CULPRIT OR CONSEQUENCE: DETERMINING THE ROLE OF GHRELIN IN SKELETAL MUSCLE DYSREGULATION

A Dissertation

by

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ABSTRACT

The lack of physical activity with or without advancing age is often accompanied by a dysregulation of skeletal muscle metabolism, leading to impaired muscle function and disease. While causes of skeletal muscle dysfunction are numerous, it is understood that the preservation/restoration of normal skeletal muscle metabolism is a consistent factor involved in the prevention, development, and progression of many of these conditions. An emerging candidate for the control of metabolic disease is Ghrelin, a multi-isoform peptide hormone that has been suggested both as a culprit and a potential therapeutic target because of its impact on inflammation and glucose uptake, both hallmarks of muscle dysfunction. Despite its documented role in the body, very little is known about ghrelin's direct impact on skeletal muscle. The purpose of this dissertation was to assess the direct action of ghrelin and its receptor on metabolic and anabolic signaling in skeletal muscle. *We hypothesized that the role of ghrelin in the control of metabolic disease was via a direct action on skeletal muscle, a major site for glucose disposal,* but our findings indicate that ghrelin-mediated actions are primarily on other tissues that subsequently influence skeletal muscle. Using healthy, cultured myotubes and genetically altered mice that did not express the ghrelin receptor, we assessed the direct action of both isoforms of ghrelin on mechanisms of insulin-dependent and independent skeletal muscle glucose uptake. Our results demonstrated, in culture, that the two isoforms do have unique actions in skeletal muscle and confirm that AG negatively impacts glucose uptake, potentially through upregulation of 4E-BP1. Further,

our data indicate that the positive influence attributed to UAG for glucose uptake is due to insulin-independent actions via AMPK but the overall response is less convincing than previous work. Global knock-out of the ghrelin receptor, GHS-R1a, in mice mitigated the metabolic dysregulation observed with advanced age. The present outcomes indicate that whole-body disruptions of glucose regulation, in part, are mediated by the presence of the acylated isoform of ghrelin, but the purported 'improvements' from unacylated ghrelin through a direct interaction in skeletal muscle may be overstated.

DEDICATION

This manuscript is dedicated to my emotional support squad, my Fiancé Eric and our dogs (Georgia, Bowie and Kira). Eric, none of this would have been possible without you. Thank you for your unwavering support, your unconditional love, and your much needed humor in my darkest moments. I love you to the moon and back.

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NOMENCLATURE

4E-BP1	Eukaryotic Translation initiation factor 4E-binding protein 1
AG	Acylated Ghrelin
AKT	Protein Kinase B
AMPK	AMP-activated Protein Kinase
BAT	Brown Adipose Tissue
FNDC5	Fibronectin Type III Domain-Containing Protein
GHS-R	Growth Hormone Secretagogue Receptor
GLUT4	Glucose Transporter Type 4
GOAT	Ghrelin-O-Acyltransferase
GSK-3β	Glycogen Synthase Kinase 3β
LCFA	Long Chain Fatty Acids
mTORC1	Mechanistic Target of Rapamycin Complex 1
PGC-1α	Peroxisome Proliferator-activated receptor-gamma Coactivator 1α
PI3K	Phosphatidylinositol 3 Kinase
T2D	Type 2 Diabetes
UAG	Unacylated Ghrelin
UCP1	Uncoupling Protein 1

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1. INTRODUCTION

Thanks to medical and technological advances, both how long we live and the way we live has changed dramatically over the last century. While aging has always been an inevitable process, life expectancy in developed countries has increased substantially. This increase in lifespan presents new medical and financial challenges because of the prevalence of age-related disorders that increases as the global lifespan increases [1,2]. Simultaneously, the overwhelming trend toward lowered physical activity and sedentary lifestyles is commonly associated with adverse health outcomes like obesity, insulin resistance, and type 2 diabetes (T2D) [3,4]. In fact, as age increases, the risk of developing T2D and other metabolic comorbidities also increases [5]. Unsurprisingly, the development of T2D increases the risk and severity of age-related adverse outcomes [6,7], confirming the interconnectedness of these debilitating diseases. There is another common feature of these two modern problems besides the overlapping consequences: the role of skeletal muscle in the development of these ailments. The prevalence of aging-related muscular deficiencies has become so commonplace that it was given its own title, sarcopenia. Sarcopenia is associated with loss of lean mass, strength, mitochondrial and metabolic dysfunction, and a reduction in overall quality of life. Similarly, peripheral insulin resistance, a hallmark of T2D, is primarily attributed to skeletal muscle because of the large percentage of post-prandial glucose uptake that occurs in skeletal muscle. It is no wonder that current avenues of research are directing efforts towards potential therapeutic targets related to the metabolic dysregulation that

occurs in skeletal muscle and leads to insulin resistance, sarcopenia, and other medical phenomena.

Ghrelin is a peptide hormone, known for its influence on appetite through growth hormone secretion, but ghrelin is purported to have many diverse functions throughout the body. This multifactorial effect has inspired lines of research into ghrelin as a potential target for various disease states, including sarcopenia and T2D. However, that same diverse activity confounds many of the results associated with the actions of ghrelin. Ghrelin has two isoforms, acylated (AG) and unacylated (UAG) ghrelin, that are known to have unique but overlapping functions. Distinguishing the outcomes of ghrelin action or ghrelin ablation is complicated by the sometimes contradictory actions of the two isoforms as well as the constitutive activity of the only known receptor. Ghrelin administration, both *in vivo* and *in vitro*, effects several positive outcomes, including a reduction in inflammation [8,9] and atrophy [8,10,11]. Conversely, ablation of the ghrelin gene [12,13] or the ghrelin receptor, a growth hormone secretagogue receptor (GHS-R) GHS-R1a [14–17], improves glucose metabolism in both aged and diabetic models, and is accompanied by reductions in obesity and inflammation. Interestingly, the circulating levels of total ghrelin with advancing age or insulin resistance are lower than in healthy individuals [18,19], which may implicate ghrelin resistance as a culprit in the development of these diseases [20]. However, the conflicting evidence of ghrelin action complicates supporting these conclusions. Aging and T2D share many similar whole-body outcomes, including low-grade inflammation, altered glucose metabolism, and decline of lean body mass, solidifying skeletal muscle as a critical player in the

development of these diseases. Despite the interest in ghrelin as a potential therapy, very little has been elucidated about the direct function of ghrelin in skeletal muscle. Currently, the literature has conflicting ideas of ghrelin action in skeletal muscle, whether the known receptor is present, and whether that receptor plays a relevant part in the skeletal muscle response. The primary purpose of this research was to assess the direct action of ghrelin and its receptor on metabolic and anabolic signaling cascades associated with muscular decline during both aging and T2D. ***The overarching hypothesis was that both acylated and unacylated ghrelin would have a direct action on skeletal muscle, independent of the only known receptor GHS-R but that GHS-R may also alter metabolic conditions of skeletal muscle either through the action of the ghrelin-GHS-R axis directly in skeletal muscle or as a response from the ghrelin-GHS-R axis in other organs during tissue cross talk.*** The following aims were used to elucidate the direct impact of ghrelin on skeletal muscle and the influence of GHS-R during the response of skeletal muscle during aging.

The first aim used *in vitro* techniques with rat skeletal muscle to systematically determine the impact of the two isoforms of ghrelin (AG and UAG) on insulin-dependent and insulin-independent glucoregulatory processes. Current research implicates that both isoforms impact metabolic and glucoregulatory processes, but there is still dissension about precisely what those actions are. This dissention most likely stems from the environmental factors of the *in vivo* work that has most commonly been done, making it impossible to tease out the direct action of ghrelin on skeletal muscle versus skeletal muscle response to other organs that have been impacted by ghrelin and

or the ghrelin-GHS-R axis. This *in vitro* model allowed us to examine the specific response of skeletal muscle with either isoform of ghrelin and whether this response has physiological relevance. We hypothesized that both isoforms would directly affect skeletal muscle and that this action would be through an AMP-activated protein kinase (AMPK) signaling cascade, implicating an insulin-independent role in ghrelin's influence on skeletal muscle glucoregulatory processes.

The second aim used an aged ghrelin receptor knockout mouse model to better understand the ghrelin signaling cascade and the ghrelin receptor function in skeletal muscle. Previous research with ghrelin receptor knockout mice has demonstrated that removing the gene protects against high-fat diet-induced obesity [21], insulin resistance during aging [17], and inflammation during aging [14,16]. These outcomes provide important insight into ghrelin action *in vivo*. Still, they do not provide the mechanistic processes behind these positive outcomes, which will help better assess where therapeutics can impact these chains of events. Recently a GHS-R, and more specifically GHS-R1a, has been proposed as a negative regulator of thermogenesis and browning of adipose tissue [16,22]. It is now known that GHS-R expression in adipose tissue increases with age [16,23]. On the other hand, the myokine irisin is known to positively regulate thermogenesis and browning of adipose tissue [24], and circulating irisin is negatively correlated with age [25,26]. Despite this tentative connection, there is no documented link between GHS-R, irisin, and the positive outcomes observed in skeletal muscle when the balance favors irisin. The purpose of this aim is to provide a potential mechanistic connection between GHS-R, irisin, and muscle health during

advancing age. We hypothesized that global ablation of GHS-R and the positive muscular outcomes observed are obtained through a concomitant rise in circulating irisin. This implicates a regulatory mechanism between GHS-R and irisin in skeletal muscle that is negatively altered during muscle aging.

The following chapter will introduce the reader to ghrelin, its isoforms, and its known function in metabolism and the metabolic dysregulation observed with advancing age and T2D. Chapter 3 will provide information related to the complexity of metabolic dysregulation in skeletal muscle with T2D in order to set up the experiments involved in chapter 4. Chapter 4 will provide experiments designed to determine the direct role of ghrelin isoforms on skeletal muscle using skeletal muscle myotubes, in culture. To address the possible role of the ghrelin receptor in skeletal muscle metabolism during advancing age chapter 5 will provide experiments using a genetically modified ghrelin receptor knockout mouse model. The final chapter will provide conclusions based on the experimental outcomes.

2. GHRELIN LIT REVIEW

Due to the multifactorial and complex nature of the metabolic dysfunction associated with both advancing age and type 2 diabetes (T2D), it is unlikely that one single factor will prove to be the causative culprit. Despite this acknowledgment, the search for compounds or cascades within the body that can alter the sequence of events that determine the advancement of these conditions is still the research equivalent of the holy grail. Ghrelin has been put forward as a possible pharmacological intervention or signaling target that could alter many diseases' negative attributes, including diabetes and the adverse outcomes associated with advancing age.

Ghrelin is a 28 amino acid peptide mainly produced by P/D1 mucosal cells of the stomach [27–29] but can also be produced in other tissues, including epsilon cells of the pancreas [30] and other tissues, including immune cells [28]. Similar to insulin, ghrelin is produced as a preproghrelin (Figure 2.1) that can then be acylated by ghrelin-O-acyltransferase (GOAT), and either acylated or unacylated forms of proghrelin are cleaved into acylated ghrelin (AG) or unacylated ghrelin (UAG). The acylated form can influence growth hormone secretion via its receptor growth hormone secretagogue receptor 1a (GHS-R1a) and has been directly linked to increased food intake [31,32], which is how it became known as the hunger hormone.

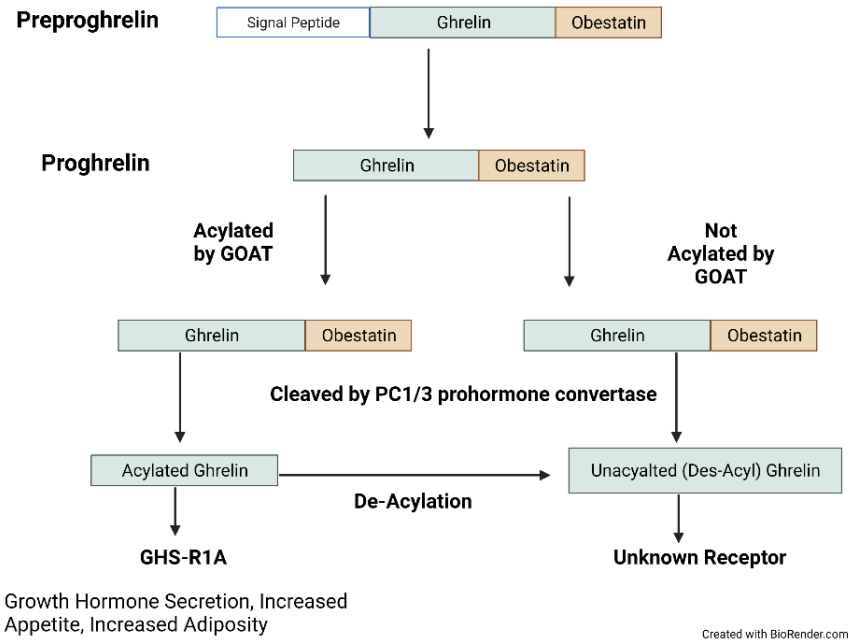

Figure 2.1 Ghrelin production, acylation and de-acylation.

While the promising nature, particularly of UAG, is hard to dispute, the mechanistic understanding of ghrelin in the body is clouded by its complex, overlapping signaling that confounds the understanding of where these positive outcomes originate. Skeletal muscle and the metabolic dysfunction that occurs take center stage in the negative effects associated with advancing age and T2D. Ghrelin has been suggested as a potential target to alter these cascades despite the limited understanding of how ghrelin imposes actions on skeletal muscle. *In vivo* work, demonstrating the most promising role of ghrelin with T2D and aging, are complicated by the involvement of ghrelin and its known receptor on a number of other organs that could then indirectly but

concomitantly act on skeletal muscle. It is therefore critical to determine the efficacy of ghrelin on what is most likely the primary instigators of these diseases, skeletal muscle.

It is now understood that the biological function of ghrelin is not dependent solely on the acylated form nor the interaction of that form with GHS-R1a [33]. That discovery expands the role of ghrelin within the body considerably; however, it also complicates the understanding of ghrelin action and its therapeutic potential. Despite this, ghrelin has been suggested as a potential therapy for multiple disease states, including T2D [34,35], cachexia [10], and sarcopenia [11,36]. In some cases, these suggestions were based mainly on its GHS-R-growth hormone axis [17], but in others, the mechanistic understanding of why ghrelin could potentially ameliorate symptoms or disease was left unresolved [35–37].

2.1. Regulation of Acylation and Acylated Ghrelin Action

Ghrelin is secreted primarily from the stomach [38], and ~95% of circulating ghrelin is UAG. Unacylated ghrelin circulates as a free peptide, while AG circulates bound to lipoproteins [39,40]. While the two isoforms are consistently discussed in tandem, it is now known that the two forms of ghrelin have different metabolic and clearance rates [41] as well as unique but overlapping actions in various tissues.

2.1.1. Ghrelin Circulation

Secretion of ghrelin, mainly represented by its unacylated form, reflects gastric production and variation throughout the day [42,43]. The synthesis and secretion of ghrelin increase with fasting and decreases with feeding [42,44], and the postprandial suppression of ghrelin is proportional to caloric load [45,46]. This implicates that

nutritional status and food intake affect the production and secretion of ghrelin which connects ghrelin back to one of the most promising lines of inquiry in healthy aging, caloric restriction. Caloric restriction alters both the secretion of ghrelin, increasing total ghrelin with chronic caloric restriction, and the presence of its receptor GHS-R1a in the pituitary [47,48], where the feeding aspect of ghrelin action is controlled. Caloric restriction is linked to positive changes with advancing age and diabetes, and because of this rise in ghrelin, the hormone has been suggested as a caloric restriction mimetic [48].

In addition to food intake itself, other signals related to nutritional status can impact ghrelin levels. Insulin, glucagon, oxytocin, somatostatin, dopamine, glucose, and dietary long chain fatty acids (LCFA) have all been shown to regulate ghrelin secretion through direct interaction with ghrelin-producing cells [49–53]. Despite these diverse influences, unacylated ghrelin concentration is folds higher than acylated ghrelin in fed or fasted states regardless of interventions [54]. The synthesis of total ghrelin appears to be controlled entirely separately from the secretion of AG [55], likely based on factors that mediate ghrelin transcription. In contrast, the ghrelin-GOAT axis that synthesizes AG specifically is sensitive to dietary fat intake [56,57]. Therefore, understanding the regulation of GOAT could be just as critical to understanding ghrelin in circulation as the knowledge of total ghrelin synthesis.

2.1.2. Ghrelin-Ghrelin-O-Acyltransferase Axis

As its name suggests, GOAT is an acyl group transferase-like enzyme that belongs to the membrane-bound O-acyltransferase family [58,59]. The production of AG via GOAT is a post-translational modification at ser3 with a medium-chain fatty acid

[60]. Ghrelin is usually modified with the 8-carbon fatty acid octanoate; however, humans can use many fatty acids to octanoylate ghrelin [61]. This enzyme appears to be the only way AG is formed, as demonstrated by GOAT-deficient mice, which completely lack octanoyl and decanoyl (acylated) forms of ghrelin [58,62]. Like ghrelin, GOAT expression is high in the adrenal cortex, spleen, stomach, pituitary [58,60] and is found in small amounts across many diverse tissues [60,61]. Since the action of ghrelin is specific to the isoform, the regulation of GOAT directly impacts ghrelin action in the body.

GOAT function to acylate ghrelin is tightly linked to lipid supply [57], and a large portion of lipids used to acylate ghrelin are recruited from the diet [63,64]. The main ghrelin-producing cells, which also produce GOAT, are located in mucosal cells of the stomach, allowing direct access to the dietary lipid supply [51]. The length of the fatty acid used for ghrelin acylation does appear to impact metabolic effects [65], with octanoyl, and decanoyl-modified ghrelin being the optimal ligands for GHS-R1a [58]. From the diet, medium chain fatty acids can be directly incorporated into acylated ghrelin [64]. However, there is some dissent as to the impact of LCFA on GOAT expression and AG production. Several studies postulate that high incorporation of LCFA into ghrelin-producing cells will increase acylation of ghrelin [56,66] via partial beta-oxidation of the LCFA into medium chains that can be incorporated into AG. Alternatively, oleic acid, an 18 carbon LCFA, and several other LCFA have been shown to reduce acylation of ghrelin *in vitro* [67] and *in vivo* [68]. This is particularly interesting when considering the documented effects of Mediterranean diets, high in

oleic acid, that reduce body weight [67,69]. There are several reasons these discrepancies could exist. The first and most prominent being the experimental differences among the studies. Studies showing an increase in AG [56,66] used different ghrelin-expressing cell lines when compared to studies using oleic acid [67]. Furthermore, the amount of fat and other dietary contributions were also quite different among studies [56,66–68]. Lastly, there may be some tissue specificity within the ghrelin-producing cell types [56] that could impact what kinds of substrates can induce or inhibit the production of acylated ghrelin. Regardless, it is clear that not all fatty acids are created equal when it comes to GOAT. The composition of available fatty acids and the amount of dietary fat could impact the circulating levels of acylated ghrelin. This is critical knowledge, especially in experimental models of diabetes where high-fat diets are used because the composition of the high-fat diet may impact circulating acylated ghrelin.

