



Unacylated ghrelin stimulates fatty acid oxidation to protect skeletal muscle against palmitate-induced impairment of insulin action in lean but not high-fat fed rats

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ABSTRACT

Background: Ghrelin is a gut hormone that spikes in circulation before mealtime. Recent findings suggest that both ghrelin isoforms stimulate skeletal muscle fatty acid oxidation, lending to the possibility that it may regulate skeletal muscle's handling of meal-derived substrates. It was hypothesized in the current study that ghrelin may preserve muscle insulin response during conditions of elevated saturated fatty acid (palmitate) availability by promoting its oxidation.

Methods and results: Soleus muscle strips were isolated from male rats to determine the direct effects of ghrelin isoforms on fatty acid oxidation, glucose uptake and insulin signaling. We demonstrate that unacylated ghrelin (UnAG) is the more potent stimulator of skeletal muscle fatty acid oxidation. Both isoforms of ghrelin generally protected muscle from impaired insulin-mediated phosphorylation of AKT Ser⁴⁷³ and Thr³⁰⁸, as well as downstream phosphorylation of AS160 Ser⁵⁸⁸ during high palmitate exposure. However, only UnAG was able to preserve insulin-stimulated glucose uptake during exposure to high palmitate concentrations. The use of etomoxir, an irreversible inhibitor of carnitine palmitoyl-transferase (CPT-1) abolished this protection, strongly suggesting that UnAG's stimulation of fatty acid oxidation may be essential to this protection. To our knowledge, we are also the first to investigate the impact of a chronic high-fat diet on ghrelin's actions in muscle. Following 6 wks of a high-fat diet, UnAG was unable to preserve insulin-stimulated signaling or glucose transport during an acute high palmitate exposure. UnAG was also unable to further stimulate 5' AMP-activated protein kinase (AMPK) or fatty acid oxidation during high palmitate exposure. Corticotropin-releasing hormone receptor-2 (CRF-2R) content was significantly decreased in muscle from high-fat fed animals, which may partially account for the loss of UnAG's effects.

Conclusions: UnAG is able to protect muscle from acute lipid exposure, likely due to its ability to stimulate fatty acid oxidation. This effect is lost in high-fat fed animals, implying a resistance to ghrelin at the level of the muscle. The underlying mechanisms accounting for ghrelin resistance in high fat-fed animals remain to be discovered.

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1. Introduction

Ghrelin is an appetite-stimulating peptide, and both acylated (AG) and unacylated (UnAG) ghrelin isoforms exhibit a large preprandial rise in the plasma [1]. By virtue of its mass, skeletal muscle clears a large proportion of postprandial substrates and is a key site for insulin action, recent research has examined whether ghrelin is able to regulate skeletal muscle's uptake and

utilization of glucose and fatty acids. In vivo studies suggest contrasting roles for ghrelin's effects on glucose metabolism, in that AG worsens [2–4] but UnAG improves [2,5] insulin-stimulated glucose disposal. However, with regards to AG infusion, confounding increases in circulating growth hormone (GH) may indirectly impair muscle's ability to uptake glucose [6]. Therefore, recent approaches have utilized isolated tissue to directly assess and compare the two bioactive forms of ghrelin. Data from our own laboratory has shown that ghrelin does not directly regulate glucose uptake, either independently or with insulin, in rat skeletal muscle [7]. However, examining ghrelin's

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direct effects on glucose uptake [7,8] has previously been assessed in the absence of fatty acids, which can alter fuel partitioning in resting skeletal muscle [9].

We and others have recently demonstrated that both ghrelin isoforms can stimulate fatty acid oxidation in myocytes and mature skeletal muscle [10,11]. Dysfunctional lipid metabolism and the accumulation of reactive lipid intermediates are regarded as causal factors in the impairments to insulin action in muscle [12,13]. As little as 1–4 h of exposure of human or rodent skeletal muscle to high physiological concentrations (~1–2 mM) of fatty acids can impair insulin signaling (e.g. activation of protein kinase B/AKT or AKT-substrate of 160 kDa: AS160) and insulin-stimulated glucose uptake, likely due in part to the accumulation of diacylglycerol and ceramides [14–16]. Given that saturated fatty acids such as palmitate can acutely impair muscle insulin signaling and insulin-stimulated glucose uptake [17], it is possible that any potential beneficial effects of ghrelin on glucose uptake would manifest during scenarios of increased fatty acid availability.

We postulated that ghrelin would promote the oxidation of saturated fatty acids and mitigate their detrimental effects on skeletal muscle insulin action. Furthermore, the interaction between obesity or high-fat diets and ghrelin's peripheral metabolic effects have been sparsely examined. More specifically, whether skeletal muscle becomes resistant to ghrelin's effects is unknown. We aimed to address this knowledge gap by also investigating ghrelin's effects in the context of a high-fat diet. The objectives of the present study were to determine whether i) ghrelin isoforms can further stimulate fatty acid oxidation when skeletal muscle is exposed to high saturated fatty acid (palmitate) concentrations (2 mM); ii) ghrelin isoforms can prevent acute high palmitate-induced impairment in skeletal muscle insulin signaling (AKT and AS160) and glucose uptake; iii) ghrelin's beneficial effects in preserving insulin action, if present, are due to its ability to stimulate the oxidation of fatty acids; and iv) whether skeletal muscle from high-fat fed rats becomes resistant to ghrelin's effects on glucose and fatty acid metabolism. We hypothesized that ghrelin would preserve insulin action in healthy skeletal muscle through its ability to stimulate palmitate oxidation, but that this protection would be lost in muscle derived from high-fat fed animals.

2. Methods

2.1. Animals

All procedures were approved by the Animal Care Committee at the University of Guelph and followed Canadian Council of Animal Care Guidelines. For experiments in healthy animals, male Sprague-Dawley rats were ordered from Charles River Laboratories (Quebec, ON, Canada) at approximately 5 weeks of age (~150–200 g). Rats were given ad libitum access to a chow diet (Teklad 2018 laboratory diet, Envigo) and water and acclimatized for one week prior to experiments. To avoid high levels of endogenous circulating ghrelin that normally occur in the morning, overnight fasted animals were allowed food access (re-fed) for a short duration at the start of their dark cycle, approximately 2–3 h prior to tissue collection, as confirmed previously [10]. Rats were anesthetized with an intraperitoneal bolus of pentobarbital sodium (6 mg per 100 g body mass) prior to all surgical procedures. A low-fat (10% kcal fat), sucrose-matched diet was used (Cat No. D12450J, Research Diets) as the control diet in a subset of animals (~3 weeks of age) during dietary intervention experiments. The other subset of rats (also ~3 weeks of age; ~50–75 g) were provided a 60% kcal fat diet (Cat No. D12492, Research Diets) ad libitum for 6 weeks.

