



# The effects of diet and chronic exercise on skeletal muscle ghrelin response

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## ABSTRACT

**Background:** Recent findings indicate that ghrelin, particularly the unacylated form (UnAG), acutely stimulates skeletal muscle fatty acid oxidation (FAO) and can preserve insulin signaling and insulin-stimulated glucose uptake in the presence of high concentrations of saturated fatty acids. However, we recently reported that the stimulatory effect of ghrelin on FAO and subsequent ability to protect insulin stimulated glucose uptake was lost following 6-weeks (6w) of chronic high fat feeding. In the current study we examined the effects of both short-term 5 day (5d) and chronic 6w high-fat diet (HFD) on muscle ghrelin response, and whether exercise training could prevent the development of muscle ghrelin resistance with 6w of HFD

**Methods and Results:** Soleus muscle strips were isolated from male rats to determine the direct effects of acylated (AG) and UnAG isoforms on FAO and glucose uptake. A 5d HFD did not alter the response of soleus muscle to AG or UnAG. Conversely, 6w of HFD was associated with a loss of ghrelin's ability to stimulate FAO and protect insulin stimulated glucose uptake. Muscle response to UnAG remained intact following the 6w HFD with chronic exercise training. Unexpectedly, muscle response to both AG and UnAG was also lost after 6w of low-fat diet (LFD) consumption. Protein content of the classic ghrelin receptor, GHS-R1a, was not affected by diet or training. Corticotropin-releasing hormone receptor-2 (CRF-2R) content, a putative receptor for ghrelin in muscle, was significantly decreased in soleus from 6w HFD-fed animals and increased following exercise training. This may explain the protection of UnAG response with training in HFD-fed rats but does not explain why ghrelin response was also lost in LFD-fed animals.

**Conclusions:** UnAG protects muscle glucose uptake during acute lipid oversupply, likely due to its ability to stimulate FAO. This effect is lost in 6w HFD-fed animals but protected with exercise training. Unexpectedly, ghrelin response was lost in 6w LFD-fed animals. The loss of ghrelin response in muscle with a LFD cannot be explained by a change in putative ghrelin receptor content. We believe that the sedentary nature of the animals is a major factor in the development of muscle ghrelin resistance and warrants further research.

## 1. Introduction

Ghrelin exists in two isoforms, an *n*-octanoyl-modified form referred to as acylated ghrelin (AG), and unacylated ghrelin (UnAG). Both isoforms exhibit a rapid pre-prandial rise and post-prandial decline in the plasma [1]. Ghrelin is best known for its orexigenic effect which is due to AG binding to growth hormone secretagogue receptors (GHS-R1a) in the hypothalamus [2]. Ghrelin's *in vivo* metabolic effects on peripheral tissues (skeletal muscle, adipose tissue, liver) have been studied primarily using AG administration over several days in rodents [3–5]. The interpretations are potentially confounded by secondary increases in growth hormone (GH) which AG potently stimulates [6]. To examine the direct effects of AG and UnAG, more recent studies have employed incubation models using isolated tissues or cells.

Skeletal muscle represents a significant depot for the clearance of substrates such as glucose and fatty acids [7,8] and is an important tissue in the etiology of metabolic diseases such as obesity and type 2 diabetes. A growing body of work from our lab and others supports a role for ghrelin in stimulating fatty acid oxidation (FAO) in cultured myocytes and isolated skeletal muscle [9–11]. Recent work from our lab suggests that ghrelin, particularly UnAG, is able to largely prevent the rapid impairment of insulin stimulated glucose uptake and insulin signaling in isolated muscle exposed to high concentrations of saturated fatty acids [9]. This is attributed to UnAG's ability to stimulate FAO, as the protective effect of UnAG is absent in the presence of etomoxir, an inhibitor of mitochondrial FA uptake [9]. We have also shown that following 6 weeks (6w) of high-fat diet (HFD) consumption (60% kcal fat), neither ghrelin isoform is able to stimulate FAO and preserve insulin stimulated

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glucose uptake in skeletal muscle when incubated with palmitate [9], indicating the presence of ghrelin resistance. Although the underlying cause of this resistance is not known, muscle from 6w HFD-fed rats showed a significant reduction in corticotropin releasing factor receptor 2 (CRF-2R) content [9]. Previous work using C2C12 cells showed that anti-sauvagine-30, a selective CRF-2R antagonist, is able to block the metabolic effects of AG [12] and UnAG [13]. This suggests that CRF-2R may be the receptor by which ghrelin exerts its metabolic effects in muscle.