Due to both the location of GOAT and ghrelin producing cells and their actions, the GOAT-ghrelin system is considered a nutrient sensor [55]. Caloric restriction decreases GOAT expression leading to reduced plasma acyl ghrelin [63,70] which is directly opposite to the total ghrelin levels mentioned earlier during caloric restriction. This was further supported by GOAT-null mice, that not only demonstrated an increase in total ghrelin, but specifically unacylated ghrelin [61,63]. Additionally, diets high in medium-chain fatty acids increased the acylation of ghrelin because they are readily absorbable, which then subsequently serves as signals to the brain via GHS-R1a that high caloric food is available [63]. Diet influence may not be the only regulating factor

for GOAT. It is now known that insulin can inhibit GOAT expression by elevating the mTOR pathway in pancreatic cells that produce ghrelin [24]. The mTOR pathway is also responsible for inhibiting GOAT expression and the production of AG in gastric mucosal cells [71]. Despite this knowledge, much of the *in vivo* work that alters circulating ghrelin levels over time either through genetic or exogenous means do not consistently measure acylated or unacylated ghrelin over the experimental time frame. The hallmark of metabolic dysregulation with T2D is alteration of lipid availability concomitant with alteration in insulin secretion. It is troubling that the current work surrounding ghrelin influence in these two models did not verify ghrelin levels throughout the study and what influence their experimental diets or models had on ghrelin levels, making outcomes from these studies difficult to interpret.

2.1.3. De-acylation of Ghrelin

Ghrelin's ability to interact with its known receptor depends on the post-translational modification that creates acylated ghrelin. As discussed above, the activity of this acylated form is dependent upon GOAT for production but is also reliant on its biological half-life. It is known that ghrelin can be broken down in circulation and tissues through different mechanisms [39,72]. In serum, various enzymes, including platelet-activating factor acetylhydrolase [73], carboxypeptidase, and cholinesterase [73,74], exhibit ghrelin deacylase activity. Several different enzymes are not necessarily distinct to the serum that de-acylate ghrelin, including butrylcholinesterases [75,76] and carboxylesterases [39]. More recently, Notum, a member of the alpha/beta hydrolase superfamily, known as a Wnt antagonist [77], has been suggested as a potential de-

acylator of ghrelin. Notum is produced in the liver and then distributed in the bloodstream and deacylated similarly to butrylcholinesterases [75,78]. Considering the various and sometimes critical functions acylated ghrelin has, the half-life in circulation is relatively short, ranging from 30 min in rats to 240 min in humans [39]. This short half-life and those of production mentioned above could help explain some of the dissention of findings in the literature since many of the papers do not record ghrelin levels throughout the experiments. With potential for alteration from both secretion patterns and de-acylation, it is no wonder there is dissension in the literature, and much of the differences might be due to discrepancies in the ghrelin levels of the animals.

2.1.4. GHS-R1a

Despite the short half-life in both rats and humans, acylated ghrelin's interaction with GHS-R1a is critical to components of its biological action. The interaction of AG and its receptor is associated with food-seeking, and reward [79,80], increased fat storage [81], and control over hepatic glucose production [82]. While some of these effects are linked to growth hormone secretion, many appear independent of the growth hormone axis. Interestingly, fasting-induced an 8-fold increase in GHS-R mRNA levels [79] implicating GHS-R1a and/or the AG-GHS-R1a interaction are essential in homeostatic maintenance during an energy deficit. This G protein-coupled receptor is present in the pituitary, hypothalamus, thyroid, pancreas, spleen, myocardium, and adrenal glands [28], with the pituitary having the highest mRNA content [27]. GHS-R1a in muscle is more controversial; however, most sources agree that if the ghrelin receptor is present in skeletal muscle, it is in small quantities [23,27,28].

GHS-R1a knockout models in diet or genetically induced obesity highlight the need for a better understanding of ghrelin signaling. Mice lacking the ghrelin receptors and provided with high-fat diets have lower body weight and food intake and have been touted as being resistant to the development of diet-induced obesity [21,83]. Additionally, these models were reported to have higher insulin sensitivity than their wild-type counterparts with lower plasma insulin levels and trends toward better glucose tolerance. The measures of glucose tolerance in these studies implicate that much of the improvements come from an increase in glucose-stimulated insulin secretion and the return of the primary phase of insulin rather than a rescue of peripheral insulin sensitivity. While the return of preliminary phase insulin secretion implicates potential alterations to pancreatic glucose sensitivity, increased insulin secretion is a natural tendency of the body during peripheral insulin resistance. It does not necessarily represent a potential reversal of insulin resistance, the hallmark of the disease. Contrary to those findings, GHS-R knockout in leptin-deficient mice worsened hyperglycemia with reduced plasma insulin and conjectured that this was due to a lack of insulin secretion [84]. They argued that the ablation of GHS-R impacted insulin secretion but had no impact on insulin sensitivity, casting further doubt on the reported positive outcomes from earlier research. It is important to note that the discrepancies between these studies could be explained, in part, by the difference in knockout backgrounds between an ob/ob leptin-deficient model and other models. Leptin involvement in these differences has been examined to a limited degree [17,84], but its definitive role on ghrelin action is undetermined. At first glance, it appears that there is a negative

connection between ghrelin signaling through its receptor, with obesity, and/or insulin sensitivity, but it is important to note that GHS-R1a is constitutively active [85], so it may not require ghrelin for the observed adverse effects, and GHS-R1a is either not expressed or in low quantity in the majority of peripheral tissues that play a role in peripheral insulin sensitivity like that of skeletal muscle. Because of the lack of studies assessing direct evidence of action we are left with speculation about how the removal of GHS-R1a or ghrelin signaling serves to increase insulin sensitivity in peripheral tissues.

2.1.5. Ghrelin Action

As previously stated, ghrelin has many actions within the body. Many of the actions relate to dysregulated systems in disease like glucose metabolism, inflammatory response, and adipose tissue accumulation. Its primary function is in the brain, leading to an increase in growth hormone secretion in response to caloric restriction or fasting. This action is guided by, but not dependent on, the GHS-R-ghrelin axis in the hypothalamus, leading to increased appetite [55,56] and food-seeking [31]. While it is best known for its influence on the brain [86], ghrelin also has a presence and activity in other organs. Ghrelin directly impacts glucoregulatory processes not only by the aforementioned reduction of glucose-stimulated insulin secretion from the pancreas [59,60] but also by increasing glucose output in hepatocytes [87]. In addition to glucose output, ghrelin is also purported to promote autophagy and induce beta-oxidation in the liver [82]. Additionally, the ghrelin signaling cascade is reported to reduce inflammation via a reduction of inflammatory cytokines like TNF-alpha [88], but this effect is only evident with UAG [5,14], and the influence of the ghrelin receptor may be opposite [16].

Ghrelin also affects adipose tissue and adipose accumulation [16]. It is clear that ghrelin has diverse functions across a wide variety of tissues. We concede that these other organs could be contributing to the observed outcomes in skeletal muscle. This dissertation focused on whether or not the aforementioned outcomes by ghrelin with aging or diabetes were a direct result of the hormone on skeletal muscle.

2.2. Ghrelin and Skeletal Muscle

While studies disagree on whether AG and UAG act similarly in skeletal muscle, the consensus is that ghrelin plays a role in muscle metabolism [11,34–37]. However, it is unclear from the current literature whether this role is due to a direct action of either isoform on skeletal muscle, some form of organ cross-talk, or a combination of the two. This section will explore what is currently known about ghrelin action in skeletal muscle, explicitly focusing on signaling cascades responsible for anabolic and glucoregulatory processes. The current literature mainly focuses on glucose homeostasis during insulin resistance and cascades responsible for atrophy in disease states.

2.2.1. Ghrelin and Glucose Uptake

Data collected regarding the role of ghrelin in whole-body glucose homeostasis is divisive at best but has provided insight into the unique and potentially oppositional actions of the two isoforms of ghrelin. In diabetic and insulin resistance models, the outcomes of the two isoforms of ghrelin begin to diverge quite strikingly. Administration of exogenous AG has been linked to weight gain, hyperglycemia with increased food intake, and fat deposition [89,90], leading to the conclusion that it is a diabetogenic hormone. On the other hand, UAG does not appear to impact food intake or

fat deposition [91] and lacks many of the hallmarks of negative glucoregulatory signaling. Unfortunately, while the current literature provides us with only a cursory glance at signaling molecules, those studies varied in experimental design, animal models, and outcomes measured, making it hard to create a complete picture of ghrelin's impact on skeletal muscle.

Several signaling molecules (Figure 2.2) can potentially give us a better understanding of skeletal muscle response to altered ghrelin signaling, but the primary focus to date has been on the phosphorylation of protein kinase b (AKT) and AMPK with downstream targets of these signaling cascades sometimes, but not always explored. Both AKT and AMPK have links to the mechanistic target of rapamycin complex 1 (mTORC1) regulation. AMPK phosphorylates tuberous sclerosis complex 1/2 (TSC1/2) and Raptor, which inhibit mTORC1 activity [92]. Inversely, AKT activates mTORC1 through phosphorylation of TSC1/2 and phosphorylation of PRAS40 [93,94].

Hyperactivation of mTORC1 has been demonstrated as a hallmark of insulin resistance in our work in obese skeletal muscle [95], and its downstream substrate, ribosomal protein s6 kinase 1 (S6K1), phosphorylates insulin receptor substrate 1 (IRS1) in an inhibitory fashion, which negatively impacts insulin sensitivity [96,97]. More detail of this connection of anabolic dysregulation and mTORC1 will be explored in chapter 3.

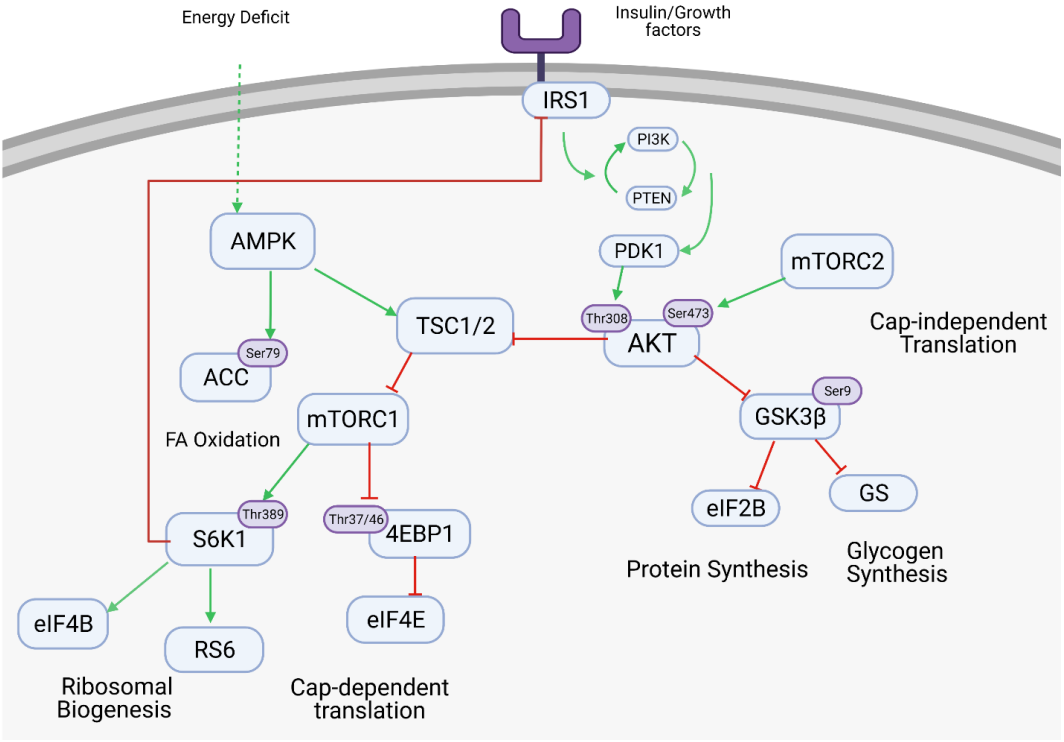

Figure 2.2 Skeletal Muscle Signaling Cascades involved in Glucoregulatory and Anabolic Processes.

2.2.2. Protein Kinase B (AKT)

Phosphorylation of AKT at ser473 is often used as a marker of insulin signaling in skeletal muscle due to AKT's influence on glycogen synthase through glycogen synthase kinase 3 beta (GSK3β) [98], and glucose transporter type 4 (GLUT4) translocation through AKT substrate at 160kDa (AS160) [99]. Previous literature has also suggested that AKT phosphorylation at ser473 is required for complete activation of AKT [100], but the evidence to support this notion is lacking. A rescue of AKT signaling through phosphorylation of ser473 is often interpreted as a rescue of insulin signaling even though phosphorylation of ser473 is accomplished by mTORC2, which does not have convincing evidence of direct insulin action. However, upregulation of

AKT phosphorylation at ser473 has been the most notable and consistent response to ghrelin treatment in models of both atrophy and T2D [10,34–37,101]. In models of T2D [34,35] and chronic kidney disease [101], the phosphorylation of AKT ser473 was credited with the observed increased insulin sensitivity and glucose uptake; however, this effect only manifested with UAG. When the experimental design was shifted towards atrophy, and atrophic environments rather than insulin resistance, the rescue of ser473 occurred with both AG and UAG [10,11,36]. mTORC2 is associated with autophagy through the AKT cascade and is also permissive of cap-independent translation. Consistent with elevated autophagy, phosphorylation of AKT on ser473 was accompanied by decreased markers of proteasomal degradation MURF and MAFBX [36] and phosphorylation of forkhead box protein 03a (FOX03a), which increases FOX03a retention in the cytoplasm and decreases transcription of the previously mentioned atrophic proteins. This ghrelin-AKT-FOX03 signaling cascade is currently implicated in the reduced apoptosis and improved muscle mass maintenance observed with ghrelin treatment in experimental models of atrophy [10,11,36]. The purported influence of ghrelin on AKT on serine 473 and downstream FOX03 only occurs using a chemically-induced model of skeletal muscle atrophy and not in a healthy state, but whether the deficit is a driving force for the response to ghrelin is unknown. It is also concerning that the disease state of the experiment can dictate a division of response between the two isoforms. Skeletal muscle has little presence of GHS-R1a [23], and both experimental models observed lowered phosphorylation of AKT before introduction of exogenous ghrelin. While some differences might be explained through

experimental design and/or lack of monitoring circulating ghrelin concentration throughout the experiment, the evidence of why these two perturbed states cause such a division of action, despite both having lowered phosphorylation of AKT has yet to be addressed.

2.2.3. Ghrelin and Autophagy

It is clear that the dominant signaling pathway of ghrelin action in skeletal muscle is mTORC2-centric. Using an *in vitro* model of uremia in C2C12 myotubes, it was discovered that silencing of autophagy related gene 5 (ATG5), a necessary component in autophagy, decreased the mTORC2-dependent phosphorylation of AKT ser473 observed with UAG administration [35,101], suggesting a role of autophagy in the beneficial outcomes of exogenous ghrelin. While the role of autophagy in skeletal muscle is still controversial [102], autophagy has been implicated as a player in homeostatic imbalances found in T2D [103,104]. AMPK is well known for its inhibitory action on mTORC1 its involvement in mitochondrial biogenesis and autophagy. AMPK positively regulates autophagy through mTORC1-dependent and -independent processes, leading to upregulation of unc-51 like autophagy activating kinase 1 (ULK1), strongly suggesting that AMPK could be the driving force behind UAG and the phosphorylation of AKT at ser473. UAG administration increased AMPK phosphorylation and AKT ser473 in skeletal muscle of diabetic mice [34]. Additionally, inhibitory phosphorylation of IRS1 at ser307 was reduced in response to UAG administration and is purported as a mechanism for the positive changes in insulin sensitivity [34]. It appears that upregulation of AMPK has a positive impact on a

diabetic model with increased autophagy through ULK1 and a reduction in mTORC1 signaling cascade, which is known to inhibit the canonical insulin signaling cascade. This implicates that UAG positively impacts the metabolic dysregulation of skeletal muscle through both anabolic and glucoregulatory means. While the phosphorylation of AKT on ser473 is strongly suggestive of a primary role of ghrelin on mTORC2, there is currently little information about ghrelin's influence on mTORC1 and the anabolic signaling cascade. The few studies that measured anabolic markers found that UAG and AG resulted in no changes in muscle mass or anabolic signaling [10,36] but both of these conclusions were made based on models of atrophy, which lack the hallmark upregulation of mTORC1 observed in insulin resistant skeletal muscle.

The complexity of ghrelin influence in the body and the intertwined signaling that occurs makes the current knowledge base both intriguing and muddling. While it appears from the most basic interpretations that AMPK signaling could be a responsible factor for ghrelin action in skeletal muscle, this promise is overshadowed by the other organs of the body where ghrelin actions are better defined. While ghrelin may act directly on skeletal muscle, it is equally likely that ghrelin actions in other organs set off a series of events that lead to responses in skeletal muscle. Despite our lack of understanding whether ghrelin directly or indirectly exerts an influence on skeletal muscle, it doesn’t necessarily negate the concept that UAG could positively affect some disease states, especially if it has no known harmful effects on glucose metabolism.

2.3. Ghrelin Action with advancing age and development of T2D

While changes observed with advancing age and the development of T2D are two distinct cascades of events, the behavior of ghrelin during the processes is remarkably similar. In young, healthy, normal-weight individuals, there is a well-established postabsorptive rise of ghrelin that continues to rise with prolonged fasting [49] followed by a decrease to baseline after a meal [42,105] which implicates a role for ghrelin in maintaining energy homeostasis. Total ghrelin levels with advancing age [86] and T2D [87–89] are lower than their younger or healthier counterparts. They also lack the ebb and flow associated with the nutrient-sensing characteristics of ghrelin in healthy individuals [18,19]. Interestingly, despite the decline of total ghrelin, circulating levels of AG are somewhat maintained [18,106]. While the literature agrees that ghrelin's impact on the brain, specifically its orexigenic impact, is dysregulated during these diseases, its role in energy homeostasis might stem from a completely different metabolic process.

Although the connection between ghrelin and AMPK signaling may be tenuous, that connection may offer an intriguing yet unexplored avenue related to muscle: thermogenesis. Despite the ongoing debate of where in the body ghrelin acts, skeletal muscle may play a role regardless of the directness of ghrelin action on it due to its involvement in certain aspects of thermogenesis and signaling molecules involved in that process.

2.3.1. Altered Thermogenesis with advancing age and T2D

Energy homeostasis is altered with advancing age and T2D, with increased adipose tissue deposition being noted with both progressions. Concurrent with an increase in adipose tissue, metabolic thermogenesis is decreased and is specifically linked to loss of brown adipose tissue (BAT) mass and activity [107]. While the influence of thermogenic activity and brown adipose tissue mass in humans is debated [108], disruption of thermogenesis has been clearly linked to obesity in humans and animal models [107,109]. That being said, BAT and the process of browning/beiging is currently believed to occur in humans [110,111], so it cannot be discounted as a potential player in energy expenditure and the thermogenic contribution to metabolic regulation.

The cascade of events that leads to increased thermogenesis is generally believed to be through a combination of increased activation of adrenergic receptors via norepinephrine and subsequent increases of uncoupling protein 1 (UCP1) in brown adipose tissue, which leads to increased mitochondrial proton leakage and an increase in heat production (Figure 2.3).

