 0.5M Tris-HCl (pH 6.8)
   - 8.2g SDS
   - 40ml glycerol
   - 500µl 1% Bromophenol Blue
   - 31mg DTT to 500µL of 4.0x Laemmeli buffer

- Bring volume to 100ml with ddH_2O and pH to 6.8

2. 10X Running Buffer
   - 30g Tris Base
   - 144g Glycine
   - 10g SDS

- Bring volume to 1L with ddH_2O, dilute 10X to 1X for day-of use

3. 1X Transfer Buffer
   - 80ml 10X Transfer Buffer
   - 160ml Methanol
   - 560ml ddH_2O

4. 10X Tris Buffered Saline (TBS)
   - 24.2g Tris Base
   - 80g NaCl

- Adjust pH to 7.6 with HCl
- 1X TBS: 100ml TBS, 900ml ddH_2O
- 1X TBST: 100ml 1X TBS, 900ml ddH_2O, 1ml Tween-20

Protocol:

Gel electrophoresis assembly:

- Clean glass plates; rinse one long and one short plate per gel well with ddH_2O. Wipe plates carefully with KimWipes and allow to dry.
- Assemble the glass plates and place into casting frame keeping the short plate facing towards the front. Ensure both plate bottoms are flush on a level surface and lock the green pressure cams to secure plates in space. Secure the casting frame in the casting stand.

Cast gel:

- Prepare running gel to be cast prior to the stacking fraction. For this thesis, 10% acrylamide gels were used for every protein target, except for ACC which used 5% gels.
- Add APS and then TEMED very quickly at the end, swirl gently to initiate

polymerization and pour between the two plates.

- Add ddH_2O to the top of the resolving gel before it sets to eliminate air bubbles and maintain a straight polymerization line.
- After the gel polymerizes, drain ddH_2O.
- Pour the stacking solution into the remaining space between the glass plates until the top of the short plate is reached. Insert the comb and allow gel to fully polymerize.

Assembly of electrophoresis module and sample loading:

- Remove casting frame from stand, place gel cassettes into green electrode assembly.
- Slide the gel cassettes into the green clamping frame with electrodes. Press down on the electrode assembly while closing the two cam levers of the frame, making sure that the gels are aligned horizontally and placed as far down as possible in the clamp. Place whole assembly into the mini tank.
- Be sure that the short plate faces inward toward the middle white barrier. Use tall electrodes if only running 1-2 gels; add the short assembly if running 3-4 in one tank. If only using one gel, be sure to use a buffer dam.
- Fill between the gels with 1X running buffer. Gently tip the mini tank to allow running buffer to escape the gel space and rinse the wells created previously by the green comb.
- Load one well of 7µL molecular weight marker on the leftmost well of each gel. Load samples slowly into each well (~10-40µg, depending on the target) from left to right. Take note of the order for sample loading.
- Fill the tank to the 2 or 4 gel line with 1X running buffer.
- Connect the apparatus to the power pack. Run at 100V until the sample exits the stacking gel, then increase to 120-150V until sample reaches the bottom of the gel.
- Remove the gels to a tray for transfer unit preparation.

Transfer blotting:

- Nitrocellulose membranes should be cut to the dimensions of the gel(s) and immersed in transfer buffer for 5 min.
- For each gel “sandwich”, soak the membrane, 6x filter paper, and 2x fiber pads in the transfer buffer.
- Prepare the gel sandwich.
    - Place one pre-wet fiber pad on the black side of the cassette
    - Place 3x soaked filter paper on the fiber pad
    - Place the gel(s) on the filter paper, either full or cut to a specific molecular weight for the corresponding protein target
    - Place the pre-wet membrane on the gel(s)
    - Gently roll to remove air bubbles
    - Place 3x filter paper on the membrane
    - Complete the sandwich by placing the final wet fiber pad on the filter paper
    - Close the cassette firmly, being careful not to disrupt the sandwich contents