2.2. Materials and reagents

Reagents, molecular weight markers and nitrocellulose membranes for western blots were purchased from BioRad (Mississauga, ON, Canada). Western lighting plus enhanced chemiluminescence (ECL) was purchased from PerkinElmer (NEL105001EA). The following antibodies were purchased from Cell Signaling: phospho-AKT Ser⁴⁷³ (Cat. No. 9271), phospho-AKT Thr³⁰⁸ (Cat. No. 9275), AKT (Cat. No. 9272), phospho-AS160 Ser⁵⁸⁸ (Cat. No. 8730), phospho-AMPK Thr¹⁷² (Cat. No. 2531S), AMPK (Cat. No. 2603S), phospho-ACC Ser⁷⁹ (Cat. No. 3661) and ACC (Cat. No. 3662). CRF-2 receptor (Cat. No. Ab104368) and GHS-R1 (Cat. No. Sc-374515) antibodies were sourced from Abcam and Santa Cruz Biotechnology, respectively. AS160 antibody (Cat. No. 07–741) was purchased from Millipore Sigma. NP40 cell lysis buffer (Cat. No. FNN0021) was obtained from Life Technologies and PMSF (Cat. No. 78830) and protease inhibitor (Cat. No. 9599) were obtained from Millipore Sigma. Insulin (Humulin rDNA origin) was purchased from Eli Lilly (Toronto, ON, Canada). Synthetic acylated (Cat. No. H-4862) and unacylated (Cat. No. H-6264) ghrelin were ordered from Bachem (Torrance, CA, USA). For skeletal muscle incubations, fatty-acid free bovine serum albumin (Cat. No. 10775835001), palmitic acid (Cat. No. P0500), Dulbecco's modified eagle's media (DMEM; Cat. No. D5030), D-glucose (Cat. No. G-8270), D-mannitol (Cat. No. M – 9546), sodium pyruvate (Cat. No. P8574) and 2-deoxyglucose (Cat. No. D8375) were all obtained from Millipore Sigma. Radio-labeled palmitic acid (Cat. No. 0172A), D-mannitol (Cat. No. 0127) and deoxy-D-glucose (Cat. No. 0103) were all purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA).

2.3. Fasting blood glucose and glucose tolerance tests

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

2.4. Fatty acid oxidation

All incubations were carried out in a heated (30 °C), gassed (95% O₂, 5% CO₂) shaking water bath as previously described [10] and the experimental design was identical for experiments with low and high-fat fed animals. Briefly, soleus muscle strips were excised tendon to tendon and pre-incubated for 30 min in DMEM supplemented with 8 mM glucose prior to being transferred into a flask containing either low (0.2 mM) or high (2 mM) palmitate. Following 2 h in buffer containing non-radiolabeled palmitate, muscles were transferred into vials containing media with labeled 0.5 mCi/mL ¹⁴C-palmitic acid for the measurement of palmitate oxidation (2 h). Following the 2 h incubation, the muscle was carefully removed from the flask, trimmed of its tendons and weighed. The muscle strip was then re-added to the flask with which 250 μL of benzethonium hydroxide was added to an eppendorf tube inside and 1 mL of 1 M sulfuric acid was added via syringe to both incubation media and muscle. The flask with acidified media and muscle was left at room temperature for 2 h to allow for the radiolabeled ¹⁴CO₂ gas to be liberated and captured in the benzethonium hydroxide solution. The eppendorf containing trapped ¹⁴CO₂ was placed in a vial containing scintillation cocktail to quench overnight prior to counting using a PerkinElmer Tri-Carb LSC 4910 TR liquid scintillation counter. Samples were counted for 5 min.

2.5. Glucose uptake

Glucose uptake was determined as previously described [7,18,19]. Briefly, incubation media were pre-gassed (95% O₂, 5% CO₂) in a heated (30 °C), shaking water bath. Given that soleus muscle can be reliably stripped lengthwise into 3 viable, 20–30 mg sections, one strip (from a leg of one animal) was used for each treatment i.e. each full set of treatments could be obtained from the muscles of one animal. Muscles were left to freely float in incubation media for the duration of the experiment. Muscle strips were pre-incubated in medium containing 8 mM D-glucose and 32 mM D-mannitol for 30 min. Following pre-incubation, muscles were transferred to medium containing either low (0.2 mM) or high (2 mM) palmitate concentrations for 4 h. Two hours into the 4 h palmitate exposure, media was re-gassed with 95% O₂, 5% CO₂ gas. Following palmitate exposure, muscles were washed in media containing 36 mM D-mannitol and 4 mM pyruvate for 30 min prior to being transferred into the final buffer containing 28 mM D-mannitol, 8 mM 2-deoxyglucose, radiolabeled 1 mCi/mL [1,2]-³H-2-deoxy-D-glucose and 0.1 mCi/mL [1]-¹⁴C-mannitol for 30 min. Acylated and unacylated ghrelin (150 ng/ml) were added to the appropriate vials for the 4 h palmitate exposure onwards, whereas insulin (10mU/ml) was only added during the wash (following 4 h palmitate) and the final radioactive glucose uptake buffer. Following the final uptake buffer, muscle strips were trimmed of their tendons, blotted dry and weighed. Muscles were subsequently boiled and digested in 1 M sodium hydroxide solution (~10 min, swirled periodically). Muscle homogenate was then sampled (200 µL) to which 5 mL of Cytoscint scintillation cocktail (MP Biomedicals, Burlington, ON, Canada) was added. Samples were shaken vigorously and left to quench overnight in complete darkness. The next day, samples were counted (5 min/sample) using a PerkinElmer Tri-Carb LSC 4910 TR liquid scintillation counter. Glucose uptake was calculated as the accumulation of intracellularly labeled glucose as previously reported [7].