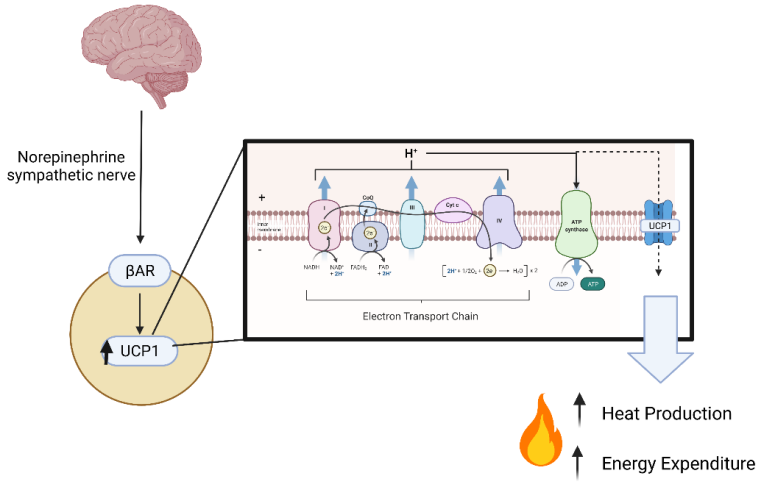

Figure 2.3 UCP1 influence on Thermogenesis in Brown Adipose Tissue

UCP1 content is reduced with aging or diabetic models, with an overall reduction in thermogenesis regardless of nutrient availability [107,112]. With ghrelin now purported to be an essential regulator of energy homeostasis, it is logical that there is some investigation into the role ghrelin plays in this phenomenon. Current research has confirmed that hypothalamic neurons expressing ghrelin reactivity can participate in the control of core body temperature and central control of thermogenesis [113], supporting the potential for ghrelin to be involved in thermogenic activity. Acylated ghrelin increases lipid accumulation [90,95,96] and suppresses norepinephrine release in BAT [114,115]. Additionally, chronic ghrelin treatment lowers UCP1 expression in BAT [17], suggesting that ghrelin signaling inhibits thermogenesis, but the authors could not specify whether those negative actions were through GHS-R. However, with GHS-R

ablation, the negative impact of ghrelin on thermogenic capacity seems to be abolished entirely [107] even though both isoforms of ghrelin are still in circulation [17]. This strongly supports a theory that ghrelin signaling, specifically through its receptor, is responsible for this inhibition (Figure 2.4). GHS-R ablation is also reported to increase UCP1 in an AMPK-dependent manner, resulting in less body fat accumulation independent of energy intake [107]. GHS-R expression in BAT increases with age in wild type animals [107] which correlates with the reduction in thermogenic activity and supports the idea that ghrelin and its receptor are integral parts of this process.

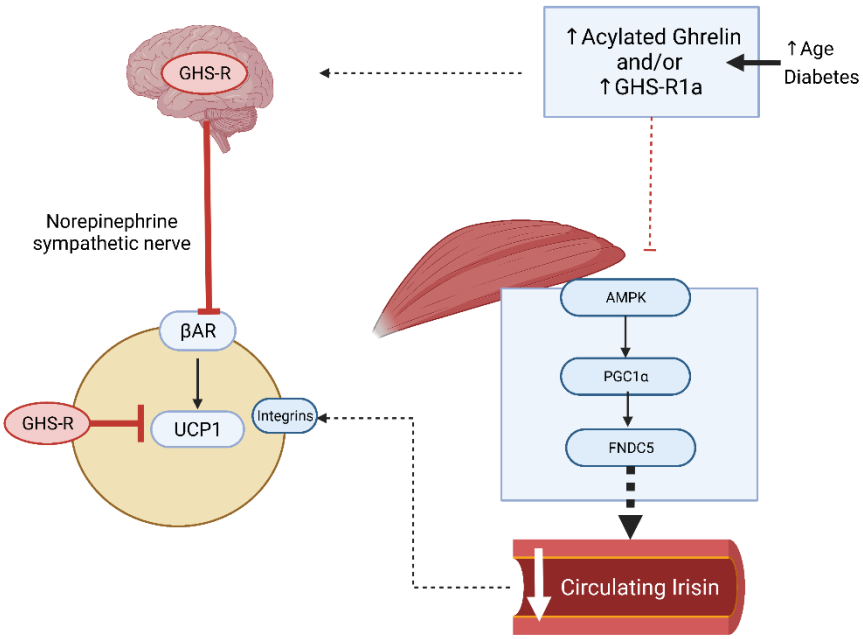

Figure 2.4 Proposed Mechanisms by which Ghrelin Signaling impacts Thermogenesis.

2.3.2. Thermogenesis and Irisin

Recently another hormone called irisin, has been proposed to be an alternative but not necessarily separate culprit in the regulation of thermogenesis and restoration of metabolic homeostasis with advancing age or obesity. Irisin was initially thought to be a myokine and is known for its increased release post-exercise [116,117]. Irisin is cleaved from fibronectin type III domain-containing protein (FNDC5) whose expression is controlled via peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PGC-1α) [118]. PGC-1α has a crucial role in skeletal muscle at the transcriptional level, specifically increasing genes related to mitochondrial biogenesis and oxidative phosphorylation [117], and is now known to control the expression of FNDC5. Irisin's relationship to exercise is through upregulation of AMPK activity, which activates PGC-1α and the subsequent cascade of mitochondrial biogenesis and irisin production. The increase of irisin post-exercise is now believed to be partially responsible for the elevation of thermogenesis during aerobic exercise [117]. Recently FNDC5 and subsequent irisin release have been identified in adipose tissue with similar induction via exercise [118], implicating that irisin may not be exclusively a myokine.. However, skeletal muscle is still considered the primary source of irisin in circulation [119]. Both upregulation of FNDC5 and exogenous irisin have been reported to reduce metabolic derangements and insulin resistance associated with diet-induced obesity [118], similarly to those observed with aerobic exercise [118,120]. Recently those positive outcomes were linked to the contribution of thermogenesis and energy expenditure observed with overexpression of FNDC5 in obese mice [118].

Irisin's ability to modulate the thermogenic pathway through UCP1 is well characterized [120–122]. The production and release of irisin by FNDC5 is believed to have both autocrine, paracrine and endocrine actions in skeletal muscle and adipose tissue, with increased heat production, oxygen consumption, and browning of adipose tissue [120–122]. The similar outcomes of exogenous irisin and GHS-R ablation begs the question of whether or not these two phenomena are related in some way (Figure 2.5). Intriguingly, in a study by Lee et al. [122], hypothalamic-specific GHS-R ablation also protected against diet-induced obesity, and strongly implicated that hypothalamic GHS-R plays a role in the central action on thermogenesis without a need for peripheral signaling. However, that study did not measure skeletal muscle involvement either through FNDC5 expression or irisin in circulation, which could help determine whether skeletal muscle is required for the positive outcomes of GHS-R ablation on thermogenesis. While our current understanding is incomplete, there is an argument for skeletal muscle involvement in this positive modulation of thermogenesis either through ghrelin's direct action on AMPK signaling leading to an upregulation of FNDC5 or indirectly through brain activity that alters FNDC5 expression and irisin production in skeletal muscle.

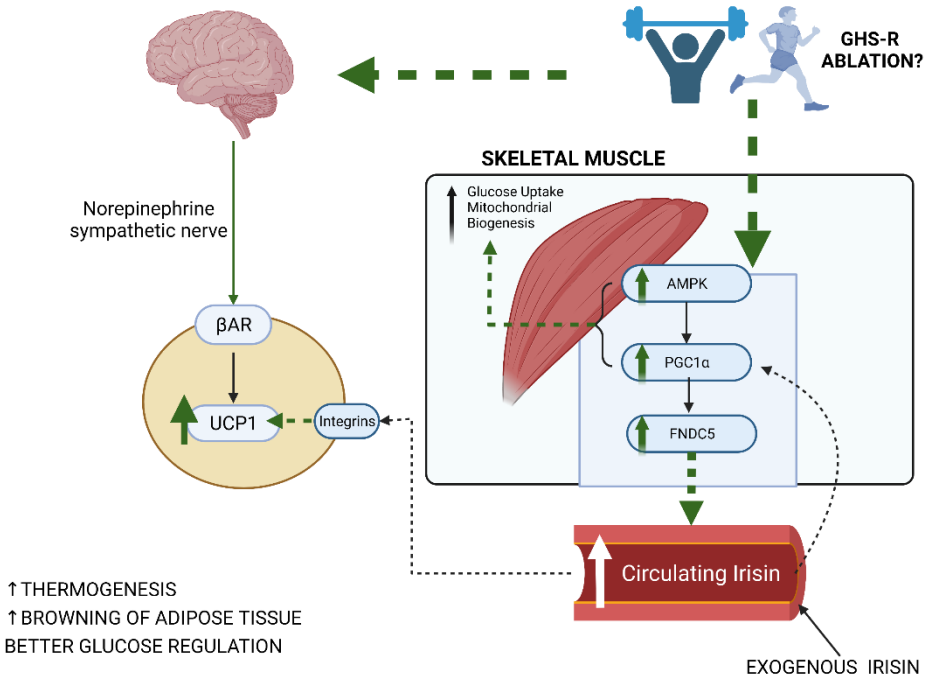

Figure 2.5 Proposed Mechanisms of Positive outcomes associated with Exercise and GHS-R ablation.

2.4. Conclusions

The ghrelin signaling cascade is complex and dynamic, making it difficult to understand ghrelin's potential as a therapeutic target. Specifically, UAG shows promise in a variety of conditions, but the impact of either isoform on skeletal muscle is neither concrete nor convincing. While the role of UAG *in vivo* may be beneficial for diseases like sarcopenia and T2D , the lack of understanding between ghrelin and skeletal muscle cannot be ignored if we are to design therapeutic strategies using the hormone. The metabolic dysregulation of skeletal muscle is not just a hallmark of both diseases; it is arguably the critical initiating factor for the design of therapeutic targets that directly promote positive outcomes. Given the complexity of ghrelin interactions on various

tissues and outcomes more research is necessary to directly assess where these actions are influencing whole body homeostasis. Studies from this dissertation were designed to specifically and directly assess the role of ghrelin on metabolic outcomes in skeletal muscle.

3. CULPRITS OR CONSEQUENCES: UNDERSTANDING THE DYSREGULATION OF MUSCLE METABOLISM IN DIABETES*

3.1. Introduction

Globally, 462 million individuals are affected by type 2 diabetes (T2D) and it is ranked as the 9th leading cause of mortality [123]. The prevalence of diabetes over the past few decades has continued to rise with no sign of this changing [123]. T2D is characterized by insulin resistance and hyperglycemia and can lead to various other outcomes and comorbidities reducing quality of life in those effected. While the pathogenesis and progression of T2D is still widely debated, it is clear that a complex interplay between the pancreas and peripheral tissues is dependent for maintenance of glucose homeostasis. Peripheral tissues account for 80-90% of glucose disposal [124,125] and of those tissues skeletal muscle is a large contributor to glucose disposal [126,127] and arguably the most important for glucose clearance [128,129]. Within skeletal muscle there is clear link to metabolic dysregulation during the progression of T2D, but the definition of causes versus consequences within the development of this disease is difficult. Identifying clear relationships, interactions and feedback loops within the insulin signaling cascade and other metabolic pathways in skeletal muscle is imperative to our understanding for the development, its progression and ultimately a cure for this disease. To that end, this review will present the canonical understanding of insulin signaling, the influential connections between the mechanistic target of

rapamycin complexes (mTORC1 and mTORC2) and the current intertwined implications of these signaling paradigms in skeletal muscle metabolic dysregulation.

3.2. Insulin Signaling

The insulin signaling cascade involves both glucoregulatory and anabolic processes which is outlined in Figure 3.1. Insulin responsive tissues have insulin receptors (IR) on the cell surface plasma membrane. These IR contain subunits where insulin can bind as well as residues that provide docking sites for downstream signaling molecules including the insulin receptor substrates (IRS). The two predominant insulin receptor substrates are IRS1 and IRS2 with similar sequences but specific signaling roles [130,131]. IRS1 appears to be the insulin receptor substrate protein whose primary responsibility is glucose regulation, including glucose transporter type 4 (GLUT4) translocation [130] with speculation that IRS2 is more involved with fatty acid metabolism, currently known to occur in adipose tissue [131]. IRS1 is a clear mediator of insulin signaling through a specific intermediate phosphatidylinositol 3 kinase (PI3K). Interaction of PI3K to IRS produces membrane phosphatidylinositol 3,4,5-triphosphates (PIP3) which is necessary for the recruitment and localization of protein kinase B (AKT) [132].

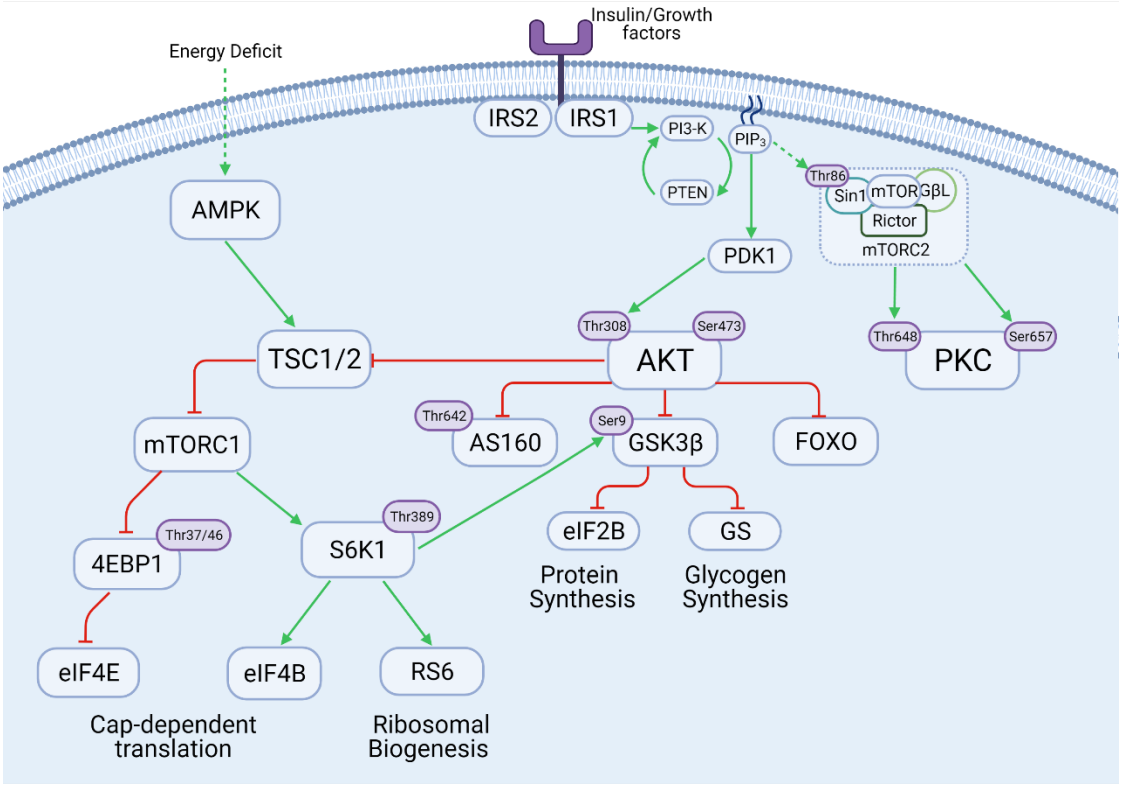

Figure 3.1 Insulin signaling cascade involving both glucoregulatory and anabolic pathways. Phosphorylation sites of interest indicated on figure. Green arrows (→) indicate activation of the substrate, red bars (⊥) indicate inhibitory action on the substrate. Figure created with BioRender.com

3.2.1. Upstream Glucose Related Substrates

This serine/threonine kinase is part of the AGC protein family and is known for its diverse function in growth, survival, proliferation and most importantly substrate metabolism [133–135]. AKT is often referred to as one molecule but actually comprises of three distinct isoforms (AKT1, AKT2, AKT3). While all isoforms are present in skeletal muscle, AKT2 is the most prevalent [134], but varies from low to immeasurable amounts in skeletal muscle [136,137]. While defining the variation and overlap between the AKT isoforms is important and needed, it is beyond the scope of this review but

what is known currently can be found in these reviews [134,138]. It is important to note that AKT2 is expressed primarily in insulin responsive tissues like fat and skeletal muscle and is the most abundant isoform in skeletal muscle [136,137,139,140]. AKT is as a critical regulator of insulin sensitive glucose uptake as well as anabolic signaling through mTORC1 making it a prime target in understanding metabolic dysregulation. The upstream regulation of AKT, in its most simple iteration, appears to be very similar across isoforms. The two common phosphorylation sites of AKT are ser473 (Ser474 in AKT2) and thr308. The insulin receptor substrates IRS1 and IRS2 will activate the PI3K-dependent conversion of PIP2 to PIP3, and PIP3 will recruit Pyruvate Dehydrogenase Kinase 1 (PDK1) and AKT to the membrane where colocalization will allow for phosphorylation at the thr308 by PDK1 [134,135]. Further, some evidence suggests that mitogen-activated protein kinase-associated protein 1 (mSIN1) of the mTORC2 complex is brought to the membrane by PIP3 (binding with the pH domain) that promotes colocalization of mTORC2 to the membrane [141,142], which is the major kinase for the ser473 phosphorylation site of AKT.

The regulation of mTORC2 activity by mSIN1 phosphorylation is controversial. It has been proposed that PI(3,4,5)P_3) promotes mTORC2 activity directly [143,144]. Recent work has indicated a positive feedback loop between AKT and mTORC2 via phosphorylation of mSIN1 [145,146]. Those studies in adipocytes and Hela cells indicated that phosphorylation of mSIN1 at thr86 by AKT (via thr308) increased mTORC2 activity and phosphorylation of AKT on ser473 [142,145]. This positive feedback loop provides an avenue for mTORC2 control via growth factors; however, the

total impact of this feedback loop on mTORC2 activity and downstream substrates like AKT ser473 is currently unknown. It is well established that PDK1 and mTORC2 are the major kinases involved upstream of AKT and that AKT is involved in a large scale, insulin sensitive pathway, but the distinct actions of these two phosphorylation sites are still not well understood.

There is also considerable debate over what the phosphorylation of specific AKT sites implicates for AKT activity and substrate specificity. Much of the early work in AKT reported a requirement of phosphorylation at ser473 for full activation [100,147–149]. However, more recent work in platelets [150], HEK cells [148,151], and skeletal muscle [152,153] demonstrated that not all downstream substrates are impacted by ser473 phosphorylation. There is some evidence to support that these changes in activity and substrate via phosphorylation site may be isoform specific [98,154] but more work needs to be done in this area.