Diet-induced dysregulation of muscle metabolism can occur quite rapidly. We have previously shown that rat skeletal muscle becomes resistant to the stimulatory effects of leptin and adiponectin on FAO in less than one week, prior to the impairment of insulin response [14]. Finally, exercise training for 2–4 weeks has been shown to protect skeletal muscle response to hormones such as leptin and insulin in rats consuming a HFD [15]. To date, no studies have examined the impact of exercise training on muscle ghrelin response. The objectives of this study were to determine i) if skeletal muscle ghrelin resistance manifests following a short term HFD (5d), and ii) whether the inclusion of endurance training with high-fat feeding can prevent the development of skeletal muscle ghrelin resistance. Given the rapid loss of skeletal muscle response to other hormones such as leptin and adiponectin induced by a HFD, we speculated that ghrelin's stimulatory effects on skeletal muscle FAO would also be impaired in less than a week on a HFD. We further hypothesized that exercise training would prevent the development of ghrelin resistance in skeletal muscle with a prolonged HFD, preserving ghrelin's ability to stimulate FAO and protect insulin stimulated glucose uptake during acute lipid oversupply. Additionally, we hypothesized that exercise training may prevent significant reductions in CRF-2R content.

## 2. Methods

### 2.1. Materials and reagents

Reagents, molecular weight markers, and nitrocellulose membranes for all western blotting were purchased from BioRad. Synthetic acylated (Cat. No. H-4862) and unacylated (Cat. No. H-6264) rat ghrelin was purchased from Bachem. Antibodies purchased were GLUT4 (Cat. No. 2213S, Cell Signaling), citrate synthase (Cat. No. ab129095, Abcam), cytochrome *c* oxidase (Cat. No. ab16056, Abcam), corticotropin releasing factor-2 receptor (Cat. No. ab104368, Abcam), growth hormone secretagogue receptor 1- $\alpha$  (Cat. No. sc-374515, Santa Cruz), and peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$  (Cat. No. AB3242, Millipore). FAT/CD36 (M025) antibody was kindly provided by Dr. Graham Holloway (University of Guelph, ON Canada). Chemicals and reagents purchased were Western lighting plus ECL reagent (NEL105001EA, Perkin Elmer), NP40 cell lysis buffer (Cat. No. FNN0021, Life Technologies), phenylmethylsulfonyl fluoride (Cat. No.78830), protease inhibitor (Cat. No. 9599, Millipore), palmitic acid (Cat. No. P0500, Millipore), *D*-glucose (Cat. No. G-8270, Millipore), Dulbecco's modified eagle's media (Cat. No. D5030, Millipore), *D*-mannitol (Cat. No. M9546, Millipore), 2-deoxyglucose (Cat. No. D8375, Millipore) and sodium pyruvate (Cat. No. P8574, Millipore). Bovine serum albumin (Cat. No. A7030) was purchased from Sigma Aldrich. Radiolabeled 14C-palmitic acid (Cat. No. 0172A), 14C-D-mannitol (Cat. No. 0127) and 3H-2-deoxy-D-glucose (Cat. No. 0103) were purchased from American Radiolabeled Chemicals Inc. Insulin (Humulin rDNA origin, Eli Lilly) was purchased from a local pharmacy.