- Lock the cassette and align black to black within the red and black transfer unit
- Add an ice pack and fill the tank with cold 1X transfer buffer
- Put on lid, plug cables into the power pack and run at 100V per tank, for 60-90 min, depending on the molecular weight of the target protein
- After the allotted transfer time, remove the sandwich, trim the membrane and place in a clean petri dish for subsequent antibody binding and development

Membrane Blocking and Incubation:

- Block the membrane with TBST and 5% w/v skim milk for at least 1 h at room temperature, shaking gently

Antibodies

- Dispose of blocking solution, rinse the membrane with TBST for 10min and add the appropriate 1º antibody – either frozen stock or prepared fresh. 1:1000 in TBST and 5% BSA or skim milk, as per manufacturer's guidelines.
- Incubate with gentle agitation at 4ºC overnight.
- The following day remove the 1º antibody using a transfer pipette and store at -80ºC until the next use.
- Rinse the membrane 2x15 min in TBST
- Add the appropriate 2º antibody (dependent on the 1º used) in TBST with 1% w/v skim milk
- Leave the membrane to shake/rock at room temp for 1 h
- Wash 2x15 min with TBST and 1x10 min with 1X TBS

ECL (Enhanced Chemiluminescence)

- Pour out all TBS from the petri dish
- Combine Western Lighting ECL (PerkinElmer) solutions in equal ratios as per directions (~5ml/membrane, 2.5ml of each clear and brown solution) in a separate container. Pour over membrane and incubate with agitation for ~30s.
- Transfer the membrane to plastic wrap on the tray within the Alpha Innotech imager, lay flat and ensure no air bubbles are present.
- Adjust light and image settings use gross and fine-tune knobs and appropriate exposure times. Collect 10-15 stacked frames per blot and save the image to the hard drive.
- Quantify the band density using Alpha Innotech software.

Appendix II: Submaximal ("moderate") and Maximal ("high") Insulin-Stimulated Glucose Uptake

Submaximal and maximal insulin-stimulated experiments represent 0.5mU/ml and 10mU/ml insulin concentrations, respectively. Attempts were made in **Study #1 and Study #3** to mimic the hormonal trends that would normally occur with a meal i.e. muscle being exposed to ghrelin before insulin, to determine whether ghrelin could prime muscle to take up glucose or alter muscle's response to insulin.

Thesis Study #2 used Krebs-Henseleit Buffer salt solutions supplemented with 1% BSA. For insulin-stimulated treatments, all vials received insulin. For muscles receiving ghrelin treatment, ghrelin was added to all vials.

In **Thesis Study #3**, while it was known that ghrelin could alter rates of fatty acid oxidation in muscle, the timepoint at which this may occur was unknown. Pilot work determined that ghrelin more effectively stimulated oxidation at t = 2-4 h, therefore palmitate tracer was added over the final 2 h of the 4 h palmitate window for oxidation experiments. Ghrelin was added for the entire 4 h exposure. For the measurement of glucose uptake/insulin signalling, ghrelin was present for the entire 4 h exposure of muscle to palmitate. Thesis Study #3 also used Dulbecco's Modified Eagle's Medium supplemented with glucose/sodium bicarb and 4% BSA to help with 2mM palmitate solubility. Only wash and final radiolabeled uptake vials received insulin for insulin-stimulated treatments. For muscles receiving ghrelin treatment, ghrelin was added to palmitate exposure, wash and radiolabeled glucose uptake/signalling vials.