The implications of ser473 phosphorylation via mTORC2 has been studied in various tissues. In mSIN1 knockout mouse embryonic fibroblasts, a regulator of mTORC2 complex formation and stability, forkhead box 01/03 (FOX01/3a) phosphorylation was inhibited but tuberous sclerosis complex 2 (TSC2) and glycogen synthase kinase 3 (GSK-3) phosphorylation was unaffected [155]. In adipose tissue [156] and liver [157], rapamycin insensitive companion of mammalian target of rapamycin (RICTOR) knockouts demonstrated tissue specific differences in mTORC2 substrate specificity. When mTORC2 inhibitors were applied in skeletal muscle, phosphorylation of AKT at thr308 was unaffected and the downstream phosphorylation

of TSC1/2, ribosomal protein S6 kinase beta-1 (S6K1) and GSK3beta, all associated with protein synthesis and growth, were also unaffected by the reduction of Ser473 phosphorylation [153]. However Akt substrate at 160 kDa (AS160), an enzyme associated with GLUT4 translocation and glucose disposal as well as proteins in the FOXO family associated with apoptosis were negatively affected by ser473 reduction [153]. That work demonstrated that there is some demarcation of substrate specificity within AKT of skeletal muscle. It may also indicate phosphorylation of thr308 focuses AKT kinase activity towards substrates involved with growth and phosphorylation of ser473 focuses on substrates involved in glucose regulation and cell survival. Alternatively, substrates unaffected by inhibition or downregulation of mTORC2 phosphorylation of AKT at ser473 may be phosphorylated by other proteins. For example GSK-3 can be phosphorylated at the same phosphorylation site that AKT does, Ser9, by S6K [158] and PKC [159]. Despite the alternative theory there is evidence for at least some context-dependent substrate specificity towards AKT's downstream targets. As for whether the activity of AKT is dependent on ser473 for full activation, a recent study in adipose tissue purports that AKT2 activity is reduced by about 50% for its substrates TSC2, PRAS40, FOX01/3a and AS160 [160]. Taken together, there may be argument for some combination of ser473 impacting substrate specificity and activity, but to our knowledge this has not been validated in skeletal muscle and would need more systematic study in both AKT1 and AKT2 to truly define this regulatory mechanism.

3.2.2. Downstream Glucose Related Substrates

As previously mentioned AKT has various downstream substrates that make the action of this kinase quite diverse in cell function. These substrates include members of the mTOR complexes Pras40 and mSIN1, glucose uptake proteins AS160 and GSK-3. Protein synthesis related TSC2, and apoptotic signaling through the FOX0 family. This section will focus on signal transduction related to glucose uptake.

GLUT4 is the predominant isoform of the glucose transporter family found in skeletal muscle, and one of insulin's primary metabolic roles is to promote the translocation of GLUT4 to the surface membrane. AKT has been linked to downstream substrates that impact insulin-dependent GLUT4 translocation including GSK-3[161] as well as AS160 [152,162,163] making it a prime target for understanding glucose uptake. GSK-3β is a well-known inhibitor of glycogen synthase, but is also an inhibitor of eukaryotic initiation factor 2B (eiF2B) which is a potent regulator of protein synthesis. When GSK-3β is phosphorylated at ser79 its activity is inhibited, which allows for the activation of both glycogen synthase and eiF2B. Interestingly GSK-3β has been linked to mTORC2 regulation via RICTOR phosphorylation at ser1235 which interferes with mTORC2 binding to AKT [164] and ser1695 [165] which marks RICTOR for degradation. AS160 is a substrate of AKT that contains a Rab-GTPase activating protein and has been associated with regulating glucose transport. In basal conditions AS160 maintains GLUT4 containing vesicles in the cytosol (intracellular) through its gap domain [166,167]. When insulin is applied AS160 is rapidly phosphorylated which disengages AS160 from the vesicles allowing them to move to the membrane for

exocytosis. In skeletal muscle, like fat [163,168], AS160 is phosphorylated in response to insulin in a dose dependent manner [169] and insulin stimulation of GLUT4 exocytosis is dependent on AS160 phosphorylation [168]. AS160 can be phosphorylated by other proteins including AMP-activated protein kinase (AMPK) making it part of both insulin dependent and insulin independent translocation of GLUT4 [152,170].

3.2.3. Anabolic Signaling

AKT phosphorylates TSC2 at thr1462 which regulates the tuberin-hamartin complex and it's activity [171–173]. Phosphorylation at this site releases the tuberin-hamartin complex inhibition of the mTORC1 complex and allows for downstream targets to be phosphorylated [171]. mTORC1 is a prolific kinase with multiple downstream substrates, but ribosomal protein S6 kinase beta-1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) are arguably the most well-known downstream targets. 4E-BP1 is known as a translation repressor protein because it inhibits cap-dependent mRNA translation by binding to eukaryotic translation initiation factor 4E (eIF4E). Phosphorylation of 4E-BP1 disrupts the interaction of 4E-BP1 and eIF4E, releasing it so that it may participate in translation by chaperoning specific cap-dependent transcripts to the translation apparatus [174]. S6K1 is best known for its action on ribosomal protein S6 (S6) which is involved in the translational control of 5' oligopyrimidine tract mRNAs [175]. Phosphorylation of S6K1 at thr389 is known to be critical for function of the protein [175], as well as correlated with kinase activity *in vivo* [176]. The subsequent phosphorylation of S6 correlates with increases in translation of cap-dependent proteins, that are necessary for the manufacture of

ribosomal machinery and peptide-chain elongation factors necessary for mRNA translation [177,178]. The regulation of S6K1 activity is diverse but S6K1 activation has been shown to be elevated by hyperglycemia [179], hyperinsulinemia [180], and high fat diet in muscle and adipose tissue [96].

3.3. Insulin Signaling and Diabetes

It is generally agreed that glucose transport is the rate limiting step of glucose uptake, and the step most impacted by the progression of T2D. The consensus in diabetes research at large is that the translocation or trafficking of glucose transport molecules in skeletal muscle is impaired in T2D [163,181] but the culprit behind this impairment is still widely debated. In skeletal muscle GLUT4 is the predominantly expressed isoform [182,183] and the localization of GLUT4 has been confirmed with insulin [184], exercise [184,185] and hypoxia [186]. The first important finding with diabetes is that the limitation in glucose transport cannot be explained by production or maintenance of the glucose transporter itself, because total GLUT4 protein is largely unchanged with T2D [187–189] . This implies that the issue is not related to GLUT4 expression, per se, but within the signaling cascades that assist in the translocation of GLUT4 to the surface membranes.

As the initial step in the insulin signaling cascade, the insulin receptor was a primary target of research related to the breakdown of the glucoregulatory signals. While current data are conflicting on IR activity with some reporting impairment [181,190,191] and others reporting normal activity [192–196], it appears that the important signaling ‘defects’ of T2D are further down the signal cascade. Signaling defects in IRS1

phosphorylation [192,196–198] and PI3K [192,196,197,199,200], activity are consistently found in the diabetic model. More controversial is the activity of AKT with studies reporting significant reductions of insulin stimulated AKT phosphorylation on ser473 or thr308 [188,194,201,202], while others report no impact of T2D on insulin dependent phosphorylation [199,200]. Downstream substrates of AKT have also been presented in the diabetic model with reduced glycogen synthase activity with protein levels of GSK-3 reported as being elevated which would inhibit glycogen synthase activity [203]. Additionally, insulin dependent phosphorylation of AS160 has also been reported to be higher in T2D [162], despite the fact that AKT phosphorylation was not different in the same study.

Despite the continued exploration and detailed understanding of what the signaling cascades are doing during diabetes, there is still no consensus on where these dysfunctions are originating. Molecular mechanisms that underlie this dysfunction of glucoregulatory processes associated with T2D as outlined above have been studied extensively, but the interaction of glucoregulatory processes with those of protein metabolism (protein turnover) are still lacking, despite the evidence that the two processes may be dependent on one another.

It is well documented that muscle mass and strength decline with T2D [6,204] and contribute to a decline in quality life over time. Interestingly despite a loss in muscle mass, there appears to be an upregulation of protein synthesis and the anabolic signal cascade in diabetic muscle [205,206]. Previously, studies assessing anabolic responses (FSR) in diabetic skeletal muscle have been inconsistent, ranging from

decreased [207,208], to normal [209,210] but more recently increased FSR has been confirmed by our lab [205,206,211,212] and others [213,214]. In fatty Zucker rats, a well-documented model for T2D, upregulated protein synthesis in specific muscle fractions and increased phosphorylation of S6K1 were observed despite an overall decrease in muscle mass. This upregulation of S6K1 appears to be linked to a loss of control of upstream mTOR activation. While the hyperactive mTOR activity may be a result of the maintained state of hyperinsulinemia with glucose intolerance, we suspect something much more sinister for the progression of T2D.

Our recent studies have demonstrated that the constitutive activation of mTOR may be a result of suppressed Dep domain containing mTOR interacting protein (DEPTOR) expression in the diabetic state. DEPTOR is one of the mTOR associated binding partners that can be a part of either mTORC1 or mTORC2 and is a negative regulator of mTOR activity. Similar to several lines of cancer [215]. DEPTOR is substantially lower in obese subjects [205,206]. Since DEPTOR is still a fairly new discovery in the mTOR signaling cascade, the implications of low DEPTOR and the regulation of mTORC1 are still speculative but the low DEPTOR appears to allow the downstream anabolic signals to go unchecked [216] which has implications for mRNA translation [217], as well as glucoregulatory signaling cascades. This unbridled mTORC1 activity without concomitant muscle mass accretion is indicative of high protein turnover [206], where it may not be warranted or wanted. It is also an important bridge between mTORC1 and mTORC2 which will be discussed in a later section.

3.4. Connecting Anabolism to Insulin Resistance

A relatively recent but important discovery in the connection of anabolic and glucoregulatory signaling paths is an inhibitory pathway that directly links S6K1 to IRS1. IRS1 can be serine phosphorylated through many pathways including JNK, IkB kinase, PKC, and S6K1 [218,219]. It is now known that the insulin receptor contains multiple phosphorylation sites [220] and even in a basal state it is highly phosphorylated [221]. Ser/thr phosphorylation of IRS1 has been linked to the degradation of IRS1 itself and the downstream signaling needed for glucose uptake. While the patterns and requirements of these phosphorylation's for the downstream signal disruption are still undefined it has been clearly demonstrated that chronic exposure of cells to insulin results in degradation of IRS1 protein [222–224]. It was later found that AKT mediated the ser/thr phosphorylation of IRS1 and that this was inhibited by rapamycin [225] . More specifically IRS1 phosphorylation at ser307 and ser636/639 were observed in moments of increased mTORC1 activation and this increase was absent in mice that were S6K1 deficient [96]. In support of this constitutive activation of S6K1 lead to IRS1 phosphorylation and degradation as well as inhibition of IRS1 transcription [226,227]. It is now a well-supported conclusion that IRS1 phosphorylation by S6K1 (Figure 3.2), decreases insulin signaling through the insulin receptor substrate [96,218,221,228,229]. This critical role is highlighted in the elevated levels of activation in liver adipose and muscle of obese animals [96,205,206,230] and is further supported by S6K1 deficient mice being protected against diet-induced obesity and insulin

resistance [96]. This clearly links mTORC1 and more specifically S6K1 to the general insulin signaling cascade making it a target molecule for alteration of insulin signaling.

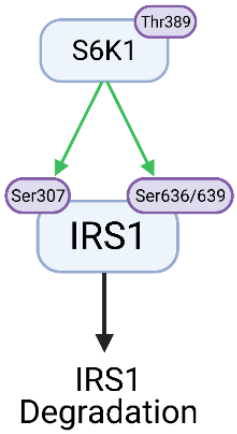

Figure 3.2 Downstream mTORC1 substrate S6K1 phosphorylation of IRS1 at 307 and Ser 636/639 leads to IRS1 degradation. Green arrows (→) indicate activation of the substrate, black arrow (→) indicates degradative pathway. Figure created with BioRender.com

While we are gaining perspective in the current literature about the interaction between mTORC1 signaling for protein synthesis and the disruption of insulin signaling for glucose disposal in skeletal muscle, far less is known about how the two mTOR complexes interact in this process. While the S6K1 connection to IRS1 is now fairly accepted, S6K1 also appears to have a role in the cross-talk between the two mTOR complexes that is not yet well defined but thought to play a role in insulin resistance. To date, very little is known about the regulation of mTORC2 [231] despite its role in phosphorylation of AKT at ser473. The role of Akt and its regulation through ser473, both upstream and downstream is still quite controversial in the literature as discussed

earlier in section 3.2.1. AKT/PKB, despite its being a widely used marker of AKT activity [100,147,148]. The downstream targets of AKT include various substrates involved in glucose uptake so the choice of this important intermediate as a marker seems obvious; however, the interpretation of what phosphorylation of AKT at ser473 truly implies remains ambiguous.

The mTORC2 complex is best known for its involvement in cell survival but is known to phosphorylate AKT through ser473 [147,232–235] as well as the PKC family [160,234–237]. This complex is composed of binding partners mSin1, DEPTOR, Protor1, mLST8 and RICTOR. While all of these binding partners play roles in mTORC2 activity, RICTOR has currently demarcated the role of mTORC2 in signal transduction [147]. RICTOR aids in localization of mTOR to the plasma membrane as well as the binding of mSIN1 to the mTORC2 complex [141], making it an important binding partner worthy of the interest it has received. While mTORC2 has been established as the kinase responsible for phosphorylation of AKT at ser473 the mechanism behind this phosphorylation is controversial. Two binding partners, RICTOR and mSIN1, have been established as important regulators of mTORC2 complex activity, and of interest is that both of these binding partners appear to be regulated by S6K1. RICTOR is prone to phosphorylation [232,238,239] and that phosphorylation may impact downstream targets like AKT, as indicated by phosphorylation at ser473 [233,240].

Work by others indicated that the muscle-specific deletion of RICTOR led to decreased ser473 phosphorylation of AKT and was accompanied by reduced

phosphorylation of AS160 at thr642 and overall glucose intolerance[241]. That work lead to speculation that regulation of RICTOR through phosphorylation was responsible for the increases or decreases in ser473 phosphorylation [233,240], and the concomitant responses of insulin-stimulated glucose homeostasis. Others determined that the phosphorylation of RICTOR at thr1135 (Figure 3.3) was responsible for inhibition of kinase activity toward AKT at ser473 [237,240,242,243]. Phosphorylation of RICTOR at thr1135 was sensitive to both growth factors and rapamycin [242] and was the direct target, established through silencing and pharmacology, of S6K1 [237]. Although the evidence connecting S6K1 to RICTOR regulation is compelling, the functional consequences of this phosphorylation are controversial. Some studies have indicated that this phosphorylation is a direct regulator of mTORC2 activity towards AKT [237,240], while others report no alteration in mTORC2 activity [242,243]. It must be noted that different experimental models were used across these studies, so it is possible that some of the differences observed were due to the differences in genetic models used to arrive at those conclusions. Despite those discrepancies, the S6K1-RICTOR interaction further supports the concept of crosstalk between the insulin glucoregulatory and protein synthesis pathways, as implicated by data demonstrating that mTORC1 regulation is important for ser473 regulation. With mTORC1 and S6K1 activity being upregulated with diabetes, this connection to the insulin signaling pathways and the direct control mTORC1 may be critically important for further understanding of the metabolic dysregulation of T2D.

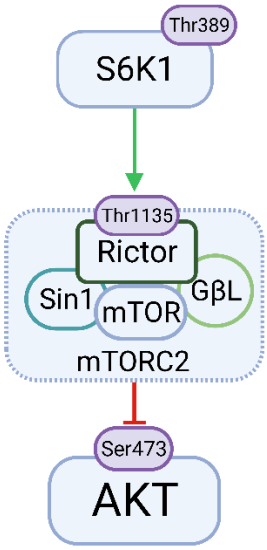

Figure 3.3 Downstream mTORC1 substrate S6K1 is the primary kinase responsible for phosphorylation of the mTORC2 component Rictor at Thr1135 which has been implicated in phosphorylation of AKT at Ser473. Green arrow (↓) indicates activation of the substrate, red bar (⊥) indicates inhibitory action on the substrate. Figure created with BioRender.com

3.5. Resistance Exercise

Exercise and physical activity are effective, low cost interventions for insulin resistance and T2D [244,245]. The benefits of aerobic exercise on glucose tolerance are well established [246–250] and the improvements are independent of improvements in general condition [250]. However, many people with T2D are overweight and/or obese, have mobility issues and other neuropathies making aerobic-type exercises difficult to accomplish [251,252]. Resistance exercise has been proposed as a more feasible activity when aerobic exercise is inaccessible and there is a growing body of evidence to support that this form of exercise can be beneficial with regard to glucose tolerance [253,254].

Much of this work attributes the glucoregulatory improvements following resistance training are due to increased muscle mass [124,255,256] which may or may not be applicable to T2D. Additionally, acute resistance exercise appears to increase insulin clearance without a change in glucose tolerance [257], which was originally attributed to increases in insulin sensitivity via receptor number or a greater liver or tissue clearance following exercise.

It is often speculated that insulin-resistant skeletal muscle is desensitized or 'resistant' to the anabolic actions of exercise [206,258,259], making it difficult to achieve gains in muscle mass. Given the aforementioned hyperactivation of mTOR with insulin resistance, the current theory is that the 'anabolic resistance' observed with diabetes/obesity may really be due to an "anabolic ceiling" in skeletal muscle that has been achieved in the hyper-insulinemic state. In healthy tissue. resistance exercise is a potent stimulator of rates of protein synthesis in muscle and repeated bouts of resistance exercise lead to skeletal muscle hypertrophy [260]. It has also been established that insulin is a necessary component in elevated protein synthesis rates after resistance exercise and it is the combination of resistance exercise and insulin that causes this modulation [261,262]. This effect of insulin appears to be through a rapamycin sensitive pathway [263–266] at least in healthy unperturbed tissue, but engaging in a moderate to high intensity exercise bouts involving eccentric muscle actions lead to a transiently-reduced capacity of insulin to elevate glucose uptake [267,268]. The mechanisms behind this alteration are still not well defined, but speculation includes a diminished capacity for glycogen synthesis and reductions in GLUT4 protein which may be fiber

type specific [268]. Further, as noted above, there are circumstances where the activation of protein anabolism requires S6K1 activation, which may feedback on upstream signals that impair glucose uptake by insulin [96,205,206,229]. More work is warranted to better define these mechanisms.

Aside from insulin sensitivity, there are benefits to regular exercise, whether it is of an aerobic or anaerobic nature. It is important to note here that there are insulin independent pathways that trigger glucose uptake that are directly related to skeletal muscle contraction. This pathway is triggered by muscle contraction and involves a distinct subset of GLUT4 [185,269–271]. These pathways can involve nitric oxide [272] and activation of AMPK [273,274] as well as cytosolic calcium [248] but these effects are distinct and additive to those of insulin mediated glucose uptake [124,275–277]. Probably most important for T2D research is that these contraction mediated glucose pathways are not only present in T2D but are fully functional [278,279].

Interestingly, in insulin resistant muscle there seems to be a difference in the control of muscle protein synthesis. It appears that in tissue where the upstream activators of the mTORC1 pathway are impaired there are alterations to the use in protein synthesis. Unlike their lean counterparts obese Zucker rats administered insulin had augmented rates of muscle protein synthesis and that these actions persisted in the presence of rapamycin [212]. This suggest that the rapamycin sensitive mTORC1 pathway is not responsible for the increased muscle protein synthesis rates observed.