### 2.2. Animals

All procedures were approved by the Animal Care Committee at the University of Guelph and followed the Canadian Council of Animal Care Guidelines. For 5d feeding experiments, male Sprague-Dawley rats were obtained from Charles River Laboratories at approximately 6w of age

(175–200 g). Rats were given access to a standard chow diet for 4d and water ad libitum, prior to beginning 5d of either high-fat diet (HFD, 60% kcals fat, Research Diets D12492) or sucrose matched low-fat diet (LFD, 10% kcals fat, Research Diets D12450J). For 6w dietary intervention and exercise-training experiments, rats were obtained at approximately 4w of age (75–100 g), received a similar 4d acclimation period on standard chow diet, and subsequently provided with ad libitum access to 10% (LFD) or 60% kcal fat diets (HFD) for 6w. For all experiments, rats were housed at 22–24 °C in groups of 3–4 with a 12 h reverse light:dark cycle. Prior to all surgical procedures, rats were fasted overnight with a brief reintroduction of food (30–45 min) at the beginning of their 12 h dark cycle and approximately 2–3 h prior to surgical interventions to prevent high levels of endogenous circulating ghrelin that would typically occur after a fast. This protocol has previously been confirmed by our lab to reduce circulating ghrelin levels prior to surgical procedures [11]. Body weights and food intake were tracked throughout the study.

Rodents were anesthetized via intraperitoneal injection of sodium pentobarbital (6 mg/100 g body weight) prior to all surgical procedures and were euthanized by intracardiac injection of anesthetic after all tissues were removed. For exercise-trained animals, a 48 h period without training was employed to prevent acute effects from the final bout of exercise.

### 2.3. Exercise training

After the initial 2w of high-fat feeding, a subset of 6w HFD rats began a 4w treadmill training protocol (Treadmill Serial No. 130254, Columbus Instruments). Rats were briefly acclimated to the treadmill during the second week of high-fat feeding and began training the following (3rd) week. Animals were trained at approximately the same time each day during their dark (active) cycle. The training protocol intensity increased in a stepwise manner beginning at 10 m/min and 0% incline for 1 h during the first week. In week two, animals trained at 15 m/min at 5% incline for 1 h; in the third week, speed remained constant at 15 m/min and incline was increased to 10% for 1 h. During the final week speed was increased to 20 m/min while the incline remained constant at 10% for 1 h. In the final two weeks of training (weeks 3 and 4), animals were subjected to 6 intermittent sprints lasting 1 min at ~30–40 m/min. This protocol is similar to those used previously by our lab [15,16].

### 2.4. Glucose tolerance tests

After 5w, rats were subjected to an intraperitoneal glucose tolerance test (IPGTT) following an overnight fast. Fasting blood glucose ( $t = 0$ ) was measured via the tail vein using a FreeStyle Lite glucometer, followed by a 2.0 g/kg body weight injection of glucose solution. Subsequent measurements of blood glucose were made at  $t = 15, 30, 45, 60, 90,$  and 120 min and used to calculate area under the curve (AUC) for the assessment of glucose tolerance.

### 2.5. Fatty acid oxidation

Incubations were carried out as previously described (33). Briefly, soleus muscle strips (~25 mg) were excised tendon to tendon and placed in sealed vials to equilibrate for 30 min in a 30 °C shaking water bath in a pre-gassed (95% O<sub>2</sub>, 5% CO<sub>2</sub>) incubation media of DMEM supplemented with 8 mM glucose and 4% BSA. Remaining soleus muscle from each animal was frozen at –80 °C to assess proteins (COXIV, CS, GLUT4, FAT/CD36, GHS-R1a, CRF-2R). For 5d diet experiments, muscles were incubated in the presence of 1 or 2 mM palmitate (with or without 150 ng/mL AG or UnAG) as we were only interested in the assessment of FAO as a marker of ghrelin response. In experiments examining the effect of 6w of diet and exercise training, muscles were incubated in buffer containing low (0.2 mM, baseline) or high (2 mM) palmitate, with or without 150 ng/mL AG or UnAG. This was designed to test the ability of ghrelin to stimulate FAO and protect the muscle from becoming less

responsive to insulin during exposure to 2 mM palmitate (see below for determination of glucose uptake). Incubations lasted for 4 h. After the first 2 h, muscles were transferred to vials for an additional 2 h containing the identical palmitate and ghrelin concentrations with the addition of 0.5 mCi/mL  $^{14}\text{C}$ -palmitic acid for the assessment of FAO. At the end of the incubation, sulfuric acid was injected into the vial and liberated  $^{14}\text{CO}_2$  was trapped in a 500  $\mu\text{L}$  Eppendorf tube containing benzethonium hydroxide. The tube containing the trapped gas was placed into a 5 mL liquid scintillation vial containing CytoScint<sup>TM</sup>-ES, allowed to quench overnight in darkness, and counted for 5 min per sample using a PerkinElmer Tri-Carb LSC 4910 TR liquid scintillation counter.