2.6. Glucose uptake with etomoxir

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

2.7. Western blotting

Incubations to assess protein signaling were carried out similarly to the functional measures of glucose uptake and palmitate oxidation. Briefly, for incubations assessing insulin-signaling, muscles were pre-incubated in DMEM for 30 min. Muscles were then transferred to their respective palmitate vials (0.2 mM vs. 2 mM) for 4 h prior to being washed for 30 min before undergoing insulin treatment (30 min). For incubations assessing fatty acid oxidation signaling, muscles were pre-incubated in DMEM for 30 min. Muscles were subsequently transferred to palmitate exposure vials (with or without ghrelin) for 1 h. Muscles were then weighed, frozen in liquid N₂ and stored at –80 °C until further analyses. ~20–30 mg muscle strips were homogenized (Qiagen TissueLyser LT) in 500 µL of ice-cold cell lysis buffer (containing Na₃VO₄ and NaF) that was supplemented with PMSF and P_i to minimize the action of both serine and threonine proteases and phosphatases. Homogenization was 3 × 3 min on ice. Samples were

then centrifuged at 1500 g for 15 min. The supernatant was removed, and protein concentrations were assessed using the bicinchoninic acid assay method [20]. Equal amounts of proteins were separated by molecular weight via electrophoresis on 10% acrylamide gels (5% for ACC, 7.5% for AS160) and then wet transferred at 4 °C onto nitrocellulose membranes for 1 h at 100V. Membranes were subsequently blocked in (5%) skim milk powder + TBST for 1 h before being washed in TBST for 10 min. Primary antibody (1:1000) was then added to the membrane and left overnight in a dark, 4 °C cold-room. The following day, membranes were washed in TBST (2 × 15 min) and incubated in secondary antibody (1:2000), shaking at room temperature for 1 h. Next, membranes were washed in TBST (2 × 15 min) and then once in TBS (10 min). Bands were visualized using ECL and quantified using Alpha Innotech Software. Vinculin was used as a loading control. Western blots are shown as the quotient of phosphorylated to total protein where appropriate.

2.8. Statistics

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

3. Results

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

3.1.1. Palmitate impairs insulin activation of AKT and downstream AS160

Similar to what has been shown previously shown [16,17], the present work using isolated rodent soleus muscle demonstrated that 4 h exposure to 2 mM of saturated fatty acids (high palmitate; HP) acutely impaired insulin signaling. Specifically, palmitate significantly impaired insulin's (10mU/ml) ability to increase phosphorylation of AKT at both Ser⁴⁷³ and Thr³⁰⁸, as well as AS160 phosphorylation at Ser⁵⁸⁸ (*p* < 0.05), when compared to low palmitate (LP; 0.2 mM) control (Fig. 1).

3.1.2. Ghrelin preserves skeletal muscle insulin action following high palmitate exposure

Insulin-mediated phosphorylation of AKT and AS160 (*p* < 0.05) following the acute 4 h exposure to HP was preserved when AG was present, such that HP+ins+AG activation of AKT and AS160 were not different (*p* > 0.05) from the insulin-stimulated control group, LP+ins (Fig. 1). UnAG also preserved insulin activation of AS160 (*p* < 0.05) and AKT at its Thr³⁰⁸ (*p* < 0.05), but not Ser⁴⁷³ (*p* > 0.05) residue.

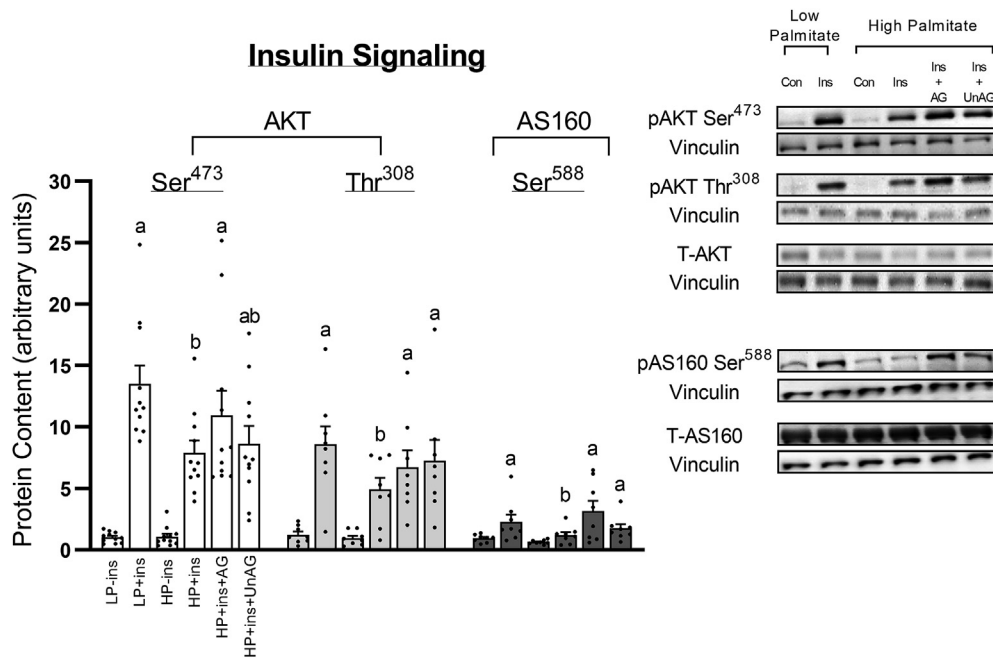


Fig. 1. Insulin Signaling: The activation of insulin signaling protein AKT at phosphorylation sites Ser⁴⁷³ and Thr³⁰⁸ as well as AS160 at site Ser⁵⁸⁸ in isolated soleus muscle following muscle exposure to low (LP; 0.2mM) or high (HP; 2mM) palmitate concentrations with or without AG/UnAG (150ng/ml) and insulin (10mU/ml). Data were analyzed using a repeated measure one-way ANOVA (n = 8-11) and expressed as individual data points and the mean \pm standard error, in arbitrary protein units (phospho/total). Data sharing a letter are not statistically different from each other. P < 0.05 was considered statistically significant.

3.1.3. Only unacylated ghrelin preserves insulin-stimulated glucose uptake in the presence of high palmitate

Insulin significantly increased glucose uptake following a 4 h exposure to LP control (p < 0.001; Fig. 2A). In agreement with insulin signaling data, 4 h exposure to HP impaired insulin-stimulated glucose uptake (p < 0.001). UnAG led to a significantly greater insulin-stimulated glucose uptake following HP exposure (p < 0.01) when compared to HP alone, indicating a protective effect. However, despite its ability to generally preserve insulin signaling, AG was unable to protect muscle's functional response to insulin i.e. insulin-stimulated glucose uptake (p > 0.05) following the acute HP exposure.

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

Fatty acid oxidation was significantly increased (p < 0.0001) in the presence of high vs. low palmitate (Fig. 2B). We built upon previous work from our laboratory which used 1 mM palmitate [10], in demonstrating that ghrelin maintains its ability to further stimulate fatty acid oxidation with a higher (2 mM) palmitate concentration (AG: p < 0.005; UnAG: p < 0.0001). Also, following the addition of ghrelin isoforms to incubation media, their stimulatory effects on oxidation were significantly greater in the latter half (i.e. t = 2–4 h; p < 0.01) of the exposure to palmitate. UnAG was a more effective stimulus for fatty acid oxidation compared to AG (p < 0.005). UnAG significantly increased rates of palmitate oxidation by 40% at t = 0–2 h (p < 0.05) and by 78% at t = 2–4 h (p < 0.01) versus HP alone, compared to the ~28% increase in rates of oxidation achieved by AG during t = 0–2 h (p < 0.01) and the ~40% increase (p < 0.001) during t = 2–4 h.