Glucose Uptake Assay

Reagents

1. Weekly Buffer (Base solution for M1, M2 & M3)
2. Medium 1 (Pre-Inc, pre-gassed with 95% O_2, 5% CO_2) – 8mM glucose, 32mM mannitol
3. Medium 2 (Wash, pre-gassed) – 36mM mannitol, 4mM pyruvate
4. Medium 3 (Radiolabelled Uptake/Experimental Buffer, pre-gassed) – 8mM 2-deoxyglucose, 28mM mannitol, 4mM pyruvate

± 0.5mU/mL (submaximal) or 10mU/ml (maximal) insulin

- insulin stock (986µl + 13.3µl Insulin = [100U/ml]) – made just prior to addition as insulin is more stable in the presence of BSA

± AG or UnAG (60µl = [150ng/ml])

Protocol

1. Incubate muscle strips (~15-30mg) for 30 min in 2ml Medium 1 (± insulin and ghrelin)
2. Wash muscle strips two times, 10min each in Medium 2 (± insulin and ghrelin)
3. Incubate muscles for 20 min (+ insulin) or 40 min (- insulin) in Medium 3 (± insulin and ghrelin)
4. Blot muscle dry, cut tendons and weigh
5. Solubilize muscle strips by boiling for 10 min in 1ml of 1M NaOH. Vortex 1-2 times during solubilization and keep the vial covered with parafilm.
6. Sample 200µl of the muscle digest into scintillation vials and add 5ml of scintillation cocktail. Prepare duplicates for each sample
7. Allow samples to quench overnight in complete darkness
8. Count samples (^{3}H and ^{14}C) using liquid scintillation counter 5 min per sample

Appendix III: Palmitate Oxidation

1. Incubate muscle strips (~15-35mg) for 30 min in 2ml DMEM supplemented with sodium bicarbonate and 4% BSA
2. Transfer muscles into new vial containing low (0.2mM) or high (2mM) palmitate solution for 2 h (± AG/UnAG)
3. Transfer muscle to new flask for measurement of oxidation over 2 h (± AG/UnAG)
4. After 2 h, use 1ml syringe to add ~300µl benzethonium hydroxide solution into small eppendorf inside larger eppendorf in flask
5. Quickly add 1ml sulfuric acid into buffer containing muscle sample to release and capture all CO_2 in the benzethonium hydroxide.
6. Allow samples to rest for ~2 h prior to collection of benzethonium-containing tube.
7. Add tube and 5ml scintillation cocktail to scintillation vial
8. Shake sample vigorously and allow to quench in the dark overnight
9. Count samples (^{14}C) using liquid scintillation counter, 5 min per sample