## 2.6. Insulin stimulated glucose uptake

Due to tissue limitations, glucose uptake and insulin signaling were determined in a separate set of animals from those used to assess FAO. Glucose uptake was assessed as previously described [9,17,18]. Briefly, soleus muscle strips from one leg were excised and equilibrated in pre-gassed DMEM supplemented with 8 mM D-glucose and 4% BSA (30 °C, 95% O<sub>2</sub>, 5% CO<sub>2</sub>) before being transferred to medium containing either low (0.2 mM) or high palmitate (2 mM) for 4 h with or without the addition of AG or UnAG. This was essentially the same as the incubations for the determination of FAO. Vials were re-gassed with 95% O<sub>2</sub>, 5% CO<sub>2</sub> gas halfway through the incubation at 2 h. Following 4 h, muscles were washed for 30 min in media containing 36 mM D-mannitol and 4 mM sodium pyruvate before being transferred to the final buffer containing 28 mM D-mannitol, 8 mM 2-deoxyglucose, 4 mM sodium pyruvate, radiolabeled  $^{14}\text{C}$ -mannitol, and  $^3\text{H}$ -2-deoxy-D-glucose for 30 min. Insulin (10 mU/mL) was added to both the 30 min wash and uptake buffers. Following this, muscles were quickly blotted and trimmed of all visible tendon before being boiled and digested at 95 °C in 1 M NaOH for 10 min. 200  $\mu\text{L}$  of muscle homogenate was sampled in duplicate and added to scintillation vials with 5 mL of CytoScint<sup>TM</sup>-ES. Samples were allowed to quench in darkness overnight before being counted.

## 2.7. Western blotting

Soleus pieces were chipped under liquid N<sub>2</sub> into 20–30 mg samples and homogenized using Qiagen TissueLyser LT in 500  $\mu\text{L}$  of ice-cold cell lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor. Samples were homogenized for 8 min and centrifuged at 1500g for 15 min at 4 °C. Protein concentrations were assessed using the bicinchoninic acid (BCA) assay [19] to allow for equal protein loading (10 $\mu\text{g}$ ). Proteins were separated by electrophoresis on 10% acrylamide gels (15% for COX IV) and wet transferred onto nitrocellulose membranes for 1 h at 100 V and 4 °C. Membranes were then blocked in 5% BSA or 5% skim milk powder plus TBST for 1 h at room temperature before a 15-min wash with TBST. Primary antibody (1:1000) was added to the membrane and shaken overnight at 4 °C. The following day, membranes were washed with TBST for 30 min before being incubated with secondary antibody (1:2000) for 1 h at room temperature. Following this, membranes were again washed for 30 min with TBST followed by a 10-min wash in TBS. Bands were visualized using ECL and quantified with Alpha Innotech Software. Alpha-tubulin was used as a loading control.

## 2.8. Statistics

All data are expressed as mean  $\pm$  standard deviation and statistical significance was accepted at  $p < 0.05$ . Body weight, caloric intake, food intake and fasting blood glucose tolerance test data in 6w LFD sedentary (LFD-SED), HFD sedentary (HFD-SED) and HFD exercised (HFD-EX) animals were analyzed using a 2-way ANOVA. 5d LFD and HFD body weights were analyzed using a repeated measures two-way ANOVA. In 5d LFD and HFD animals, total food and caloric intake was analyzed

using an unpaired *t*-test. IPGTT AUC was calculated as the area above baseline blood glucose concentration at  $t = 0$  min for each animal. Western blot data in 6w LFD-SED, HFD-SED and HFD-EX animals were analyzed within each group using a one-way ANOVA. Glucose uptake and palmitate oxidation in 6w LFD-SED, HFD-SED, and HFD-EX animals, as well as FAO in 5d LFD and HFD sedentary animals were analyzed using a repeated measures two-way ANOVA. Tukey's multiple comparisons post hoc testing was used if significance was detected with the ANOVA.