3.1.5. The ability of UnAG to preserve insulin-stimulated glucose uptake depends on its stimulation of fatty acid oxidation

We sought to determine whether ghrelin's beneficial effects on insulin-stimulated glucose uptake were dependent on its ability to

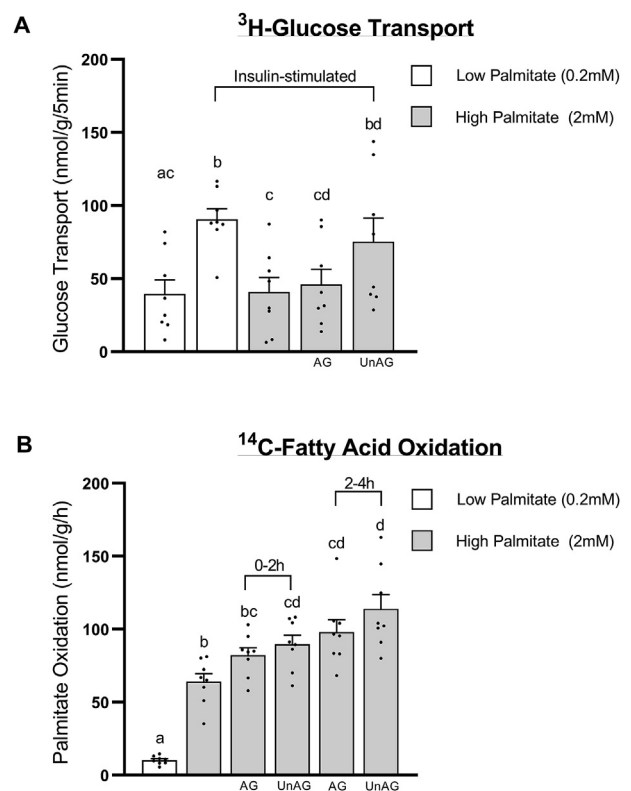


Fig. 2. Glucose Uptake and Palmitate Oxidation: Rates of basal and insulin-stimulated (10mU/ml) glucose uptake (A) and palmitate oxidation (B) following the exposure of isolated soleus muscle to either low (LP) or high (HP) palmitate concentrations with or without AG/UnAG (150ng/ml). Data were analyzed using a repeated measure one-way ANOVA (n=8 for each) and expressed as individual data points and the mean \pm standard error. Data sharing a letter are not statistically different from each other. P < 0.05 was considered statistically significant.

stimulate fatty acid oxidation, by utilizing etomoxir, an irreversible inhibitor of CPT-1 [21,22]. Pilot work with etomoxir demonstrated that, in our isolated muscle incubation preparation, 100 μ M was an effective dose to reduce palmitate oxidation by ~50% ($p < 0.05$; Fig. 3A). Furthermore, etomoxir inhibited fatty acid oxidation ($p < 0.01$) during the latter 2 h of the (4 h total) muscle exposure to palmitate (Fig. 3B), while the initial 2 h exposure (0–2 h) to etomoxir was ineffective ($p > 0.05$). Consistent with initial findings, HP exposure significantly reduced insulin-stimulated glucose uptake versus LP ($p < 0.01$; Fig. 3C). During HP exposure, soleus muscle concurrently exposed to UnAG ($p < 0.005$; Fig. 3C), but not AG ($p > 0.05$; Fig. 3C) demonstrated significantly higher rates of insulin-stimulated glucose transport versus HP alone. However, UnAG was no longer able to preserve insulin-stimulated glucose uptake in muscle exposed to HP when co-treated with etomoxir, compared to HP alone ($p > 0.05$; Fig. 3C). Etomoxir's presence had no effect on rates of insulin-stimulated glucose transport during HP

exposure with AG ($p > 0.05$; Fig. 3C).

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

3.2.1. 6-Weeks of high-fat diet causes weight gain and glucose intolerance

There were significant main effects of both diet ($p < 0.0001$) and time ($p < 0.0001$) on both food and energy intake over the 6-week diet intervention (Fig. 4A and B). Low-fat diet (LFD) animals consumed more food in grams than high-fat diet (HFD) animals ($p < 0.05$), at all timepoints. However, apart from week 1 ($p < 0.05$), HFD animals had significantly greater energy intake (kcal) during each week of the dietary intervention ($p < 0.001$). Over the 6-week dietary protocol, there was a statistically significant interaction between the effects of diet and time on body weight ($p < 0.0001$; Fig. 4C). There was a significant main effect of both time