One key player that may have an impact on muscle protein synthesis in response to insulin is a serine/threonine kinase called protein kinase C (PKC). PKC has long been

considered as a regulatory contributor during mRNA translation in a number of tissues [280,281] but more recently specific isoforms of PKC have been implicated in the regulation of glucose uptake. Specifically, the conventional family of PKCs (α,β,γ) lead to attenuated insulin receptor tyrosine kinase and PI3K activity [282,283] which leads to reduced glucose disposal. It has been discovered that in diabetic tissue, when insulin complexes with its receptor PKC is activated which then impairs downstream insulin signal [211]. This phenomenon is not observed in muscle from lean humans who have normal glucose response, mirroring the observed changes in insulin induced protein synthesis not present in lean counterparts [212]. Additionally inhibition of PKC activity through pharmacology has been demonstrated to partially restore signal transduction and glucose disposal in otherwise insulin resistant muscle [282,284]

The regulation of PKC, like many of the enzymes related to insulin signal transduction and glucose uptake is complex. It is known that PKCα is a downstream substrate of mTORC2 at both its turn motif (thr638) and is hydrophobic motif (ser657) both of which are required for PKCα stability [160,234–237]. Deletion of RICTOR, abolishes phosphorylation of the hydrophobic motif of PKCα [232,233] and deletion of either RICTOR or mSIN1 dramatically reduces PKCα protein content [235], implicating that RICTOR, a component of mTORC2, plays a role in PKC activation much like it does for the activation of AKT at ser473. This draws mTORC2 further into the complex crosstalk that impacts insulin signaling and provides a feasible opportunity for mTORC2 to assist in the bypass of normal insulin signaling with the upregulation of PKC. It is

important to note that PKC activation does not rely on mTORC2 however because it can also be activated by diacylglycerol [235] which would be high in the obese state.

3.6. Conclusions

Dysregulation of mTOR signaling is a key player in the development of many disease states including diabetes. While decades of research have been dedicated to understanding the insulin signaling cascade, many aspects of its regulation and control remain elusive. It is becoming clear that crosstalk between the two mTOR complexes is adding considerable complexity by impacting both hormone-mediated glucose uptake and the underlying pathogenesis of this disease. This emerging evidence now blurs their roles and responsibilities of fixtures in protein homeostasis. Research in this area has focused on specific culprits in the glucoregulatory pathway that are thought to cause the manifestation of the disease, but with all of the newly emerging anabolic/glucoregulatory cross talk that are involved with the manifestation of this disease, it is possible that the factors once viewed as culprits for this disease may actually be the consequence of anabolic/glucoregulatory cross talk. These recent findings offer exciting new targets for the control of insulin resistance.

4. DETERMINING GHRELIN ACTION ON SKELETAL MUSCLE *IN VITRO*

4.1. Introduction

Type 2 Diabetes (T2D) is a rampant disease with an impact on worldwide health that continues to rise despite continuous efforts to combat this disease [285]. The complex and highly regulated relationship between insulin secretion and insulin-mediated glucose disposal continues to be examined and debated; however, the molecular mechanisms that define the development of this disease remain unresolved. While their relative contributions to T2D are still widely debated, it is clear that maintenance of insulin sensitivity of peripheral tissues and beta-cell function are critical in the prevention and treatment of T2D [286]. Ghrelin, a peptide hormone with two isoforms, acylated (AG) and unacylated (UAG), is purported to impact both insulin sensitivity and beta-cell function, making it a clear target molecule for T2D. Additionally, total circulating ghrelin levels are lower in diabetic and insulin-resistant obese subjects [18,106]; but AG remains unaltered [106], providing an argument for ghrelin involvement in the progression of the disease.

Exogenous ghrelin has been reported to decrease glucose-stimulated insulin secretion [287,288], and circulating ghrelin levels are inversely related to insulin levels [50,51]. Additionally, ghrelin can increase glucose production and subsequent release by the liver [87], potentially contributing to the hyperglycemia associated with insulin resistance and T2D. While the influence of ghrelin in the pancreas and liver are well defined, a direct impact of ghrelin on glucose uptake in skeletal muscle is less clear.

Skeletal muscle plays a significant role in insulin-stimulated glucose disposal, commanding up to 80% of postprandial glucose uptake [289]. Therefore, defining the role ghrelin has on glucose uptake in this tissue may be vital to understanding the underlying cause of whole-body insulin resistance.

Ghrelin receptor (GHS-R) knockout models have resistance to diet-induced obesity and trends towards glucose tolerance [34,101]. It is postulated that the removal of the Ghrelin-GHS-R axis is responsible for these improvements. Additionally, in models of both T2D and chronic kidney disease, the introduction of exogenous UAG but not AG was associated with increased insulin sensitivity and glucose uptake [34,35,101]. The negative impact of AG on glucose uptake is fairly well accepted, but the mechanisms behind either isoforms outcomes are difficult to discern because of the complexity of ghrelin signaling *in vivo*.

The purpose of the current study was to confirm and provide insight into the impact of AG or UAG on the insulin-dependent and insulin-independent glucoregulatory processes of skeletal muscle *in vitro*. Our working hypothesis was that the decrease of insulin stimulated glucose uptake observed with AG is through the upregulation of anabolic signaling, a hallmark of diabetic skeletal muscle and that the observed oppositional increases in glucose uptake observed with UAG will be through AMP-activated Protein Kinase (AMPK) dependent signal cascade, implicating an insulin-independent dominance of signal transduction for the unacylated isoform.

4.2. Methods

4.2.1. Cell Culture

Rat L6 myoblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Unless otherwise specified, all cell culture supplies were obtained from Corning Inc (Corning Inc, Corning, NY, USA). Myoblasts were grown in Dulbecco's modified Eagle's medium with sodium pyruvate supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Cytiva Life Sciences, Marlborough, MA, USA). Cells were differentiated with Dulbecco's modified Eagle's medium without sodium pyruvate and supplemented with donor horse serum for six days. Once fully differentiated myotubes were treated with 500nM AG, UAG obtained from Anaspec (Anaspec, Fremont, CA, USA) or DMSO with and without the presence of inhibitors obtained from APExBIO (ApexBio Technology, Houston, Tx, USA) for 48h. Media was changed at 24h, but the same treatments were applied with the media change.

Small aliquots of media were collected for glucose and ghrelin assessment at the initiation of ghrelin administration and 3h, 12h, and 24h. The media was spun at 3000RPM for 5 min and frozen in separate aliquots for glucose or ghrelin assessment. The media for ghrelin assessment was acidified with 1M HCL to a final concentration of 0.1M HCL to stabilize acylated ghrelin in the samples [290].

4.2.2. Pharmacological Inhibition

Cells were incubated with or without ghrelin for a total of 48h as previously described [10] with a media change at 24h. The 48h incubation was used to determine ghrelin influence on myofibrillar fractional synthesis rates, 24h glucose clearance, and

signaling with or without the presence of LY294002 (10μM) or Compound C (10μM). These inhibitors were applied to the media in DMSO in the last hour of treatment with ghrelin. Ly294002 (10μM) functions as a PI3K inhibitor and downregulates AKT by binding to its ATP-binding domain [291–293]. Compound C (10μM) served as a selective AMPK inhibitor and was used to assess ghrelin's action on AMPK and downstream events [294]. After the 48h incubation period was complete, including the hour of pharmacological intervention, the media was removed, cells were washed in ice-cold PBS and then harvested in 400μl ice-cold Norris buffer [25mM HEPES, 5mM β-glycerophosphate, 200μM ATP, 25mM benzamidine, 2mM PMSF, 4mM EDTA, 10mM $MgCl_2$, 100mM NF, 10mM Na_3VO_4, Sigma protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis, MO, USA), and 1% TritonX100, pH 7.4] and frozen until analysis. When ready for analysis, tissue samples were thawed on ice, vortexed vigorously, and spun at 14,000 RPM for 30 min at 40^0C to separate the cytosolic and myofibrillar protein content. The myofibrillar pellet was then used to analyze protein synthesis rates, and the cytosolic were immunoblotted.

4.2.3. Fractional Synthesis Rates

Twenty-four-hour fractional synthesis rates (FSR) for myofibrillar fractions were assessed in myotubes using deuterium oxide (^2H_2O). During the second 24h of the 48h ghrelin treatments, the media was additionally enriched with 4% ^2H_2O. The precursor pool (culture media) and tissue (myofibrillar fraction of myotubes) were analyzed by gas chromatography-mass spectrometry as previously described [205].

FSR was calculated using the following equation:

$$EA \cdot [EBW \times t(d) \times 3.7)\text{-}1 \times 100$$

EA = Protein-bound 2H-alanine EBW=quantity of $^{2}H_{2}0$ in precursor pool

t(d)= total time of incorporation in days 3.7=exchange coefficient between body water and alanine

4.2.4. Glucose Clearance

Glucose at 0h and 24h was assessed by an Accucheck guide glucometer (Roche Diabetes Care, Indianapolis, Indiana, USA) as previously described [295] with slight modifications. Briefly, the glucose samples were thawed and vortexed thoroughly, and 125µL of the sample was diluted with 375µL of reverse osmosis water for a 1:3 ratio of sample to water. A glucose curve was created, with the same dilution applied that ranged from 1.8 to 4.8mg/mL glucose, and was used to determine glucose in each sample. Glucose readings for samples and standards were performed in duplicate and were only considered valid if variation remained within 5mg/dL on the glucometer. The glucose amount in the media at 24h was then subtracted from 0h to determine glucose clearance in 24h. This was then normalized across plates by protein content determined by Pierce BCA Protein Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

4.2.5. Western Blot Analysis

Total protein and phosphorylation of glucoregulatory and anabolic signaling molecules were assessed via western blotting as previously described [296,297]. Briefly, whole tissue was pulverized at the temperature of liquid nitrogen. Then 40 mg

of tissue was homogenized in cold Norris buffer [25mM HEPES, 5mM β-glycerophosphate, 200μM ATP, 25mM benzamidine, 2mM PMSF, 4mM EDTA, 10mM $MgCl_2$, 100mM NF, 10mM Na_3VO_4, Sigma protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis, MO, USA), and 1% TritonX100, pH 7.4]. Homogenates were then spun at 14,000 RPM for 30 min at 4^0C to separate myofibrillar rich from cytosolic rich fractions. The cytosolic fractions were denatured in 4x laemmli buffer at 95^0 C, and identical quantities of protein were loaded onto polyacrylamide gels. Following 1.5h electrophoresis at 20mA in standard electrode buffer, a semi-dry 1 h transfer ($7.5mA/cm^2$) was used to transfer proteins onto 0.2μm PVDF membranes soaked in methanol.

Membranes were then blocked for 1h in blocking buffer (5% dried milk (w/v) in Tris Buffered Saline) and incubated overnight in a heat-sealed plastic bag containing 1:1000 primary antibody/buffer (5% BSA (w/v) in TBS). Blots were probed with Cell Signaling primary antibodies for phospho AMPK (#2531), total AMPK (#5832), phospho ACC (#11818), total ACC (#3662), phospho 4E-BP1 (#2855) and Total 4E-BP1 (#9452), phospho AKT (#4058), and total AKT (#2920). After a serial wash step with 1xTBS (3X5min), membranes were incubated for 1h at room temperature with 1:2000 secondary antibody/buffer (5% milk (w/v) in TBS). After another serial wash step, membranes were incubated for 5 min in ECL (Pierce) and bands were developed with a CCD camera mounted in a FluorChem SP imaging system (Alpha innotech, San Leandro Ca, USA). Optical Density was determined using the Studio Lite software (LI-COR Biosciences, Lincoln, NE) and was automatically set to subtract nonspecific

binding from densitometry values. All bands were normalized to total protein from Ponceau S staining and expressed as arbitrary units.

4.2.6. Ghrelin ELISA

Media was collected and acidified with 1N HCL for AG and UAG concentrations. The media was thawed, and ghrelin concentrations were assessed via EIA Sandwich Elisa Kits from Cayman Chemical (Cayman Chemical Company, Ann Arbor, MI, USA). Due to the high concentrations of the ghrelin treatments, dilutions ranging from 1:1000 – 1:50,000 were completed for AG and UAG with EIA buffer as per the manufacturer's instructions. The completion of the Elisa kits followed the manufacturer's instructions, and the plate was read every 30 min for 1.5h at an absorbance of 405.

4.2.7. Glucose Uptake

A second time course over 4h was used to determine the acute effect of ghrelin on glucose uptake. Cell culture was performed identically to the previous study until treatment with inhibitors. Due to the short nature of incubation, this time course included overnight serum starvation prior to incubation. One hour before the end of serum starvation and the beginning of ghrelin incubation, the same inhibitors from the 48h study were applied. When media was changed and ghrelin incubation started, the same inhibitors were also present in the media for those treatment groups. Media and tissue samples were harvested at 15 min, 30 min, 1.5h, and 3h to determine the short-term effects of ghrelin on glucose uptake.

Glucose uptake of the acute ghrelin treatment was assessed using tritiated 2-deoxy-D-glucose (2-DG) (Moravek Inc, Brea, CA, USA). A combination of 2-DG (0.5μCI/mL) and insulin (900nM) was added to the media 10 min prior to harvest; a media sample was collected prior to this addition for glucose levels at the time of 2-DG addition and the plates placed back into the incubator. At harvest, media was carefully collected for assessment of tritiated 2-DG on the scintillation counter (Beckman Coulter, Brea, CA, USA) and glucose concentration as described earlier in glucose clearance. The cells were then washed with ice-cold PBS and harvested in Norris buffer to assess tritiated 2-DG concentration and protein content. Mixed protein content was determined by Pierce BCA Protein Assay, and then 100ul of tissue sample was prepared for the scintillation counter with a combination of 4mL of scintillation cocktail (Ready Safe Liquid Scintillation Cocktail, Beckman Coulter, Brea, CA, USA) and 500μL of DI water. The samples were shaken vigorously and then allowed to settle overnight. The abundance of tritium for each sample was then assessed the liquid scintillation counter for 10 min. Media samples for precursor pool presence of tritium and glucose were assessed similarly with 500μl of media, 200μl of DI water, and 4mL of scintillation cocktail. The samples were treated the same as the tissue samples in preparation for the counter.

Glucose uptake was determined by normalizing tritiated 2-DG to the glucose present in the media and determining the ratio of glucose to tritium present in the media and the tissue samples. That was then divided by the total protein of each sample to determine how much glucose was taken up over the 10 min.

$$\frac{\text{SA of Media}\left(\frac{\text{ng glucose}}{\text{DPM}}\right) \Big/ \text{DPM of Tissue}}{\text{Protein Content of Tissue Sample (ug)}} = \text{Glucose uptake (ng/ug)}$$

4.3. Statistical Analysis

One-way analysis of variance was used to compare ghrelin groups using Graph Pad Prism software (Graph Pad Software, San Diego, California, USA, www.graphpadcom). When Significant F-ratios were present, a Student-Newman-Keuls (SNK) post-hoc measure was used to evaluate differences among group means. Significance was predetermined at $p<0.05$. Groups not sharing the same letter are statistically different (from Student-Newman-Keuls; $p<0.05$)**.** No letters within a graph indicates no statistical differences.

4.4. Results

4.4.1. Glucose Clearance and Protein Synthesis

Glucose clearance from the media was lower ($p<0.001$) with AG (Figure 1a). Glucose clearance from the media with UAG was not different from control ($p>0.05$).

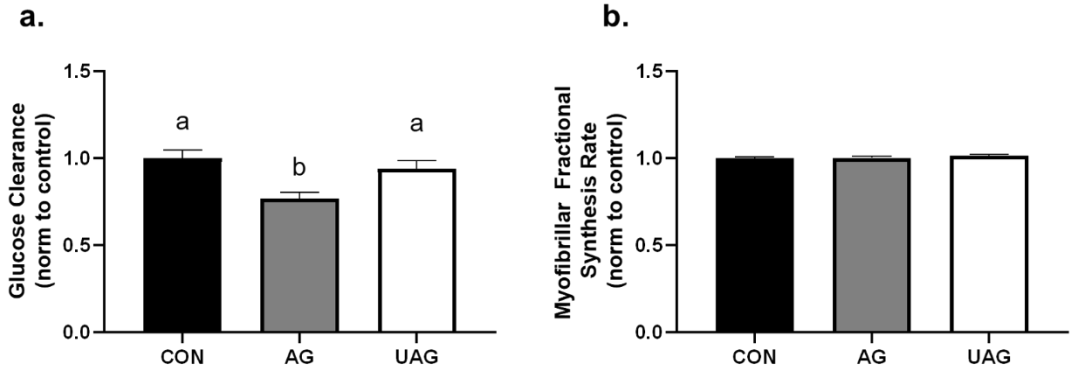

Figure 4.1 Glucose Clearance and Protein Synthesis rates of Cultured L6 Rat Myotubes with 48h incubation of DMSO (CON), 500nM Acylated Ghrelin (AG), or 500nM Unacylated Ghrelin (UAG). (a) Glucose clearance relative to control. (b) Myofibrillar fractional synthesis rates of L6 myotubes. Data are presented as means ± Standard error (n=8). Groups not sharing the same letter are statistically different (from Student-Newman-Keuls; $p<0.05$). No letters within a graph indicates no statistical differences.

Previously our laboratory demonstrated dysregulated protein synthesis in diabetic models, with an upregulation of protein synthesis despite a loss of muscle mass [95]. To assess whether the introduction of exogenous ghrelin contributes to this dysregulation, myofibrillar fractional synthesis rates were evaluated by deuterium incorporation into protein with 500nM of AG or UAG. There were no differences among the groups (Figure 4.1) implicating ghrelin action is not involved in altering myofibrillar protein synthesis rates.

4.4.2. Anabolic Signaling

Anabolic signaling cascades measure mTORC1 activity through the downstream targets of eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), ribosomal protein S6 kinase beta-1 (S6K1) and dep domain containing mTOR interacting protein (DEPTOR) a known mTOR inhibitor (Figure 4.2). 4E-BP1 is a rate-

limiting protein in the regulation of protein translation. Phosphorylation of 4E-BP1 at thr37/46 was higher with exogenous AG than both DMSO or UAG. The introduction of the PI3K inhibitor LY294002 (Figure 4.3a) depressed ratios of all three treatments, completely abolishing the increase observed with AG without pharmacological inhibition. Intriguingly, when amp-activated protein kinase (AMPK) was inhibited with Compound C, the phosphorylation declined as with PI3K inhibitor (See inset, Figure 4.3). Still, the phosphorylation of 4E-BP1 with AG was higher than both CON and UAG mimicking, at a reduced level, what was observed in the control groups.