## 3. Results

### 3.1. 5d LFD and HFD experiments

#### 3.1.1. Body weight and caloric intake

HFD rats consumed significantly more calories ( $p < 0.0001$ ; Table 1), but body weight did not differ between groups ( $p > 0.05$ ; Table 1).

#### 3.1.2. Stimulation of FAO by UnAG

In the absence of ghrelin, rates of FAO were significantly increased in 2 vs. 1 mM palmitate concentration ( $p = 0.0190$  LFD,  $p = 0.0001$  HFD; Fig. 1). UnAG, but not AG, significantly stimulated FAO at medium (1 mM) ( $p = 0.0678$  LFD,  $p = 0.0324$  HFD) and high (2 mM) palmitate concentrations ( $p = 0.0437$  LFD,  $p = 0.0400$  HFD), indicating that UnAG's stimulatory effects are still present following 5d of HFD feeding.

### 3.2. 6w diet and exercise training experiments

#### 3.2.1. Body weight, caloric intake, and whole-body glucose tolerance

HFD-sedentary (HFD-SED) rats had significantly greater body weight beginning after one week of diet ( $p = 0.0079$ ; Fig. 2C). HFD-SED rats had significantly increased body weight compared to HFD-exercised (HFD-EX) animals for the duration of the 4w training period ( $p = 0.0003$  w3,  $p = 0.0014$  w4,  $p = 0.0001$  w5,  $p = 0.0032$  w6; Fig. 2C). Body weights were not significantly different between LFD-sedentary (LFD-SED) and HFD-EX rats following training ( $p = 0.4124$ ; Fig. 2C). LFD-SED animals consumed significantly more food for the duration of the study ( $p < 0.005$ ; Fig. 2A), but energy intake was significantly greater in the HFD-SED group relative to LFD-SED rats for the duration of the study ( $p < 0.0001$  at all time points; Fig. 2B), and HFD-EX rats for the first 3w of training ( $p = 0.0218$  w3,  $p = 0.0035$  w4,  $p = 0.0035$  w5; Fig. 2B).

HFD-SED rats had significantly higher blood glucose concentrations during the IPGTT (Fig. 2D) compared to LFD-SED rats at 30 ( $p = 0.0034$ ), 45 ( $p = 0.0014$ ), 60 ( $p = 0.0105$ ), 90 ( $p = 0.0102$ ), and 120 min ( $p = 0.0082$ ). HFD-SED rats had significantly higher blood glucose AUC (Fig. 2E) than both LFD-SED ( $p = 0.0020$ ) and HFD-EX rats ( $p = 0.0357$ ).

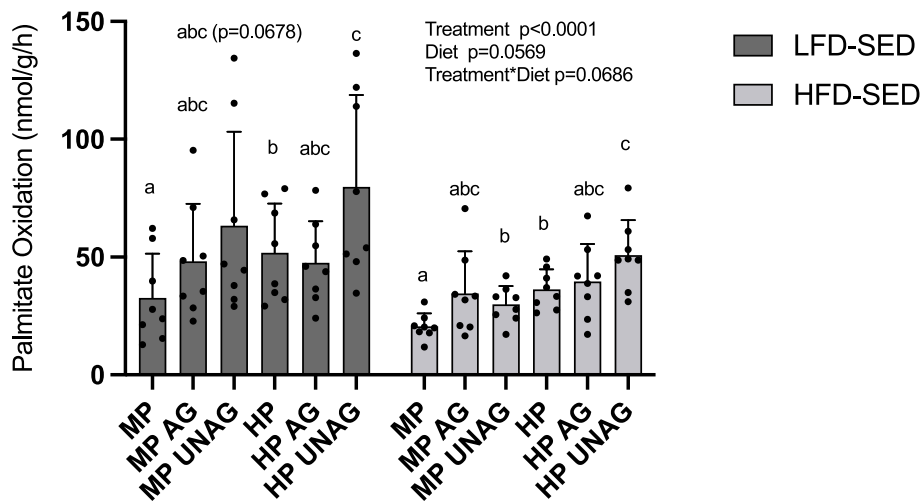
#### 3.2.2. Mitochondrial markers, GLUT 4 and FAT/CD36 transporter content in LFD-sedentary, HFD-sedentary, and HFD-exercised rats