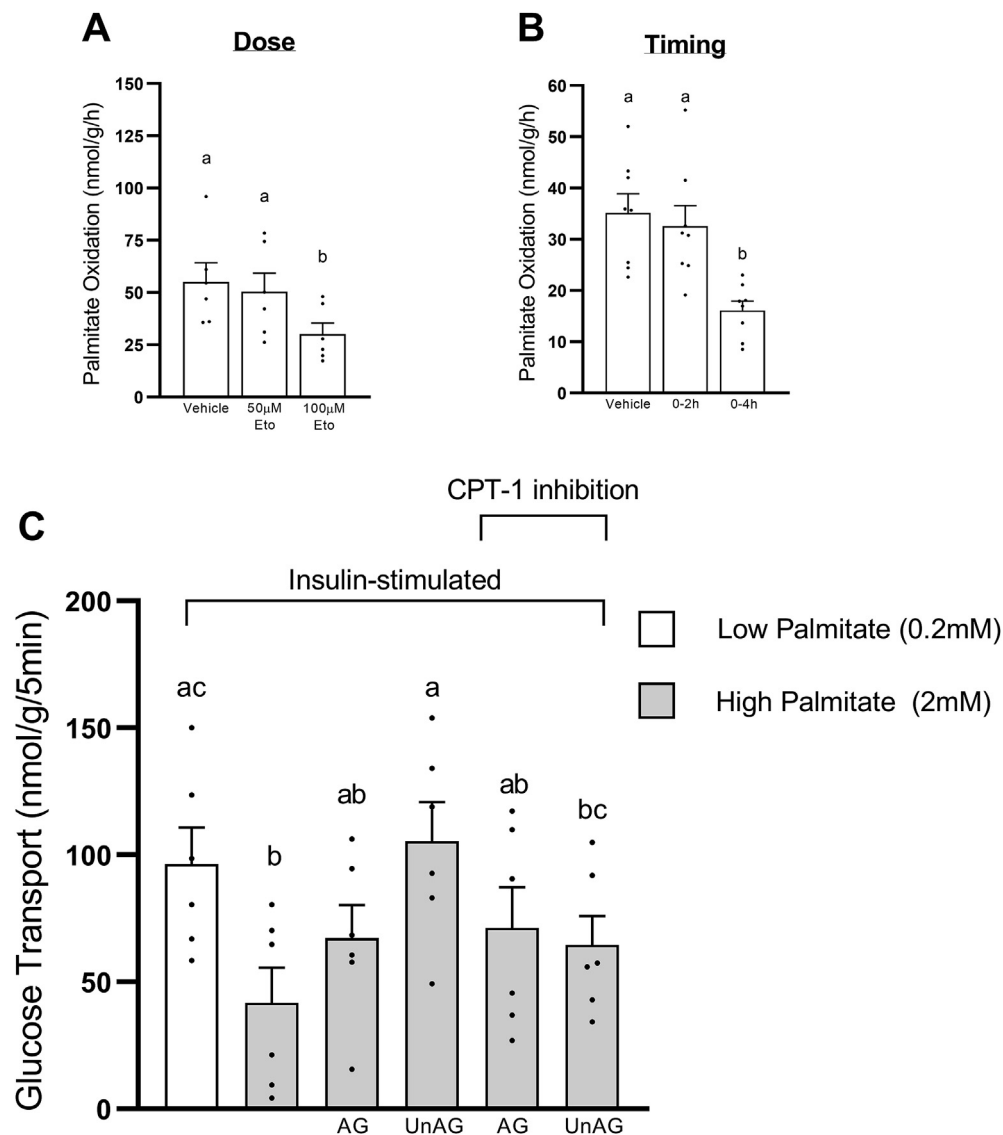


Fig. 3. Inhibition of Fatty Acid Oxidation: The effects of etomoxir (CPT-1 inhibitor) dose (A) and exposure time (B) or vehicle (DMSO) on rates of palmitate oxidation in isolated soleus muscle during an exposure to 2mM palmitate. Rates of insulin-stimulated (10mU/ml) glucose transport (C) were also assessed following 4 h low (LP) or high (HP) palmitate exposure with or without AG/UnAG (150ng/ml) in the presence or absence of 100 μ M etomoxir (4 h). Data were analyzed using a repeated measure one-way ANOVA ($n = 6-8$) and expressed as individual data points and the mean \pm standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

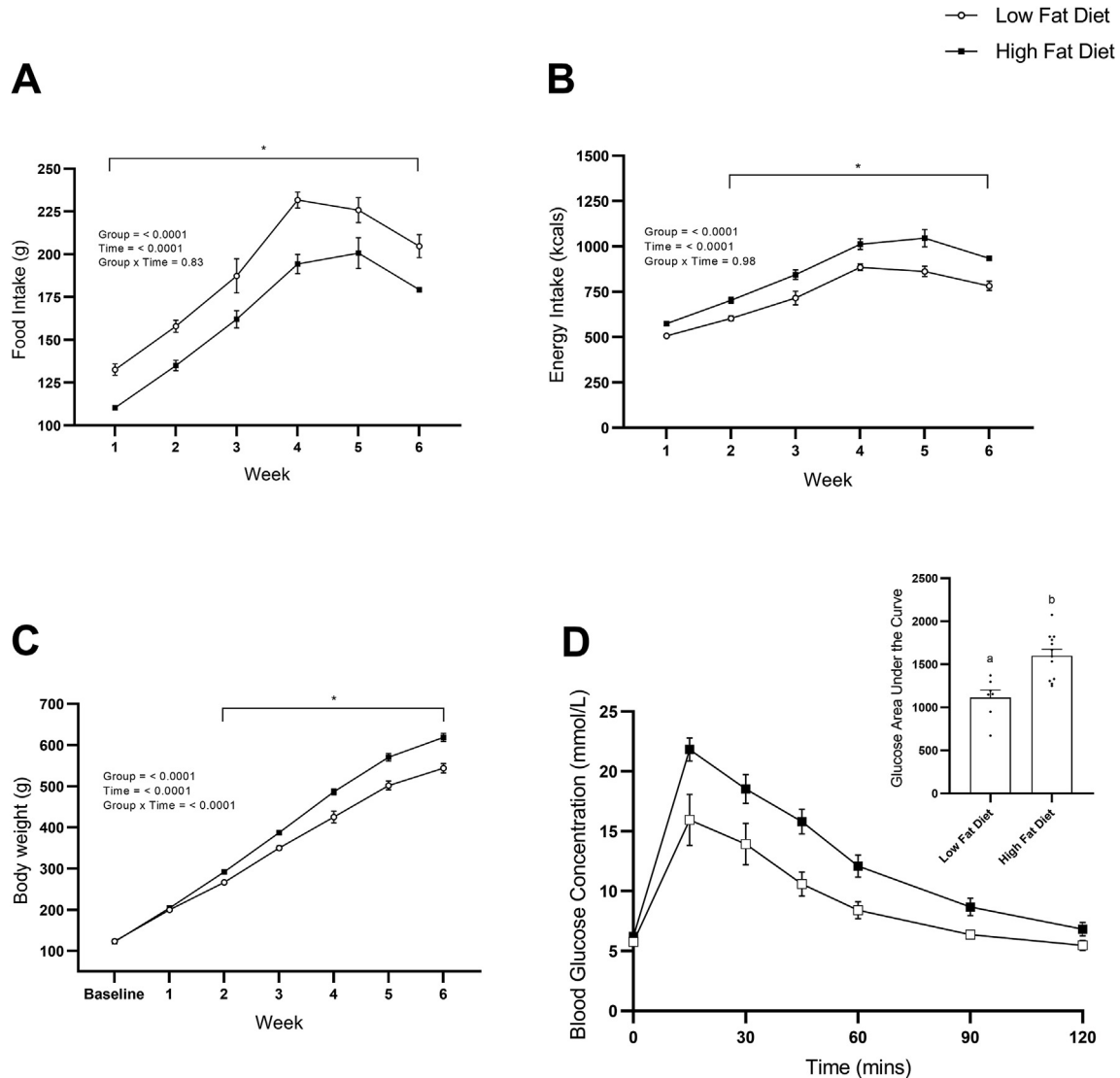


Fig. 4. Whole-Body Measurements: Food (A) and energy (B) intake as well as body weights (C) during a 6-week dietary intervention providing rats with diet either low (10%) or high (60%) in kcal from fat, ad libitum. Following an overnight fast, blood glucose response was tracked following an IPGTT (D) at the end of 6-weeks on specified diet. Food, energy intake and body weight data were analyzed using a two-way (main effects and interaction displayed in text) ANOVA ($n=8-12$). Glucose AUC data were analyzed using an unpaired t test (LFD: $n=7$; HFD: $n=12$). All data were expressed as individual data points and the mean \pm standard error. An asterisk (*) depicts a significant difference at a given week between dietary groups (main effect; HFD vs. LFD). $P < 0.05$ was considered statistically significant.