Appendix IV: Lipolysis Following (8h) CL and Ghrelin Treatment in ATOC

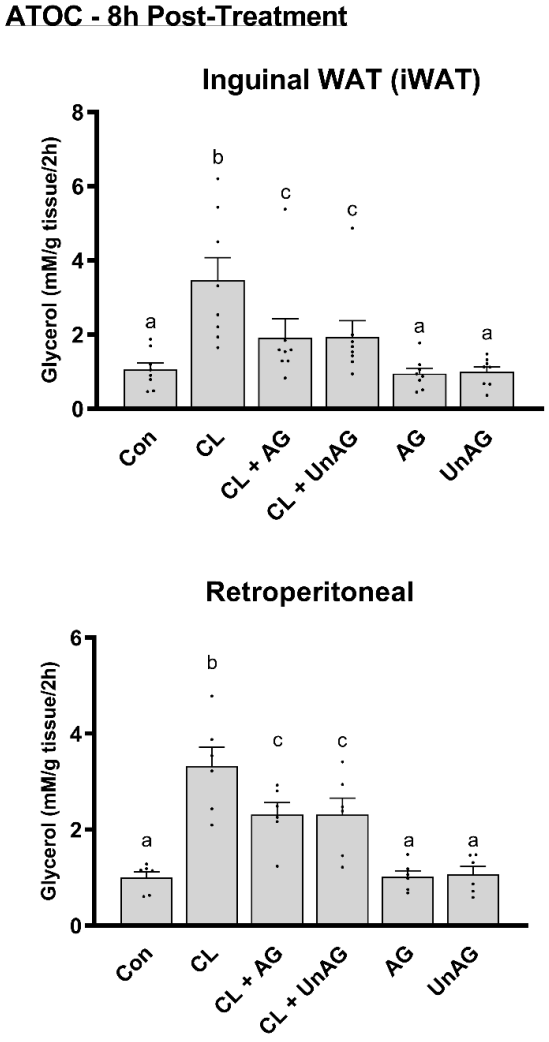
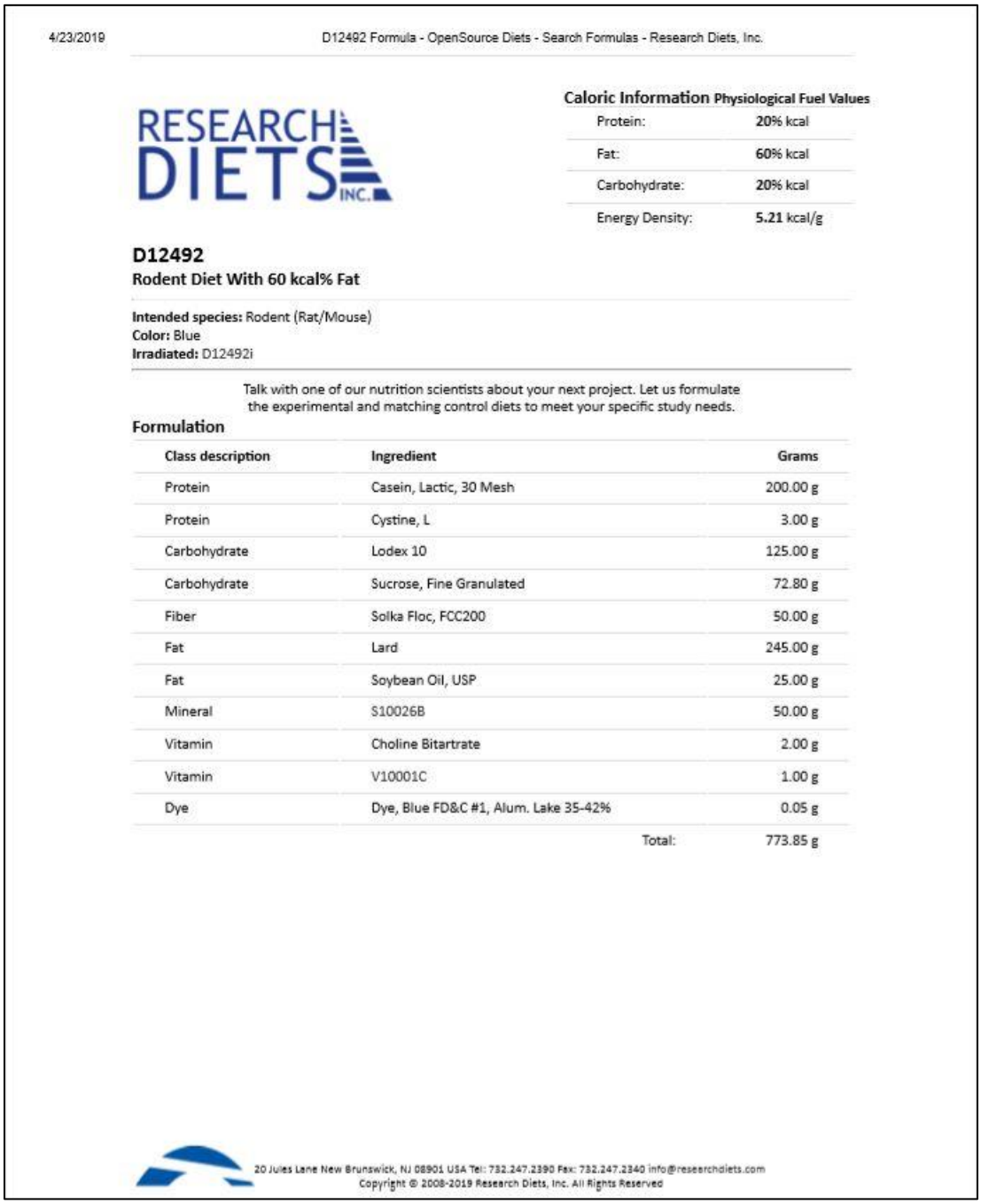