COX IV (Fig. 5A), and CS (Fig. 5B) protein content was significantly increased by exercise training relative to sedentary rats (COX IV:  $p = 0.0001$  HFD-EX vs. HFD-SED,  $p = 0.0037$  HFD-EX vs. LFD-SED; CS:  $p =$

**Table 1**  
5-day LFD and HFD sedentary rat body weight and caloric intake.

	LFD	HFD	p-value
Initial body weight (g)	154.0 $\pm$ 2.8	152.8 $\pm$ 1.9	$p = 0.9999$
Final body weight (g)	229.3 $\pm$ 3.2	238.1 $\pm$ 4.0	$p = 0.0985$
$\Delta$ Body weight (g)	75.3 $\pm$ 8.0	85.4 $\pm$ 14.9	$p = 0.1126$
Total caloric intake (kcal)	326.8 $\pm$ 4.5	457.3 $\pm$ 40.9	$p < 0.0001$

Initial, final, and  $\Delta$  body weight, and total caloric intake in 5d LFD and HFD rats. Body weights were analyzed using a repeated measures two-way ANOVA. Caloric intake and  $\Delta$  body weight were analyzed using an unpaired *t*-test. Data is expressed as the mean  $\pm$  standard deviation.



**Fig. 1.** Muscle fatty acid oxidation following 5d diets. Rates of palmitate oxidation in isolated soleus muscle from 5d LFD and HFD animals following medium palmitate (MP; 1 mM) or high palmitate (HP; 2 mM) exposure +/- AG/UnAG (150 ng/ml) treatment. Data were analyzed using a repeated measures two-way ANOVA ( $n = 8$  for each) and expressed as individual data points and the mean  $\pm$  standard deviation. Data sharing a letter are not statistically significant from each other within each diet condition.

0.0132 HFD-EX vs. HFD-SED,  $p = 0.0395$  HFD-EX vs. LFD-SED). HFD-EX rats tended to have greater PGC-1 $\alpha$  content (Fig. 5C) relative to LFD-SED ( $p = 0.0640$ ). There was no significant difference between HFD-SED and HFD-EX animals, which may be due to the tendency of a high-fat diet to increase PGC-1 $\alpha$ . There were no significant differences in total GLUT 4 ( $p = 0.6917$  LFD-SED vs. HFD-SED,  $p = 0.8241$  LFD-SED vs. HFD-EX,  $p = 0.9712$  HFD-SED vs. HFD-EX; Fig. 6A) or FAT/CD36 transporter protein content in skeletal muscle between groups ( $p = 0.8812$  LFD-SED vs. HFD-SED,  $p = 0.4490$  LFD-SED vs. HFD-EX,  $p = 0.7369$  HFD-SED vs. HFD-EX; Fig. 6B).

### 3.2.3. UnAG improves insulin-stimulated glucose uptake during high palmitate exposure, but only in exercised rats

Insulin-stimulated glucose uptake in soleus was significantly decreased following a 4 h exposure to high (2 mM) palmitate in all experimental groups ( $p = 0.0391$  LFD-SED,  $p = 0.0437$  HFD-SED,  $p = 0.0020$  HFD-EX; Fig. 4.). Neither ghrelin isoform was able to improve insulin-stimulated glucose uptake in the presence of 2.0 mM palmitate in HFD-SED rats, as glucose uptake was actually lower with AG ( $p = 0.0181$ ) and UnAG had no effect ( $p = 0.2853$ ; Fig. 4). As hypothesized, a protective effect of UnAG ( $p = 0.0489$  UnAG; Fig. 4) was observed in HFD-EX rats. However, to our surprise, there was no protective effect of UnAG on glucose uptake in LFD-SED rats ( $p = 0.9964$ ). This was completely unexpected.