($p < 0.0001$) and diet ($p < 0.0001$) on body weights. There were no differences in body weights between groups of animals at baseline ($p > 0.05$). By week 2, rats consuming the HFD weighed more than LFD animals ($p < 0.05$) and this difference persisted until week 6.

At the end of the 6-weeks, animals on the HFD had impaired glucose tolerance as evidenced by a significantly greater glucose AUC following an IPGTT compared to LFD controls ($p < 0.0001$; Fig. 4D). HFD ($n = 11$; 6.3 ± 0.20 mM) animals also had significantly higher ($p < 0.05$) fasting blood glucose levels compared to LFD ($n = 7$; 5.8 ± 0.16 mM).

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

Ghrelin's metabolic effects in the context of obesity or high-fat feeding are relatively unknown. Therefore, we examined ghrelin's ability to stimulate palmitate oxidation in soleus skeletal muscle from high-fat fed rats. As expected, exposure of muscle to HP

significantly increased rates of fatty acid oxidation compared to LP ($p < 0.0001$; Fig. 5A). In contrast to ghrelin's effects in healthy muscle, neither isoform was able to further stimulate fatty acid oxidation in the presence of HP in muscle derived from high-fat fed animals ($p > 0.05$). A maximal insulin concentration was still able to significantly increase glucose uptake ($\sim 46\%$) in muscle exposed to LP from high-fat rats ($p < 0.05$; Fig. 5B). However, insulin no longer stimulated glucose uptake in the presence of HP ($p > 0.05$); neither AG nor UnAG ($p > 0.05$) had any significant effect in mitigating the HP-induced impairment in insulin-stimulated glucose uptake.

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

AMPK and its downstream target ACC are important regulators of fat oxidation and their phosphorylation at key residues significantly alters their activity [23]. Our group has previously demonstrated that ghrelin can activate the AMPK axis, coinciding with an increase in fatty acid oxidation [10]. Exposure of soleus to HP

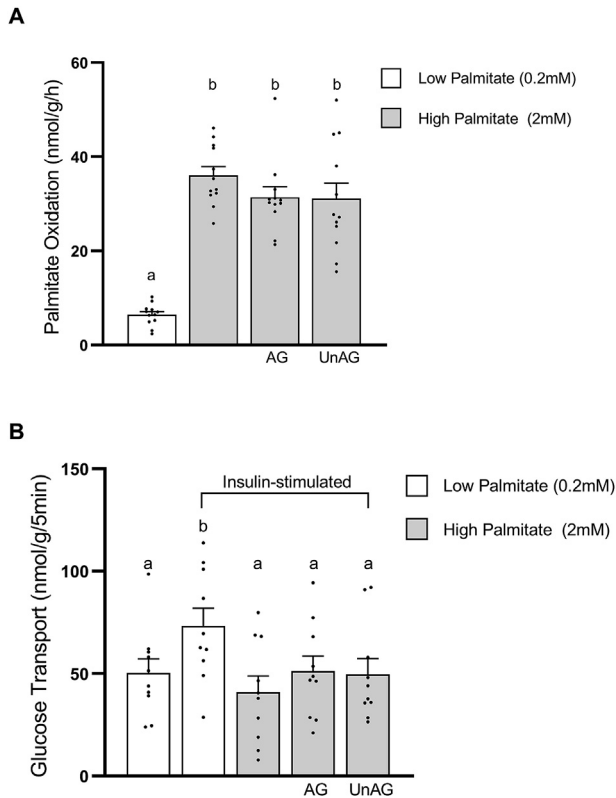


Fig. 5. Glucose Uptake and Palmitate Oxidation in High-Fat Fed Animals: Rates of palmitate oxidation (A) and basal or insulin-stimulated (10mU/ml) glucose transport (B) following either low (LP) or high (HP) palmitate exposure either with or without AG/UnAG, in isolated soleus muscle from 6-week high-fat fed animals. Data were analyzed using a repeated measure one-way ANOVA ($n=10-12$) and were expressed as individual data points and the mean \pm standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

resulted in increased phosphorylation/activation of AMPK compared to LP ($p < 0.05$; Fig. 6A), although this did not translate to an increase in ACC phosphorylation ($p > 0.05$). Neither AG nor UnAG had any effect on the phosphorylation of AMPK or ACC in soleus muscle from high-fat fed rats following HP exposure ($p > 0.05$).

3.2.4. 6 weeks of high-fat diet significantly reduces CRF-2R protein content in soleus muscle

Studies indicate that ghrelin may exert some of its metabolic effects in skeletal muscle by acting through the corticotropin-releasing hormone receptor-2 (CRF-2R) [8]. Interestingly, CRF-2R protein content was significantly lower in soleus skeletal muscle from animals after 6-weeks of high-fat diet feeding ($p < 0.05$; Fig. 6B). Also of interest was the GHS-R1 subtype (GHS-R1a) through which AG exerts many of its classical effects i.e., GH-release and appetite-stimulation [24,25]. GHS-R1 content was unchanged in soleus muscle from high-fat fed animals compared to low-fat fed animals ($p > 0.05$). The fatty acid transporter FABPpm was similarly unchanged in muscle from high-fat animals ($p > 0.05$).