Figure 23: Lipolysis Following (8h) CL and Ghrelin Treatment in ATOC

Glycerol release (index of lipolysis) in subcutaneous iWAT and visceral RP adipose tissue depots harvested from chow-fed rats following treatment with CL 316,243 (1µM) and AG or UnAG (150ng/ml). Data were analyzed using a repeated measure one-way ANOVA (n=6-8) and were expressed as individual data points and the mean ± standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

Appendix V: Low and High Fat Diet Composition

D12492, High-fat diet (60% kcal FAT)



4/23/2019 D12492 Formula - OpenSource Diets - Search Formulas - Research Diets, Inc.

RESEARCH DIETS INC.

Caloric Information **Physiological Fuel Values**

Protein:	**20**% kcal
Fat:	**60**% kcal
Carbohydrate:	**20**% kcal
Energy Density:	**5.21** kcal/g

D12492
Rodent Diet With 60 kcal% Fat

Intended species: Rodent (Rat/Mouse)
Color: Blue
Irradiated: D12492i

Talk with one of our nutrition scientists about your next project. Let us formulate the experimental and matching control diets to meet your specific study needs.

Formulation

Class description	Ingredient	Grams
Protein	Casein, Lactic, 30 Mesh	200.00 g
Protein	Cystine, L	3.00 g
Carbohydrate	Lodex 10	125.00 g
Carbohydrate	Sucrose, Fine Granulated	72.80 g
Fiber	Solka Floc, FCC200	50.00 g
Fat	Lard	245.00 g
Fat	Soybean Oil, USP	25.00 g
Mineral	S10026B	50.00 g
Vitamin	Choline Bitartrate	2.00 g
Vitamin	V10001C	1.00 g
Dye	Dye, Blue FD&C #1, Alum. Lake 35-42%	0.05 g
	Total:	773.85 g

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D12450J, Low-fat diet (sucrose-matched to D12492; 10% kcal FAT)

RESEARCH DIETS INC.

Caloric Information	Physiological Fuel Values
Protein:	20% kcal
Fat:	10% kcal
Carbohydrate:	70% kcal
Energy Density:	3.82 kcal/g

D12450J

Rodent Diet With 10 kcal% Fat (Matching Sucrose to D12492)

Intended species: Rodent (Rat/Mouse)
Color: Yellow : Blue = 4:1
Irradiated: D12450Ji

Formulation

Class description	Ingredient	Grams
Protein	Casein, Lactic, 30 Mesh	200.00 g
Protein	Cystine, L	3.00 g
Carbohydrate	Starch, Corn	506.20 g
Carbohydrate	Lodex 10	125.00 g
Carbohydrate	Sucrose, Fine Granulated	72.80 g
Fiber	Solka Floc, FCC200	50.00 g
Fat	Soybean Oil, USP	25.00 g
Fat	Lard	20.00 g
Mineral	S10026B	50.00 g
Vitamin	Choline Bitartrate	2.00 g
Vitamin	V10001C	1.00 g
Dye	Dye, Yellow FD&C #5, Alum. Lake 35-42%	0.04 g
Dye	Dye, Blue FD&C #1, Alum. Lake 35-42%	0.01 g

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Appendix VI: Intraperitoneal Glucose Tolerance Test Protocol (IPGTT)

1. Provide each rat a clean cage and remove available food to begin the 6-8h fast (water ad libitum)

2. On the morning of experiments, weigh each rat to determine the volume of glucose to inject

3. Preparation of glucose solution:

- Dissolve 5g D-glucose in 9ml H_2O then adjust to 10ml with ddH_2O, yielding 0.5g/ml solution
- Multiply the body weight (in grams) by 4 to obtain the desired dose (in µl) of 2g/kg, i.e. For a 500g rat, inject (500x4) = 2000µl.