### 3.2.4. Improvement in insulin-stimulated glucose uptake by UnAG coincides with further stimulation of FAO

FAO was significantly greater with 2 vs. 0.2 mM palmitate in all experimental groups ( $p = 0.0015$  LFD-SED,  $p < 0.0001$  HFD-SED,  $p = 0.0419$  HFD-EX; Fig. 3). Neither ghrelin isoform further stimulated FAO during the high palmitate condition in muscle from LFD-SED or HFD-SED rats ( $p = 0.9911$  AG,  $p = 0.9804$  UnAG LFD-SED,  $p = 0.8962$  AG,  $p = 0.8788$  UnAG HFD-SED; Fig. 3). In HFD-EX, UnAG significantly increased rates of palmitate oxidation relative to high palmitate alone ( $p = 0.0171$ ; Fig. 3). AG did not further stimulate FAO ( $p = 0.7746$ ; Fig. 3).

### 3.2.5. Ghrelin receptor content in skeletal muscle of LFD-SED, HFD-SED, and HFD-EX rats

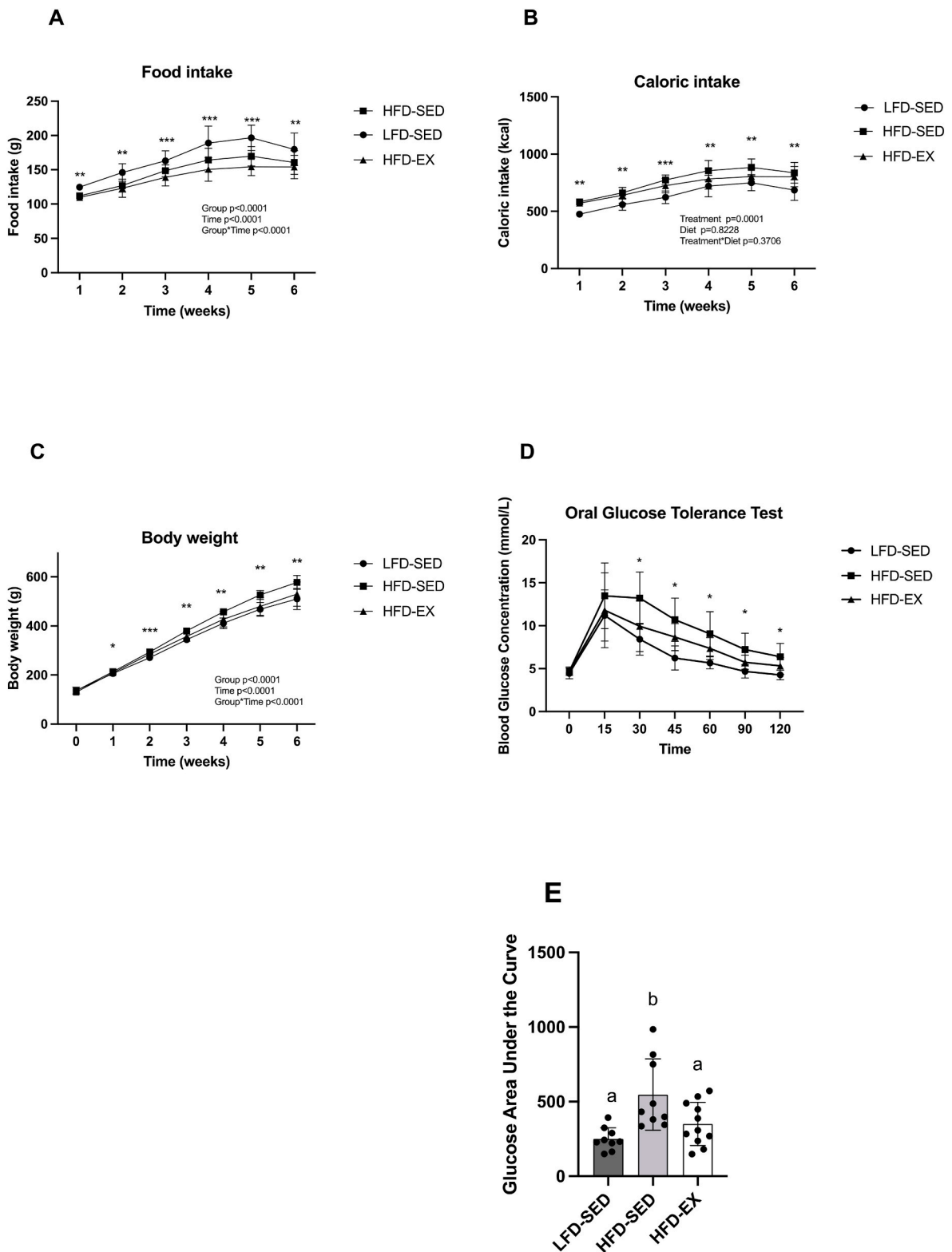
There were no significant differences in soleus GHS-R1a protein content between the groups ( $p = 0.3411$ ; Fig. 7A). CRF-2R content was significantly reduced in HFD-SED vs. LFD-SED rats ( $p = 0.0055$ ). In 6w HFD-EX animals CRF-2R content was not reduced relative to LFD-SED ( $p = 0.4773$ ). Exercise tended to increase CRF-2R content relative to HFD-SED animals ( $p = 0.0944$ ), however this did not reach statistical significance.