4. Discussion

Current research interest in ghrelin extends beyond its orexigenic effects and has begun to explore its regulatory role on peripheral tissue glucose and fatty acid metabolism. Both acylated and unacylated ghrelin peak in the circulation immediately prior to anticipated mealtime, suggesting that ghrelin may affect substrate

uptake and metabolism in tissues such as skeletal muscle, which is a major sink for glucose and fatty acids. However, ghrelin's direct metabolic effects on muscle remain poorly understood. We have shown previously that ghrelin isoforms do not independently affect muscle glucose uptake [7] in the absence of fatty acids. However, ghrelin's direct effects on muscle metabolism have yet to be examined in the context of concurrent glucose and lipid availability, similar to a typical mixed meal. Recent work has demonstrated that ghrelin stimulates fatty acid oxidation [10,11]. Therefore, in the present study, we postulated that ghrelin's stimulation of fatty acid oxidation could protect muscle from lipid-induced impairment of insulin-stimulated glucose uptake. The current results demonstrate that both AG and UnAG further stimulate fatty acid oxidation in the presence of elevated palmitate and preserve muscle insulin signaling. While both ghrelin isoforms largely preserved insulin's activation of insulin signaling proteins (AKT, AS160), UnAG was exclusively responsible for preserving insulin-stimulated glucose uptake during the acute exposure to high palmitate concentrations. The protective effects of UnAG on muscle glucose uptake appear to be mediated through its greater ability to stimulate fat oxidation, as this protection was no longer evident with the CPT-1 inhibitor, etomoxir. Ghrelin's ability to preserve insulin signaling and glucose uptake in the presence of high palmitate was impaired in skeletal muscle derived from high-fat fed rats. This may be due to a decrease in skeletal muscle expression of CRF-2R protein content, a receptor that is thought to contribute to ghrelin's peripheral metabolic effects [8].

4.1. Ghrelin, lipids and their regulation of skeletal muscle insulin action

Skeletal muscle is important for blood glucose homeostasis, primarily due to its overall mass. Acute lipid oversupply can impair insulin-stimulated glucose uptake in muscle [26–28], which can last for several hours [28]. In the current study, 4 h of exposure to high palmitate impaired insulin-stimulated glucose uptake in muscle concurrent with reduced activation of the signaling proteins AKT and its downstream target AS160. AG consistently preserved insulin-stimulated AKT activation during the high palmitate exposure, whereas UnAG's effects were more variable on this marker. However, in terms of preserving a functional increase in insulin-stimulated glucose uptake, only UnAG was protective. Previous studies have also shown a dissociation between impaired insulin signaling and reductions in insulin-stimulated glucose uptake/clearance, in vivo [29,30]. This difference may potentially be explained by UnAG's ability to more effectively stimulate fatty acid oxidation. Numerous contributors have been proposed to mediate muscle insulin resistance following lipid oversupply. Accumulation of LCFA-CoA intermediates, as well as diacylglycerol (DAG) and ceramide have been implicated [31–33]. It is plausible that UnAG maintains an increase in insulin-stimulated glucose uptake during high palmitate exposure through its ability to stimulate the oxidation of palmitate and reduce intracellular accumulation of some of these lipid intermediates. However, in a previous study from our laboratory, UnAG did not divert labeled palmitate away from TAG or DAG [10]. The effect of ghrelin on muscle ceramide synthesis has yet to be examined during exposure to high fatty acids. Overall, UnAG appears to influence muscle insulin signaling through its stimulation of fatty acid oxidation, although it is worth noting that there are previous reports suggesting that UnAG is capable of directly modulating insulin action in the absence of lipid availability [5]. The effects of AG are more difficult to interpret. Insulin signaling, but not glucose uptake, was preserved in the presence of high palmitate. AG was also able to stimulate fatty acid oxidation, albeit less so than UnAG. This implies that ultimately, the

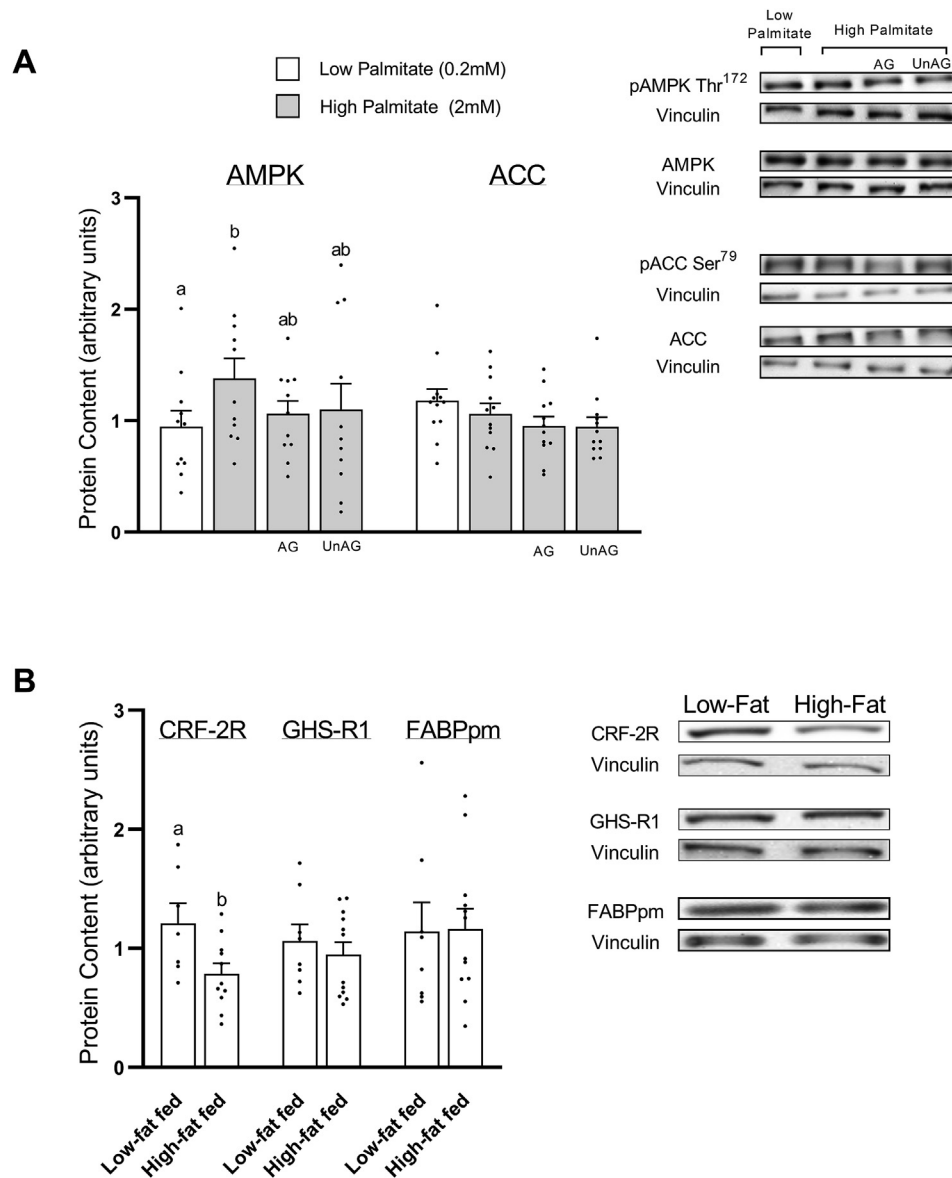


Fig. 6. Muscle Signaling in High-Fat Fed Animals: The phosphorylation (activation) of the cellular energy-sensing protein AMPK (Thr¹⁷²) and its downstream target ACC (Ser⁷⁹) following low (LP) or high (HP) palmitate exposure either with or without AG/UnAG treatment, in isolated soleus muscle from 6-week high fat-fed rats. Data were analyzed using a repeated measure one-way ANOVA (n=11-12) and were expressed as individual data points and the mean \pm standard error. Also shown, are CRF-2R and GHS-R1 receptors, as well as the fatty acid transporter FABPpm from soleus muscle of the same 6-week high-fat fed rats. Data were analyzed using an unpaired t test (n=7-12) and were expressed as individual data points and the mean \pm standard error. Data sharing a letter are not statistically different from each other. $P < 0.05$ was considered statistically significant.