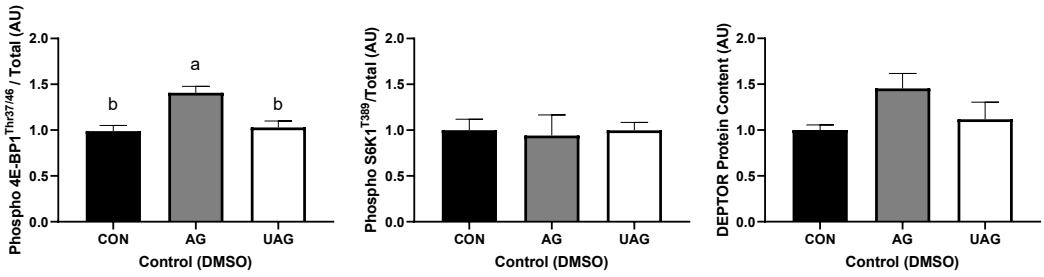

Figure 4.2 Anabolic Signaling of L6 Myotubes with 48h incubation of of DMSO (CON), 500nM Acylated Ghrelin (AG), or 500nM Unacylated Ghrelin (UAG). Phosphorylated to total protein ratios are presented for 4E-BP1 (n=8) and S6K1 (n=4) along with total protein content of the mTOR inhibitor DEPTOR (n=4). Data are presented as means ± standard error**.** Groups not sharing the same letter are statistically different (from Student-Newman-Keuls; $p<0.05$). No letters within a graph indicates no statistical differences.

4.4.3. Glucoregulatory Signaling

To assess ghrelin's influence on phosphorylation of protein kinase B (AKT) on skeletal muscle in an unperturbed state, the activity of AKT was assessed through phosphorylation of AKT at ser473 (Figure 4.3b) and no differences between groups were observed. When the PI3K inhibitor LY294002 was added, the phospho/total ratios were

depressed, implicating that the PI3K inhibitor effectively decreased the phosphorylation of AKT at ser473, but there were no differences in response between the ghrelin treatments. With the addition of Compound C, the phospho/total ratios of all groups were upregulated, but no differences were observed between the ghrelin treatments.

Phosphorylation of AMPK was significantly reduced with the introduction of AG or UAG (Figure 4.3c). The introduction of LY294002 had a large variation, resulting in no differences across the board. As expected, incubation with Compound C, a potent AMPK inhibitor, the phosphorylation was downregulated with no differences observed between groups.

Despite the downregulation of AMPK phosphorylation observed with AG and UAG, there were no differences between ghrelin treatments for phosphorylation of acetyl coA carboxylase (ACC) (Figure 4.3d). There were also no differences across the board with PI3K inhibition. As expected, the inhibition of AMPK through Compound C had a profound reduction of ACC phosphorylation on ser79, but no indication of differences between groups was observed.

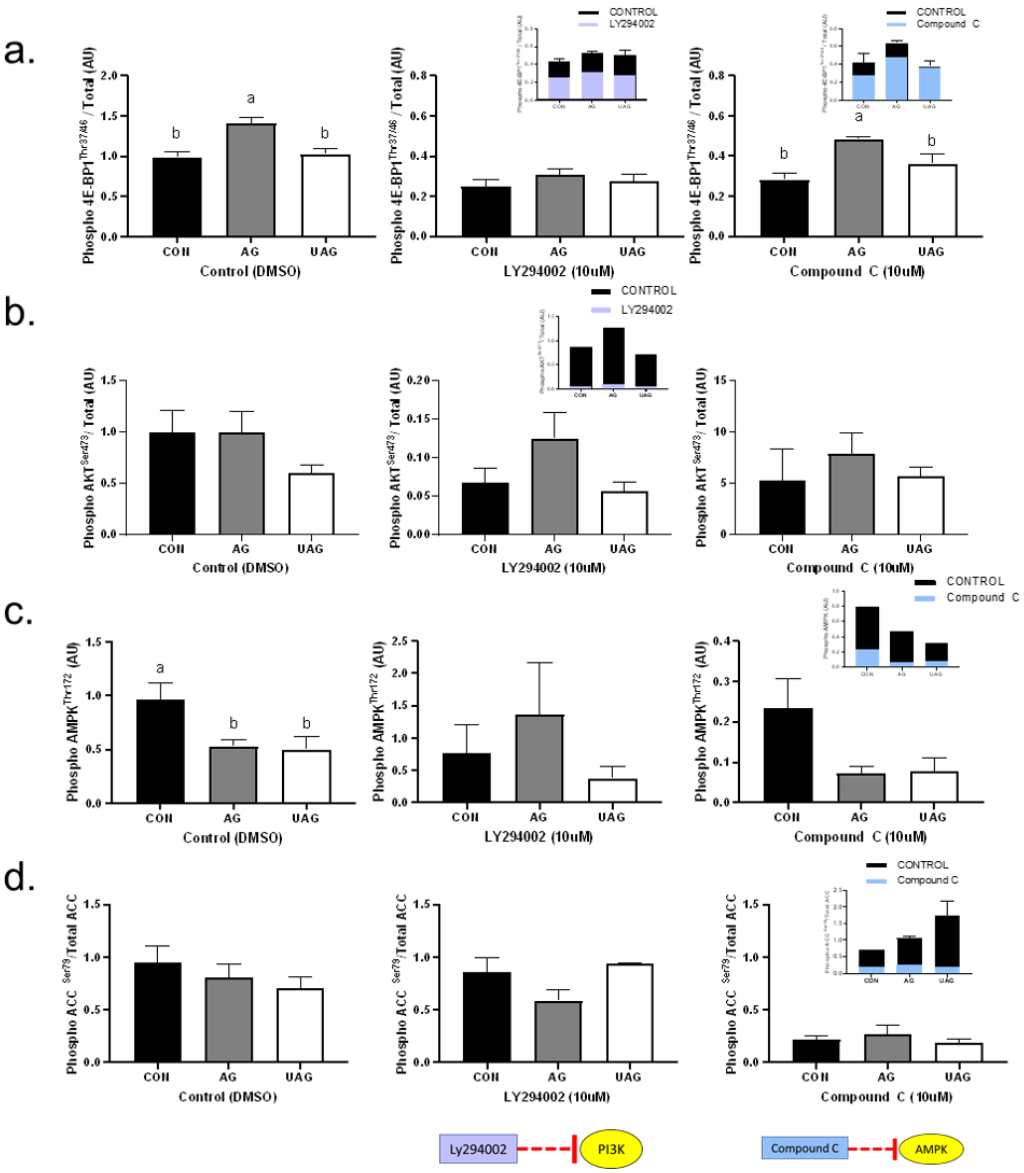

Figure 4.3 Glucoregulatory signaling with 48h incubation of ghrelin and 1 hr of pharmacological inhibition. 48h incubation of DMSO (CON), 500nM acylated ghrelin (AG) or 500nM unacylated ghrelin (UAG) (n=8), and pharmacological inhibition of DMSO (CON), LY294004 (10μM), and Compound C (10μM) for the last hour prior to harvest (n=4). Phospho to total AKT at ser473, AMPK phosphorylation at thr172, and Phospho to total ACC at ser79 are presented. Inset graphs show visual confirmation of the efficacy of pharmacological compounds on relevant proteins. Data are presented as means ± Standard error. Groups not sharing the same letter are statistically different (from Student-Newman-Keuls; $p<0.05$). No letters within a graph indicates no statistical differences.

4.4.4. Ghrelin Concentration over Time

Elisa kits for acylated ghrelin were used to determine ghrelin concentration in media samples taken at Pre, 3h, 12h, and 24h. It is clear that AG disappears from cultured media rapidly, with almost all AG deacylated or degraded by 12h post introduction (Figure 4.4a). UAG concentration is also reduced over time in media cultured with L6 myotubes (Figure 4.4a), but the reduction is not as significant or as fast as AG.

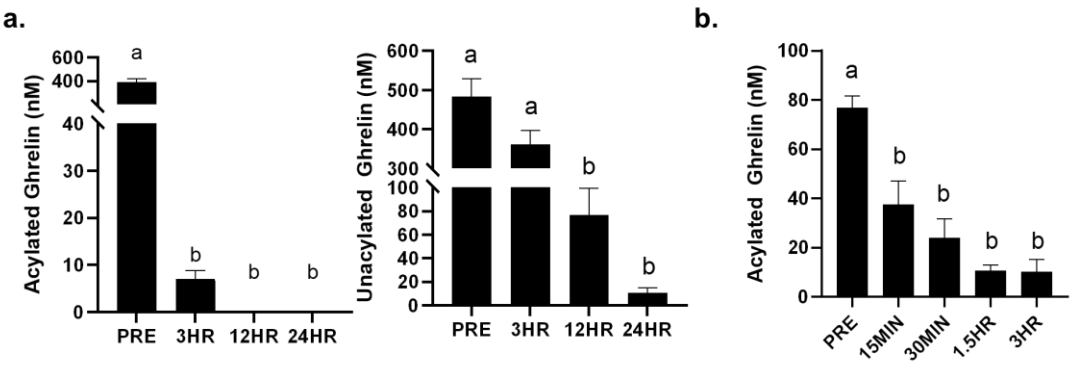

Figure 4.4 Ghrelin concentration in media during myotube culture. (a) Concentration determined by ELISA of acylated and unacylated ghrelin over 24 hours (n=2) (b) Concentration determined by ELISA of acylated ghrelin over 3hours (n=2). Data are presented as means ± Standard error. Groups not sharing the same letter are statistically different (from Student-Newman-Keuls; $p<0.05$). No letters within a graph indicates no statistical differences.

Acylated Ghrelin concentration was assessed during an acute time course with media collected pre-incubation and post-harvest at 15 min, 30 min, 1.5h, and 3h. The acute ghrelin concentrations confirmed the data from the 48h incubation with less than 20nM concentration of AG available after 3h of incubation (Figure 4.4b).

4.4.5. Glucose Uptake

Glucose uptake was assessed between 15 min and 3h post ghrelin introduction in the media, with or without a pretreatment with pharmacological inhibitors of PI3K and AMPK (Figure 4.5). Control groups at both 15 min and 1.5h appeared similar to the 24h glucose clearance (Figure 4.1) with AG significantly lower (Figure 4.5). At 15 min, both the PI3K and Compound C inhibitors demonstrated lower glucose uptake across the board (See inset graphs; Figure 4.5), with no differences across groups. Samples taken at 1.5h also had similar group trends with AG lower than control. The lower glucose uptake that was observed with the PI3K inhibitor at 1.5h was similar to data collected at 15 min. With the addition of Compound C, glucose uptake was lower across all three treatments, with both AG and UAG being significantly lower than control, but similar to each other.

Glucose uptake assessed at the 3h time point, revealed no differences across groups when incubated without pharmacological inhibitors. The addition of the PI3K inhibitor resulted in a universal reduction of glucose uptake (see inset graph; Figure 4.5) but unlike other time periods, UAG was significantly lower than control. The addition of Compound C created an opposite image to the early time points of glucose uptake without pharmacological intervention. With Compound C, AG had higher glucose uptake in comparison to both control and UAG. Control and UAG were not different ($p>0.05$).

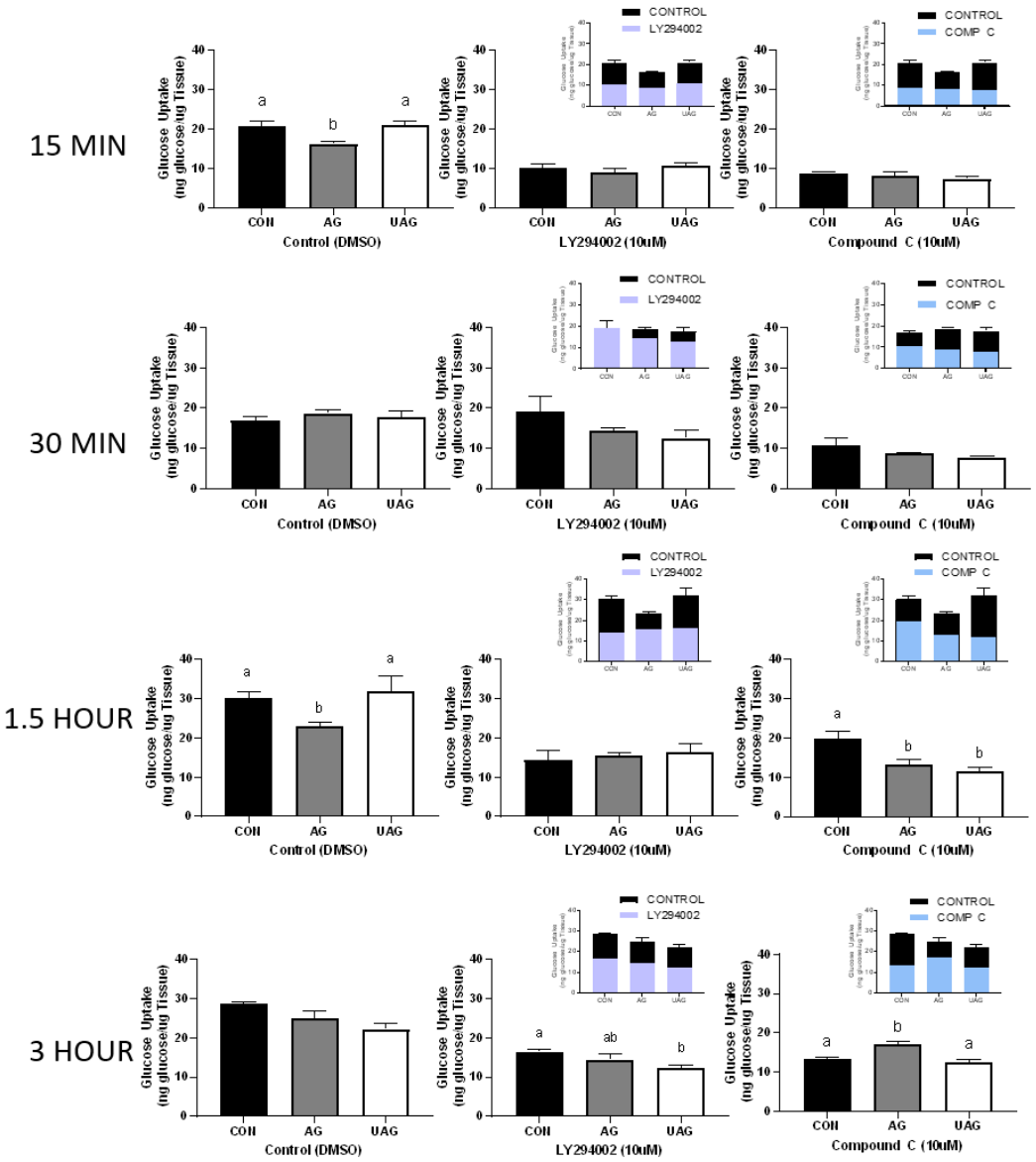

Figure 4.5 Glucose uptake measured by tritiated 2-DG with incubation of ghrelin with or without the presence of pharmacological inhibition. Incubation with DMSO (CON), 100nM acylated ghrelin (AG), or 100nM unacylated ghrelin (UAG), and pre-incubation pharmacological inhibition of DMSO (CON), LY294004 (10uM), and Compound C (10uM). Inset graphs show differences between pharmacologically inhibited and DMSO groups of the same time point to visually demonstrate the effect of each inhibitor. Data are presented as means ± standard error (n=4) Groups not sharing the same letter are statistically different (from Student-Newman-Keuls; $p<0.05$). No letters within a graph indicates no statistical differences.

4.5. Discussion

The purpose of this study was designed to assess the capacity of AG or UAG to directly exert a glucoregulatory or anabolic biological action on skeletal muscle *in vitro*. While our data do not support any conclusions that these hormones play a role on anabolic capacity in muscle, the current data suggest there may be a subtle shift toward mTORC1 anabolic signaling (as evidenced by 4E-BP1 phosphorylation) in the presence of AG that could lead to insulin resistance over time. The current data also support the postulation that acylated ghrelin lowers skeletal muscle glucose uptake with both 24h glucose clearance (Figure 4.1) and acute glucose uptake (Figure 4.5) lower with AG when compared to control or UAG but do not show convincing evidence of the positive impact measured in the *in vivo* models of UAG. Lastly, our data also reveal that there is a rapid de-acylation of AG over a 3h window and a substantial loss of total ghrelin within a 24h period in culture (Figure 4.4), suggesting that it is important to monitor the status of AG and UAG during experimental periods in order to have interpretable results.

Previous research from our lab has implicated that rampant and unregulated mTORC1 activity results in upregulation of anabolic signaling and protein synthesis, leading to the dysregulation of insulin-stimulated glucose uptake [95,205]. We postulated that the inhibitory action of AG might be through a promotion of anabolic signaling that would result in a similar impact on insulin-stimulated glucose uptake. Myofibrillar fractional synthesis rates were not different between groups (Figure 4.1). The lack of differences in protein synthesis could be partially explained by the use of the myofibrillar fraction since the most convincing evidence of protein synthesis

upregulation in diabetic animals was found in mixed fraction samples [95], but the current FSR in combination with the anabolic signaling provides a compelling argument for lack of AG involvement in the development of anabolic dysregulation observed in T2D. There were no differences among groups for either S6K1 activity or DEPTOR total protein (Figure 4.2), hallmarks of the anabolic dysregulation, and subsequent insulin resistance [95,216]. Incubation with AG did increase phosphorylation of 4E-BP1, another downstream marker of mTORC1 activity. Phosphorylation of 4E-BP1 at thr 37/46 stops 4E-BP1 from binding with eukaryotic translation initiation factor 4E (eIF4E), allowing cap-dependent translation to continue. Whole-body ablation of 4E-BP1 resulted in a significant reduction in adipose tissue and an increase in whole-body energy expenditure in mice [298], supporting the concept that 4E-BP1 signaling specifically may be involved in metabolic dysregulation. Additionally, when the binding of 4E-BP1 to eIF4E is enhanced through inhibition of mTORC1 binding sites in mouse skeletal muscle, there was an increase in oxidative metabolism and protection against diet-induced obesity and metabolic dysfunction [299]. Those results, along with the current study, support the conclusion that the observed inhibition of glucose clearance could be attributed to the upregulation of 4E-BP1, but the cascade responsible for this upregulation is unclear. These studies of different cancer cell lines have linked glycogen synthase kinase 3β (GSK-3β) [300], P38-MAPK [301], and others [302] with phosphorylation of 4E-BP1 at thr37/46, but the evidence supporting these kinases is limited, and none to our knowledge have been demonstrated in skeletal muscle. Despite this, it provides alternative cascades that could result in the phosphorylation of 4E-BP1

that are usually attributed to mTORC1. Further, it has been found that inhibition of PI3K completely blocked the insulin-stimulated 4E-BP1 phosphorylation [303], which is consistent with our findings that the PI3K inhibitor Ly294002 not only reduced phosphorylation of 4E-BP1 in all three groups but also abolished any differences across the ghrelin treatments (Figure 4.3a). This observed increase in 4E-BP1 phosphorylation implicates there is a subtle shift toward mTORC1 signaling with AG that could contribute to insulin resistance over time.

The phosphorylation of AKT on ser473 has been commonly used as a marker for maximal insulin-stimulated signaling for glucose uptake. However, that concept may be overstated, as phosphorylation of ser473 occurs via insulin independent activation of mTORC2. As such, data from the current study diverged from the current literature with regard to ghrelin action on skeletal muscle. In models of both atrophy and T2D, disease states associated with a significant reduction of AKT activity in skeletal muscle, there was increased phosphorylation of AKT at ser473 observed with incubation of either AG or UAG [34–36,101,304]. Contrary to these findings, when ghrelin was applied to healthy myotubes, we found no significant difference of AKT status among the two isoforms of ghrelin or the control group (Figure 4.3b). This supports current speculation that ghrelin requires a perturbed metabolic state to impact signaling [23] but perhaps more importantly calls into question the use of ser473 as a marker of insulin-stimulated glucose uptake or insulin signaling decrements. It is widely accepted that mTORC2 phosphorylates ser473, a complex responsible for the maintenance of autophagy [234] with little involvement in the canonical insulin signaling cascade.