4. Set-up bench with the following:

- pair of scissors to cut tails and gauze
- two glucometers, timers

5. Following the fast, measure blood glucose (t=0min) and then inject the rat with diluted glucose following the steps below:

- fill syringe with correct dose of diluted glucose and fill syringes for ALL cages first
- inject intraperitoneally after restraining the rat with a large cloth/towel
- measure blood glucose at t= 15, 30, 45, 60, 90, 120 min
- as soon as the rat has been injected, begin timer (count from 0:00), record the time and blood glucose concentrations

6. At the end of 120 min, return rats to their normal cages and ensure adequate food and water

Appendix VII: Blood Collection and Ghrelin ELISA

Ghrelin is extremely sensitive to degradation upon collection and previous literature suggest that large discrepancies in circulating blood concentrations exist within and across species (8, 223, 245). As such, the first set of pilot experiments for this thesis were aimed at determining whether we could avoid the morning peak in ghrelin levels that may confound our metabolic measures in isolated experiments. We also wanted to ensure that ghrelin was stable in a heated, shaking incubation bath for upwards of ~4 h, which would be a time-course for upcoming experiments. Therefore, rats were either re-fed or overnight fasted prior to being anesthetized with an intraperitoneal injection of sodium pentobarbital (6 mg/100g b.w.). Blood samples were collected in tubes containing Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich; 1mg/ml) and aprotinin (Phoenix Pharmaceuticals Inc.; ~600-700kIU/ml). Blood was immediately acidified with 1.0M HCl (100µl per 1ml blood) and centrifuged at 9500g for 5 min. Supernatant was then collected in separate tubes and samples were frozen at -80℃ for future analysis. Initially, supplied ghrelin standards were reconstituted with 2ml deionized water, and inverted gently. A serial dilution was performed for ghrelin standards using provided assay buffer. Final standard concentrations were 5, 2.5, 1.25, 0.6, 0.3 and 0.16 ng/ml, respectively. Pipette tips were changed for every dilution and each tube was vortexed. Small aliquots of unused portions were stored at -80℃. Quality controls (1 and 2) were reconstituted in 0.5ml deionized H_2O and inverted. Unused portions were stored in small aliquots (-80℃). Supplied wash buffer was diluted 10-fold by pouring entire contents of both bottles (50ml each) into 900ml deionized H_2O.

The ELISA was performed as follows:

The necessary number of plate strips were assembled in an empty plate holder and filled with diluted wash buffer. The plate was then decanted, inverted and tapped dry on absorbent towel several times. This was repeated two more times. Prior to well's drying, 20µl of matrix solution was added to "blank", "standard" and "quality control" wells. Then, 30µl assay buffer was added to "blank" and "unknown" wells, whereas 10µl assay buffer was added to "standard" and "quality control wells". In duplicate, ghrelin standards and quality controls were added (20µl) to appropriate wells. Sequentially, 20µl of unknown sample was added to the remaining wells. 50µl of detection antibody was added to every well, after which, the plate was covered and incubated at room temperature for 2 h on an orbital plate shaker. Following this, all wells were decanted and then washed (3 times) with wash buffer as done previously. 100µl of enzyme solution was then added to each well, the plate was again covered and incubated for an additional 30 min. Seal was then removed, wells decanted, and washed (6 times). 100µl was then added to each well, the plate was sealed and shaken for approximately 15 min, whilst monitoring colour development. Stop solution was added at the point where the lowest concentration ghrelin standard's colour development was faintly visible and distinct. Finally, 100µl of stop solution was added to all wells, and the plate was shaken by hand to mix. The plate was then placed into a spectrophotometric plate reader and analyzed by measuring sample absorbance (enzyme activity, proportional to the amount of captured total rat ghrelin) at dual wavelengths of 495 and 590nm. The output data was fit to a 4-parametric curve. The assay was considered accepted when quality control samples fell within the provided range and the coefficient of variation between duplicates of a sample was < 15%.