ability to stimulate fatty acid oxidation may be critical to preserve glucose uptake, and that there is a disconnect between the degree of phosphorylation of key insulin signaling proteins and functional glucose uptake during muscle's acute exposure to high palmitate concentrations.

4.2. Ghrelin's regulation of substrate utilization

There are multiple points of regulation modulating skeletal muscle's reliance on fatty acids for fuel [34,35]. The literature assessing ghrelin's effects on muscle substrate oxidation and underlying mechanisms is sparse. Following a standardized meal, the *in vivo* infusion of AG reduces insulin sensitivity in humans, whereas the co-administration of both AG and UnAG may improve it [2]. Interestingly, the improvement in whole-body insulin sensitivity was paralleled by a reduction in circulating free fatty

acid levels [2], suggesting a ghrelin-mediated reduction in lipid mobilization, which has been observed previously [36]. Alternatively, ghrelin may be stimulating fatty acid uptake/clearance into peripheral tissues like skeletal muscle [2]. Since the effects of AG *in vivo* are often assessed secondary to subsequent rises in GH, and possibly the incretin GLP-1, the ability to draw conclusions on the direct action of ghrelin is partly confounded [2,37]. A single study has demonstrated that as little as 4 d of AG injection can reduce gastrocnemius TAG content and increase the expression of genes associated with skeletal muscle lipid utilization e.g. ACC, UCP2 [38]. However, the underlying mechanisms for this reduction in TAG content were not pursued [38]. Recently, research has aimed to uncover the direct effects of ghrelin in the regulation of lipid metabolism. Han et al. [11] and work from our own lab [10] demonstrate that ghrelin can stimulate fatty acid oxidation. Han et al. observed increases in AMPK activation in myoblasts treated

with AG [11], which contributed to the increase in fatty acid oxidation. Initially, we did not observe any changes to muscle AMPK activation when ghrelin's effects were assessed in the absence of fatty acid availability, although downstream ACC activation was not measured [7]. However, in another study, ghrelin isoforms stimulated the AMPK/ACC axis to a similar extent as AICAR (AMP analog), during exposure to 1 mM palmitate [10]. More work will be required to determine whether AMPK is an essential signal for the positive effects of ghrelin on fatty acid oxidation.

4.3. Ghrelin signaling in skeletal muscle and changes with high-fat feeding

The ability of ghrelin to stimulate fatty acid oxidation and preserve insulin-stimulated glucose uptake was no longer observed in muscle isolated from high-fat fed animals. This suggests that chronic muscle adaptations to the high-fat diet impacted either receptor content or sensitivity to ghrelin. AG signals through GHS-R1a in the central nervous system, anterior pituitary gland and other tissues [39,40]. However, there has yet to be any definitive receptor attributed to the widespread effects of UnAG [41–43]. Skeletal muscle exhibits relatively low expression of GHS-R1a, and although there are studies to suggest that GHS-R1a content may be altered in other peripheral tissues e.g. adipose tissue during aging [44], data depicting whether changes to receptor content occurs as a consequence to different dietary interventions is lacking. Results from the current study suggest that GHS-R1 content is unaltered in skeletal muscle following 6-weeks of high-fat feeding. However, our GHS-R1 analysis does not distinguish between *a* and *b* isoforms of the receptor. Since AG only binds and signals through the GHS-R1a receptor isoform, more specific GHS-R1 antibodies are required to draw definitive conclusions. More recently, some of ghrelin's direct metabolic effects have been attributed to the CRF-2R [8]. Interestingly, the total CRF-2R content was significantly lower in muscle from high-fat fed rats. Future investigations examining the contribution of this receptor to AG and UnAG's metabolic effects in peripheral tissue's like skeletal muscle are merited.

5. Limitations and considerations

In the current study, high-palmitate exposure was utilized to acutely induce defects in insulin action in skeletal muscle, as done previously [17]. However, the specific roles of LCFA-CoAs and ceramides, and potentially reactive oxygen species, were not assessed in the current study. Future investigations may aim to target the relative contribution of each of these insulin-desensitizing components and whether they are affected by ghrelin. Next, the exact signaling transducers for ghrelin's effects in skeletal muscle remain uncertain. As such, no cellular measurement was made to directly confirm the presence of ghrelin resistance which was observed in the functional outcomes of fatty acid oxidation and glucose transport. Lastly, while isolated incubations attempt to replicate *in vivo* scenarios of substrate and hormonal concentrations, due to tissue limitations, there was no treatment assessing the effects of simultaneous AG and UnAG muscle exposure, as would be observed prior to a meal, *in vivo*.

6. Conclusion

Taken together, these findings provide further evidence for ghrelin isoforms as direct regulators muscle substrate metabolism. UnAG is a stimulator of muscle fatty acid oxidation which likely contributes to its protective effects on insulin-stimulated glucose uptake and AKT/AS160 activation. Skeletal muscle from high-fat fed

rats is resistant to ghrelin's stimulatory effects on fatty acid oxidation and preservation of insulin-stimulated glucose transport. These findings agree with work in humans that demonstrate a role for UnAG in the lowering of systemic FFAs, particularly when co-administered with AG, which may link ghrelin's role in peripheral fatty acid metabolism to improvements in whole-body glucose homeostasis [2].

Declaration of competing interest

This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada under Grant #400535. No conflicts of interest declared.

CRedit authorship contribution statement

Daniel T. Cervone: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Barbora Hucik:** Investigation, Writing - review & editing. **Andrew J. Lovell:** Investigation. **David J. Dyck:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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DTC and DJD contributed to the conception of the study and designed the experiments. All authors assisted with measurements. DTC and DJD analyzed and interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.metop.2020.100026>.

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