Despite this, phosphorylation of AKT at ser473 as a marker of full AKT activation and insulin signaling is still widely used in various research venues. The current study shows no differences in phosphorylation of AKT on ser473 despite the reduction in glucose clearance and glucose uptake observed in the AG group, implicating that this may not be an accurate or viable indirect measure for insulin signaling or glucose uptake.

AMPK has been cited as a critical component of ghrelin signaling in various tissues [305–307], and AMPK phosphorylation in skeletal muscle has been connected to the rescue of muscle mass and glucose regulation of unacylated ghrelin in both atrophy [36] and diabetes [34]. In our hands, the addition of either AG or UAG reduced the phosphorylation of AMPK when compared to control but had no impact on the phosphorylation of one of its prominent downstream targets, ACC (Figure 4.3d). The implication is that upregulation of AMPK and its downstream targets, would lead to increased potential for beta-oxidation through its action on ACC and mitochondrial biogenesis through peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α). These processes would then result in increased glucose intake, or at the very least, a rescue of decrements. However, the current immunoblotting data do not support a direct influence of ghrelin on AMPK activity or its downstream substrates in a long-term incubation, implicating that the upregulation of AMPK observed in other experiments, generally *in vivo*, may be from some indirect action of ghrelin or other organs. The differences observed in acute glucose uptake, especially that of 15 min and 3h (Figure 4.5), suggest a tenuous connection between UAG and AMPK. With no

pharmacological inhibitors, UAG and control groups remain remarkably similar across the four time points measured. Furthermore, with the addition of AMPK inhibitor Compound C, glucose uptake was also similarly reduced, without the same influence over the glucose uptake observed with AG. This tenuous connection suggests AMPK may be responsible for some of UAG action on glucose uptake in skeletal muscle, but the lack of differences between DMSO and UAG with Compound C may indicate that this pathway becomes more important with decrements observed in skeletal muscle dysregulation like advancing age or T2D. These glucose uptake alterations are quite minor, at least in the unperturbed state of this *in vitro* model, and would need to be confirmed from a signaling perspective and in models of dysregulation.

It is clear from both our 24h and acute measures that ghrelin is deacylated and/or degraded in cell culture, similar to the processes found *in vivo*. Yet, to our knowledge, current published studies have not measured ghrelin concentrations during their experimental time frame. The rapid reduction of AG and, to some extent, UAG over time may implicate an early influence of ghrelin on skeletal muscle glucose uptake as observed with both glucose clearance and glucose uptake in the current findings. While these early actions were critical to the *in vitro* 24h glucose clearance measured in our study, it may be less relevant *in vivo* because of the involvement of other organs (like the liver) in glucose homeostasis.

There are a few considerations about the current cell culture model that could explain some of the differences observed. The present study was completed on healthy cultured myotubes. In contrast, most of the existing research has been conducted on

models where decrements in both signaling and glucose uptake are present before the introduction of exogenous ghrelin. While the current literature suggests a function of ghrelin in skeletal muscle, the limited impact on healthy tissue, combined with the inability to distinguish ghrelin function on skeletal muscle versus other tissues in previous research, makes it hard to determine if ghrelin action during these perturbed states is a direct consequence of ghrelin interaction with skeletal muscle and arguably more importantly if these proposed direct actions are physiologically relevant when compared to the responses from other organs.

Through the use of cell culture, we established that AG directly acts on skeletal muscle directly and reduces glucose uptake, potentially through a subtle shift toward mTORC1 substrate 4E-BP1. While we did not observe the same positive influence on signaling observed in vivo with UAG, we did find subtle changes in glucose uptake implicating that UAG action, while limited in healthy tissue were directed through AMPK. Overall, our data indicate that any direct positive influence of ghrelin on skeletal muscle glucoregulation, at least in the healthy state, is negligible. However, it agrees with current literature that AG may impact skeletal muscle glucose uptake and suggests that this may be through a yet to be defined cascade involving AKT/mTORC1/4E-BP1 signaling cascade. While the current data tenuously supports the concept that UAG action in skeletal muscle supports the positive effects observed with *in vivo* dosing of UAG, it is likely that the small direct action that UAG plays on skeletal muscle through AMPK is overshadowed by actions on other organs. These findings do not negate UAG potential as a therapeutic target for various disease states

but does call into question how active ghrelin is in the development of diseases like diabetes and whether the use of UAG as therapy will alter any of the known dysregulation of skeletal muscle during this disease progression.

5. ABLATION OF GHRELIN RECEPTOR MITIGATES THE METABOLIC DECLINE OF AGING SKELETAL MUSCLE

5.1. Introduction

Aging is associated with a reduction in lean body mass, and consequent losses of physical strength and mobility, leading to a decreased quality of life. Sarcopenia, the loss of muscle mass during aging, is a critical factor in these negative outcomes and has been directly linked to increased mortality [308]. While skeletal muscle has a primary role in locomotion and maintenance of posture, it also has a clear role in organism-wide metabolic homeostasis. The loss of strength and function of skeletal muscle with advancing age often overshadows the metabolic dysfunction that occurs simultaneously, including mitochondrial dysfunction [309–311], glucose intolerance and insulin resistance [5,311,312], and anabolic resistance [313–315]. Another feature of advanced aging is a shift in skeletal muscle fiber type from faster to slower phenotypes [316–318]. Although not completely understood, these are key contributors of metabolic dysregulation that lead to the deleterious progression of sarcopenia with aging.

Ghrelin is an acylated 28 amino acid peptide predominantly produced in the X/A-like enteroendocrine cells of the stomach [319]. Ghrelin, mainly known for its orexigenic effects, is now increasingly recognized as a key regulator of energy homeostasis, as well as a regulator of muscle health. The only currently known, biologically relevant receptor of ghrelin is growth hormone secretagogue receptor-1a (GHS-R1a), and its canonical function is to exert an orexigenic effect in the

hypothalamus [319,320]. GHS-R1a is known to be constitutively active [321–323] and has been suggested to also have noncanonical functions [324,325]. Previous research from our lab and others have shown that knockout of the growth hormone secretagogue (GHS-R) gene in rodents protects against high fat diet induced obesity [21], insulin resistance during aging [326], and inflammation in both aging and obesity [14,16].

It has recently been proposed that ghrelin signaling is involved in thermogenic activity [22,326] and that GHS-R works as a metabolic thermostat in brown adipose tissue [327]. The peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) dependent myokine irisin has been implicated as a major player in the browning or beiging of adipose tissue [24]. Although the current understanding of irisin and its function are debated, skeletal muscle is a contributing factor in irisin content in circulation [25]. The purpose of this work was to establish the potential connection between GHS-R, irisin and muscle health of advanced aging.

5.2. Materials and Methods

5.2.1. Animals

Ghsr$^{-/-}$ mice in C57BL/6J background were generated as we previously described [91,326]. Animals were housed under controlled temperature and lighting (75 ± 1^0 F; 12 h light dark cycle) with free access to food and water. Data-relevant age cohorts were developed as previously described [326] and the age groups were described as young (4–5m), middle-aged (12–14m) and old (18–26m). All experiments were approved by the Animal Care Research Committee at the Baylor College of Medicine.

5.2.2. Real-time RT-PCR

Real-time RT-PCR was completed as previously described [16,326]. Briefly, total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA was treated with DNAse and run on gels to validate purity and quality. The cDNA was synthesized from 1μg RNA using the Superscript III First-Strand synthesis system for RT-PCR (Invitrogen). Real-time RT-PCR was performed on ABI 7900 using the SYBR green PCR master mix or the TaqMan gene expression master mix (Invitrogen). *18S* RNA and *β-actin* were used as internal controls. All primer and probe information are available upon request.

5.2.3. Lipid Content

Lipid content of the gastrocnemius muscle was completed as previously described [328]. Gastrocnemius muscle samples (about 50mg) were minced in liquid nitrogen and transferred to ice-cold Teflon-lined screw-cap tubes. 1mL of chloroform: methanol (2∶1 v/v) mixture was added to each tube. After homogenized by sonicator for 5s, the tubes were placed in a rotary mixer at room temperature for 24h. The lower organic phase was transferred to another tube and washed with PBS twice. After evaporation, the residue was weighted. The lipid content was further normalized by the tissue weight.

5.2.4. Western Blot Analysis

Western blot analysis was completed on gastrocnemius muscle as previously described [296,297]. Briefly whole tissue was pulverized at the temperature of liquid nitrogen and then 40mg of tissue was homogenized in cold Norris buffer [1:10

tissue/buffer (mg/µl); 2mM HEPES, 5mMβ-glycerophosphate, 200µM ATP, 25mM benzamidine, 2mM PMSF, 4mM EDTA, 10mM $MgCl_2$, 100mM NF, 10mM Na_3VO_4, Sigma protease inhibitor cocktail P8340 (Sigma-Aldrich, St. Louis, MO, USA), and 1% TritonX100, pH 7.4]. Homogenates were then spun at 14,000 RPM for 30 min at 4^0C to separate myofibrillar rich from cytosolic rich fractions. The cytosolic fractions were denatured in 4x laemmli buffer at 95^0 C and identical quantities of protein were loaded onto 8% polyacrylamide gels. Following 1.5h electrophoresis at 20mA in standard electrode buffer, a semi-dry 1h transfer (7.5 mA/cm^2) was used to transfer proteins onto 0.2µm PVDF membranes soaked in methanol.

Membranes were then blocked for 1h in blocking buffer (5% dried milk (w/v) in Tris Buffered Saline) and incubated overnight in a heat-sealed plastic bag containing 1:1000 primary antibody/buffer (5% BSA (w/v) in TBS). Blots were probed with Phospho AMPK (Cell Signaling, #2531) Total AMPK (Cell Signaling,#5832), Phospho ACC (Cell signaling, #11818), Total ACC (Cell Signaling, #3662), UCP3 (Cell Signaling, #97000) and GLUT4 (Cell Signaling, #2213) antibodies. After a serial wash step 1xTBS (3X5min), membranes were incubated for 1hr at room temperature with 1:2000 secondary antibody/buffer (5% milk (w/v) in TBS). After another serial wash step membranes were incubated for 5 min in ECL (Pierce) and bands were developed with a CCD camera mounted in a FluorChem SP imaging system (Alpha innotech, San Leandro Ca, USA). Optical Density was determined using the Studio Lite software (LI-COR Biosciences, Lincoln, NE) and was automatically set to subtract nonspecific

binding from densitometry values. All bands were normalized to total protein from Panceau S staining and expressed as arbitrary units.

5.2.5. Fiber Type Analysis

Fiber type analysis was completed using SDS-PAGE and silver staining as previously described with modifications [329–331]. The myofibrillar rich pellets obtained from the 40mg tissue were resuspended in 300µl of Norris Buffer and homogenized. An aliquot of the resuspended myofibrillar fraction was denatured with 4x laemmli buffer at 95^0C and 2µl was applied to 8% polyacrylamide gels for 20h at 40V. Silver stain was completed using PierceTM Silver Stain Kit (Thermo Scientific 24612) following the manufacturer's instructions. Gels were imaged using Alpha Innotech imager (Alpha Innotech, San Leandro Ca, USA) and myosin heavy chains were identified according to their apparent molecular weights compared with those of marker proteins as described previously [332] . The percentage of each myosin isoform was determined through densitometry with Image Studio^{TM} Lite software (LI-COR Biosciences, Lincoln, NE).

5.2.6. Treadmill Endurance Test

A treadmill endurance protocol was performed using an Exer-3/6 open treadmill (Columbus Instruments, Columbus, OH) similar to previously described [333]. Mice started the test at 6m/min. Treadmill speed was then increased by 2m/min every 2 min, until the mice are exhausted. Exhaustion is defined as spending more than 10 seconds on the shocker without attempting to reenter the treadmill

5.2.7. Plasma Irisin Content

To determine irisin content in plasma, a commercially available irisin ELISA kit was used (EK-067-16, Phoenix Pharmaceuticals Inc, Burlingame, CA). Samples were prepared according to manufacturer's instructions with a 5x dilution of the mouse plasma.

5.2.8. Statistical Analysis

Two-factor ANOVA was used to evaluate the significance of interaction between genotype and age, and *post* hoc tests were used to follow up. During experiments containing old mice only two-tailed Student's t-test were used to determine statistical significance between genotypes. The results are expressed as mean ± standard error of the mean. Statistical significance was set as $p<0.05$.

5.3. Results

5.3.1. Muscular Aging is accompanied by an increase of GHS-R expression

It has been previously shown that there is an increase of GHS-R in adipose tissue of aging mice along with metabolic alterations [16,23,326]. To assess whether GHS-R is correlated with skeletal muscle metabolic disfunction, GHS-R expression, common markers of mitochondrial function, and glucose uptake were assessed in young, middle-aged and old mice using real time RT-PCR (Figure 5.1). Relative expression of uncoupling protein 3 (UCP3) and Sirtuin-1 (SIRT1) were significantly lower in the middle-aged and old mice, consistent with the known mitochondria functional decline associated with sarcopenia (Figure 5.1a). Additionally, the relative expression of PGC-1α, a potent stimulator of mitochondrial biogenesis and central mediator of energy

metabolism, was also reduced in the aged mice (Figure 5.1a). Insulin receptor substrate 1 (IRS1) and glucose transporter-4 (GLUT4) were also lower in the middle and old age groups when compared to young, supporting the concept of metabolic functional decline with aging in skeletal muscle (Figure 5.1b). There was also an increase in GHS-R expression in old mice when compared to younger cohorts (Figure 5.1c). These results indicate that there is an increase in GHS-R expression in aging muscle, and GHS-R expression is inversely correlated with metabolic function of skeletal muscle in aging.

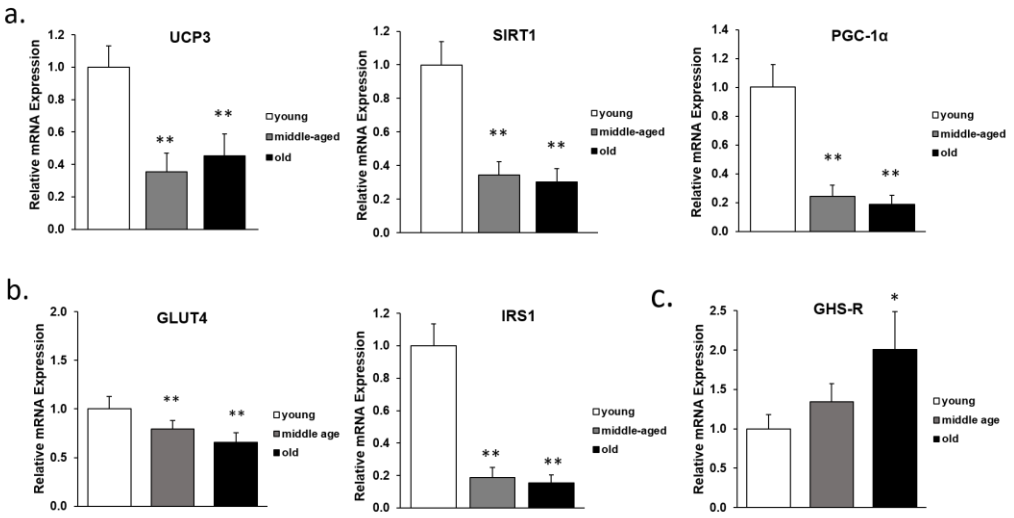

Figure 5.1 Relative mRNA expression in gastrocnemius muscle of young (4–5m), middle-aged (12–14m) and old (18–26m) C57BL/6J mice. a) Relative mRNA expression of mitochondrial functional markers UCP3, SIRT1 and PGC-1α; (b) Relative mRNA expression of glucose transporter and insulin signaling markers GLUT4 and IRS1; (c) Relative mRNA expression of GHS-R. Data are presented as means ± standard error. (n = 6). *p < 0.05, **p < 0.001.

5.3.2. Gene expression of old GHS-R knockout mice reveals improved lipid metabolism, mitochondrial function, and insulin sensitivity

Our previous work in ghrelin receptor knockout mice shows that GHS-R ablation reduces obesity and improves insulin sensitivity [326]. Here, we found that global ablation of GHS-R protected against the decrements of mitochondrial and glucose uptake genes in old mice (Figure 5.2). The gastrocnemius of the old *Ghsr*$^{-/-}$ groups had higher expression of *UCP3* and *PGC-1α* and a trend to higher acetyl-CoA carboxylase 1 (*ACC1*) mitochondrial genes required for metabolic function and mitochondrial biogenesis. *PGC-1a* expression was also higher ($P \leq .05$) in the soleus muscle, along with the *UCP3*, fatty acid synthase (*FASN*), and *ACC1* (not pictured). Consistently, lipid content (Figure 5.2b) of the gastrocnemius was reduced in the old *Ghsr*$^{-/-}$ mice when compared to old wild type, suggesting an increase in β-oxidation in the muscle of *Ghsr*$^{-/-}$ mice. Additionally, *GLUT4* and *IRS1* expression were both increased in the aged knockout mice (Figure 5.2c) implicating a potential for improved glucose uptake and insulin response.

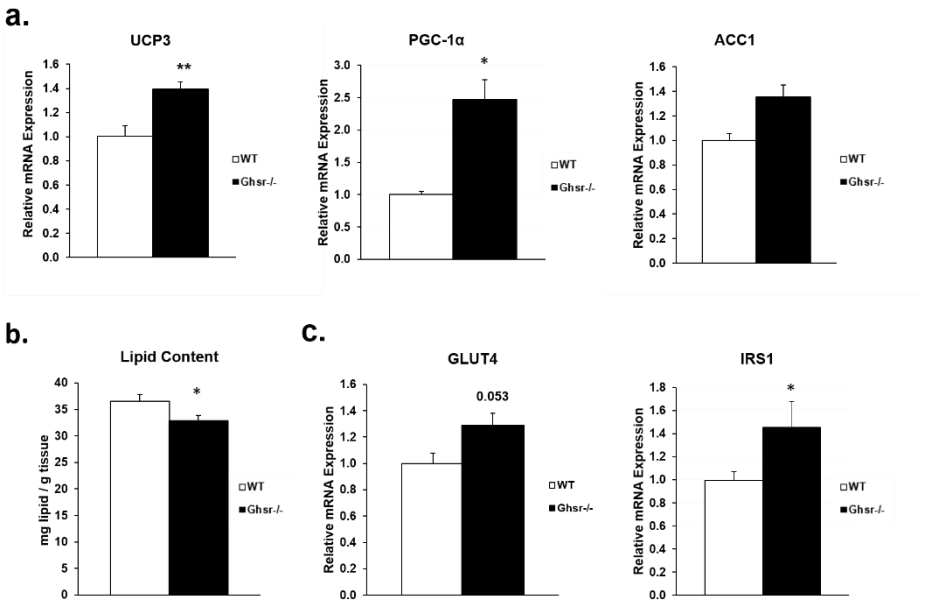

Figure 5.2. Effects of GHS-R ablation on metabolic dysfunction of old (18–26m) wild type (open bar) and GHS-R-/- (black bar) gastrocnemius muscle in mice. (a) UCP3, PGC-1α and ACC1 mRNA expression. (b) Lipid content in gastrocnemius muscle tissue. (c) Relative expression of IRS1 and GLUT4 in gastrocnemius muscle. Data are presented as means ± standard error. (n = 9). *$p < 0.05$, **$p < 0.001$.

While not as robust as data obtained for mRNA, trends observed in mRNA were confirmed by western blot analyses of several of markers (Figure 5.3). Phosphorylated to total ratios (as an indication of active versus nonactive, respectively) of AMPK and ACC as well as UCP3 content were all consistent with mRNA content. Total GLUT4 protein content was not different between groups.

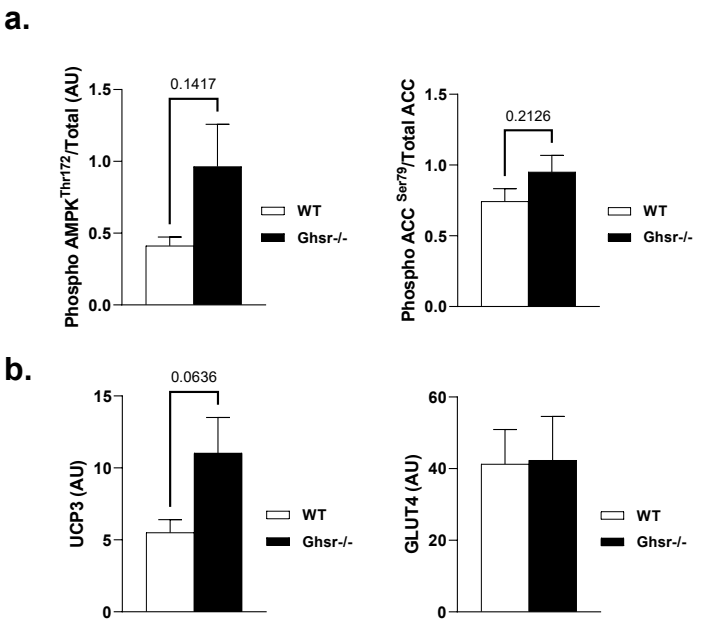

Figure 5.3 Effects of GHS-R ablation on protein markers of metabolic dysfunction of old (18–26m) wild type (open bar, n=4) and GHS-R-/- (black bar ,n=3) gastrocnemius muscle in mice. (a) Activation of AMPK and ACC expressed as phosphorylated to total protein ratios. (b) Total protein expression of UCP3 and GLUT4 in gastrocnemius muscle. Data are presented as means ± standard error.

5.3.3. GHS-R ablation alters expression of myosin heavy chain in skeletal muscle and improves treadmill performance of old mice

In aging populations there is a consistent shift in skeletal muscle fiber type to a more oxidative fiber type, i.e. Type 2 to Type 1 [316–318]. In both the gastrocnemius (Figure 4a) and soleus muscle (Figure 5.4b), mRNA content was higher for myosin heavy chain IIa (MHC-IIa) in the old *Ghsr*$^{-/-}$ compared to wild type. Fiber typing of the gastrocnemius myofibrillar rich fraction (Figure 5.4d) is consistent with our mRNA data demonstrating that there is a phenotypic shift toward MHC-IIa (with lower in MHC-IIb) in the *Ghsr*$^{-/-}$ group when compared to their WT counterparts (Figure 5.4d).

Interestingly, in soleus muscle myosin heavy chain I (MHC-I) was lower with GHS-R ablation when compared to wild type. Taken together, this implicates a maintenance of a more oxidative (fast) fiber type in the old *Ghsr*$^{-/-}$ mice in both gastrocnemius and soleus muscles. The observed mRNA alterations and fiber type analysis are further supported by the muscle function treadmill test (Figure 5.3c). While there were trends of increased (no statistical significance) running time and distance traveled between the genotypes, *Ghsr*$^{-/-}$ mice were able to generate higher work output than the aged wild type in a treadmill test.

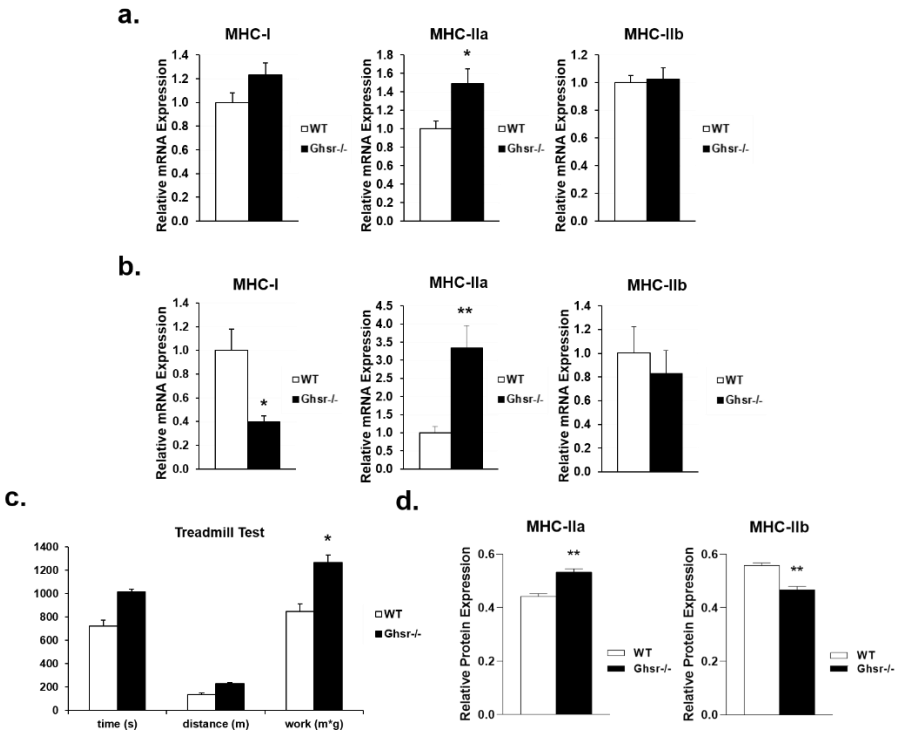

Figure 5.4 Relative mRNA expression of myosin heavy chain subtypes and treadmill work output of old (16–24 m) wild type (open bar) and Ghsr-/- (closed bar) mice. (a) Relative mRNA expression of myosin heavy chain in gastrocnemius (n = 9). (b) Relative mRNA expression of myosin heavy chain in soleus muscle (n = 6). (c) Treadmill time, distance and work of old wild type and Ghsr-/- mice (n = 10). (d) Protein expression of myosin heavy chain isoforms MHC-IIa and MHC-IIb relative to total Myosin heavy chain expression of old wild type (n=4) and Ghsr-/- (n=3) mice. Data are presented as means ± standard error. *$p < 0.05$, **$p < 0.001$.

5.3.4. Irisin/FNDC5 expression is higher in old GHS-R knockout mice

Irisin, a cytokine found in multiple tissues, is the product of the gene FNDC5. Skeletal muscle is currently touted as the largest reservoir for FNDC5, therefore, muscle is considered a major source of circulating irisin and irisin is called a myokine. It is known that an increase in irisin can directly modulate skeletal muscle and other tissues, and irisin is associated with exercise performance due to its effects on mitochondrial function and glucose uptake [334,335]. Our results indicate that FNDC5 is reduced in

skeletal muscle of middle-aged and old mice when compared to young animals. Interestingly, there was an increased FNDC5 mRNA expression in the gastrocnemius muscle and elevated circulating Irisin levels in old *Ghsr*$^{-/-}$ mice (Figure 5.5), which is in line with the improved metabolic profiles observed in the aged *Ghsr*$^{-/-}$ model.

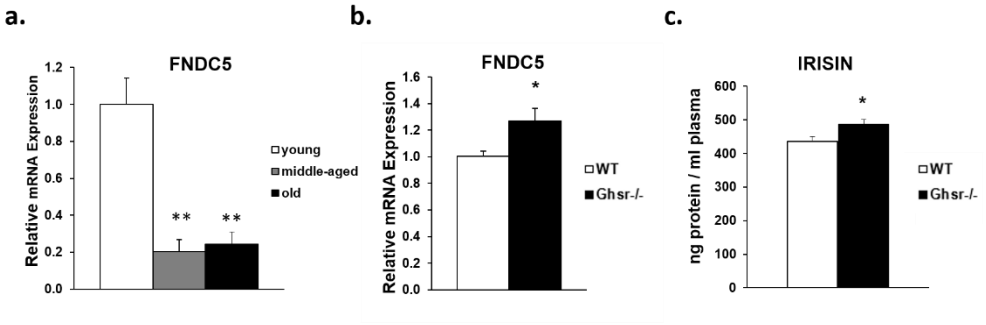

Figure 5.5 Relative mRNA expression of FNDC5 in gastrocnemius muscle and plasma irisin content. (a) FNDC5 mRNA expression in young (4–5m), middle-aged (12–14m) and old (18–26m) wild-type mice (n = 6). (b) FNDC5 mRNA expression in gastrocnemius muscle of old wild-type (open bar) and Ghsr-/- (filled bar) mice. (c) Plasma irisin levels in old wild-type (open bar) vs. Ghsr-/- (filled bar) mice. Data are presented as means ± standard error. *$p < 0.05$, **$p < 0.001$.

5.4. Discussion

While it is clear that muscular aging often leads to losses of muscle mass and metabolic dysfunction, the understanding of how this progression occurs is still widely debated. Unlike ghrelin, which is ubiquitously expressed, the ghrelin receptor GHS-R is much more restricted in where it is expressed and in what quantities it is expressed [23,28]. Despite this restricted expression, it appears in our current study as well as previous reports [16,23,326] that GHS-R expression increases in aging. This suggests

that ghrelin signaling may be involved in the functional decline of aging. Previous work in our lab has implicated that GHS-R is involved in the adiposity and insulin resistance that occurs with advancing age [326], but much of our work was focused on adipose tissue [22,326,327]. The current study specifically underscores that the ablation of GHS-R impacts the metabolic profile of skeletal muscle, showing a rescue of mitochondrial and glucose uptake genes in aged skeletal muscle of GHS-R knockout mice.

A common factor in aged skeletal muscle is the change in fiber composition to a relatively slow, more oxidative fiber type [316–318]. This appears to be from a preferential atrophy and eventual loss of type 2 muscle fiber types with advancement of age [336,337]. In the present study, we found that the aged *Ghsr*$^{-/-}$ animals had higher MHC-IIa mRNA content in both gastrocnemius and soleus muscle, suggesting that the knockout of GHS-R mitigates the loss of these fiber types with advancing age. This was supported through the fiber type analysis implicating an increase in the more oxidative MHC-IIa fiber type in the *Ghsr*-/- aged mice compared to wild type. However, we also found that *Ghsr*$^{-/-}$ blocked the potential transition toward slower muscle types, as evidenced by the loss of type I mRNA in soleus muscle. This suggest that the shift of fiber types and potential loss of type II fibers is attenuated with the ablation of GHS-R. Recent work reported similar results with ghrelin deletion, with aged ghrelin KO mice having an increased number of type IIa muscle fibers [12]. Similarly, observation of higher type IIa muscle fiber with *Ghsr*$^{-/-}$, was further supported by the treadmill function data, which showed the *Ghsr*$^{-/-}$ animals had a higher overall work output. It has been suggested that reprogramming of transcription factors involved in mitochondrial

biogenesis are linked to both white to brown fat beiging [110] as well as a fiber type shift [299,338,339] supporting the links between GHS-R ablation, a rescue of MHC-IIa fiber type and the thermogenic shifts observed potentially through irisin.

This relationship of skeletal muscle FNDC5 and circulating irisin in GHS-R ablated mice is perhaps the most interesting finding from this data. First, we found that FNDC5 is down-regulated on old animals, suggesting irisin is a pathogenic regulator for muscle with aging. Findings from the current work are consistent with work by other showing lower serum irisin levels observed in middle-aged and old human subjects compared to their younger counterparts [25,26]**.** Second, the effect of irisin on skeletal muscle metabolism is linked to ghrelin signaling through GHS-R. Irisin is a current target molecule for obesity and insulin resistance which often accompanies the aging process. Recent studies argue that irisin is somehow linked to exercise despite the continued debate on whether exercise impacts FNDC5 [340–342]. It is generally agreed that irisin concentration is linked to muscle mass, strength and skeletal muscle metabolism [340] with muscle mass being the best known predictor of irisin plasma circulation in humans [25]. The relationship to exercise is still unclear but it has been reported that exogenous irisin has similar whole-body effect to that of free wheel running [341]. That concept is further supported by *in vitro* studies , which have observed increased gene expression for both glucose uptake and fatty acid oxidation with exogenous irisin [334,343] similar to what was found in the present study in GHS-R ablated old mice. This modulation is proposed to be through AMPK phosphorylation and its downstream cascade which includes PGC-1α. This may indicate that irisin's

positive effects in skeletal muscle may be part of a positive feedback loop of GHS-R - Irisin - AMPK- PGC-1α.

The best known effects of irisin are related to its impact on the browning or beiging of white adipose tissue [25,121]. It was originally proposed that a proteosome cleaved irisin from FNDC5 [24,121], allowing it to be released into the blood to the adipose tissue, where it activates thermogenesis which leads to increased energy expenditure. Recently, however, the role of muscle as the main source of irisin has been called into question due to the observation that adipose tissue-expressed FNDC5 is more reflective of circulating irisin [340,342]. We previously reported that *Ghsr*$^{-/-}$ mice have increased energy expenditure and enhanced thermogenesis [327] and that GHS-R knockdown in brown adipocytes activates thermogenic signaling [22], indicating that thermogenesis is regulated by GHS-R and GHS-R has cell-autonomous effects at least in brown adipose tissue. While the focus of the current study is not adipose tissue, this study demonstrated the novel finding that GHS-R ablation increased FNDC5 in skeletal muscle and elevates circulating irisin in aged mice. These data support the current hypothesis that the increase in FNDC5 and subsequent increase in circulating irisin increased AMPK signaling in the muscle of old *ghsr*$^{-/-}$ mice, which has also been observed with the administration of exogenous irisin *in vitro* [344]. It appears that regulation of circulating irisin levels is multifaceted but that GHS-R may be one negative regulator of this cascade as evidenced by the increase of circulating irisin with GHS-R ablation While more research is warranted, the current data provide a

potentially new mechanism that connects GHS-R to irisin in muscle, and thus may indirectly affect muscle metabolism with aging.

While our novel observation that GHS-R at least partially regulates the irisin pathway in aging is novel, we recognize there are several limitations. The first limitation is that much of the data provided are relative mRNA content, which may or may not reflect the protein content of these specific target molecules. While the protein content data provided reinforces the mRNA data, further studies would need to expand upon the current data to further bolster the current work. Additionally, given that the current understanding of irisin is very fragmented and the concern that rodent and human models store and respond differently to irisin and its precursor FNDC5 [25,345], more studies are warranted to further understand whether the GHS-R and irisin connection here translate to humans. While the current study did not provide the complete picture of how GHS-R regulates muscle metabolism in aging, the data do provide a foundation for future inquiry.

In conclusion, the present work confirms that GHS-R is at least partially responsible for the metabolic decline observed in old skeletal muscle. Secondly, the suppression of GHS-R mitigates the metabolic decline of skeletal muscle with advancing age. Further studies are warranted to verify the mechanistic network between GHS-R, irisin, and muscle metabolism.

6. CONCLUSIONS

This dissertation was designed to determine the direct action of the two isoforms of ghrelin, acylated (AG), and unacylated (UAG), on skeletal muscle and whether that action is related to the development or slowing of the metabolic disturbances associated with advancing age and type 2 diabetes (T2D). These results indicate that the negative impact of AG on glucose metabolism *in vivo* is at least partially due to direct action on skeletal muscle glucoregulatory signaling, most likely through alterations of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). This increase in 4E-BP1 activity was not accompanied by an increase in phosphorylation of ribosomal protein s6 kinase 1 (S6K1), nor an increase in myofibrillar protein synthesis rates, supporting our conclusion that this increase in phosphorylation of 4E-BP1 was through an albeit unknown mTORC1-independent pathway. Additionally, these experiments called attention to the necessity of monitoring ghrelin presence for the duration of an investigation when attributing outcomes to ghrelin action due to the rapid de-acylation and degradation observed. Time course data revealed a short-lived but still substantial impact of acylated ghrelin on skeletal muscle glucose uptake that was correlated with its availability in the media. This discovery highlights that AG action is dependent on its availability over time, which implicates alterations that increased secretion and/or decreased degradation of AG could directly impact the severity of ghrelin-associated adverse outcomes.

The observed positive actions attributed to unacylated ghrelin *in vivo* were less convincing in our cell culture model. While the data, specifically the acute time course data, alluded to AMP-activated protein kinase (AMPK) playing a role in the unaltered glucose uptake observed with UAG compared to control, we did not see definitive evidence of insulin-independent signaling for glucose uptake with our 48h incubations. While the lack of compelling evidence for direct action of UAG on skeletal muscle glucoregulatory signaling differed from previous work, it was at least consistent with previous work that normal glucose tolerance was observed in the presence of UAG. This suggests that UAG action toward the restoration of glucose tolerance may become more pronounced in skeletal muscle experiencing dysregulation and could explain the lack of differences observed in our model.

This dissertation also provides preliminary evidence for alternative explanations of ghrelin involvement in the metabolic dysfunction observed with advancing age. Ablation of the ghrelin receptor gene GHS-R, which encodes the only known receptor for AG, attenuated the development of mitochondrial and glucoregulatory dysfunction in old mice. This work provides evidence that GHS-R regulates FNDC5 expression and circulating irisin, an adipo-myokine associated with the positive outcomes of aerobic exercise, including increased glucose uptake, mitochondrial biogenesis, and thermogenesis. We also found that GHS-R ablation and the concurrent rise of irisin inhibited the typical fiber type shift observed with advancing age. Future investigations to discern the primary facilitators of these outcomes are warranted. Specifically, determining whether a skeletal muscle-specific GHS-R ablation, in culture, produces

similar outcomes to our *in vivo* studies are needed. Further, mechanistic studies designed to determine if these outcomes are dependent on the observed rise in FNDC5/irisin would provide definitive evidence of which organs/tissues the ghrelin-GHS-R signaling axis is critical and whether these same positive outcomes observed with GHS-R ablation could be rectified with exogenous irisin regardless of GHS-R presence.

In summary, these studies indicate that ghrelin action on skeletal muscle is present but may be less influential than ghrelin action on other organs. These data do not question the positive outcomes observed *in vivo* with UAG in perturbed states but suggests that the response of skeletal muscle is primarily due to signaling originating from other organs that subsequently impact skeletal muscle. It also supports the concept that skeletal muscle has a crucial role in some of the positive benefits associated with GHS-R ablation but again points to the lack of GHS-R signaling in other organs as being the primary instigator of whole-body positive outcomes.

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