## 4. Discussion

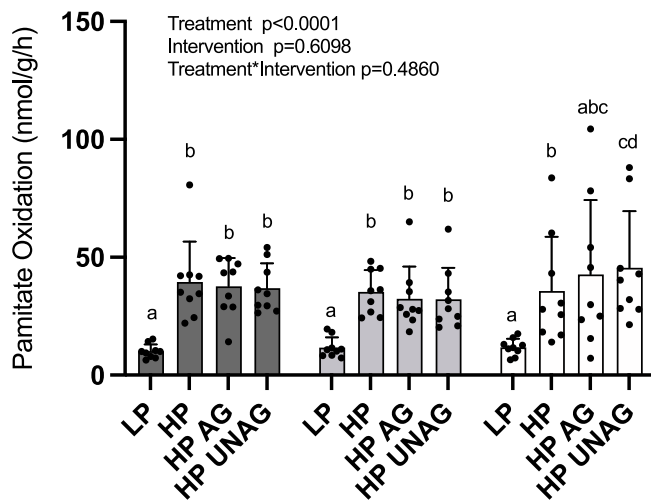
### 4.1. Overall findings

A growing body of evidence supports a role for ghrelin as a regulator of glucose and fatty acid metabolism in peripheral tissues such as skeletal muscle and adipose tissue [3,4,9,12,20–22]. Findings from our lab and others indicate a direct stimulatory effect of ghrelin (AG and UnAG) on FAO [9–11], with some evidence of a role for the AMPK-ACC axis [11, 12]. Importantly, ghrelin's ability to stimulate FAO protects the muscle from acute impairments in insulin signaling and glucose uptake induced by lipid oversupply [9,10]. In a recent study from our lab, we investigated whether the ability of ghrelin to stimulate FAO and protect muscle from palmitate-induced impairment in insulin signaling and glucose uptake remained intact in chronically high-fat fed rats [9]. Our findings indicated that muscle developed a resistance to ghrelin; however, this comparison was made as part of a larger study and did not include a chronic low-fat fed group as a control. Furthermore, we did not examine how quickly resistance to ghrelin occurs in muscle, or whether exercise training can prevent this. We have previously shown that skeletal muscle can become resistant to hormones such as leptin and adiponectin in as early as 3 to 7d of HFD feeding [23]. Furthermore, exercise training can restore the response of muscle to hormones such as leptin and insulin [15]. To our knowledge, no studies have examined exercise as a potential intervention to modulate ghrelin response in skeletal muscle. Therefore, in the present study we sought to determine i) if ghrelin's stimulatory effect on muscle FAO was impaired following short-term (5d) high-fat feeding, and ii) if the development of skeletal muscle ghrelin resistance induced by chronic (6w) high-fat feeding could be prevented with concurrent exercise training.

In the current study, the ability of ghrelin to stimulate FAO and protect insulin stimulated glucose uptake remained intact following 5d of high-fat feeding. Following 6w of a HFD, neither AG nor UnAG stimulated FAO or protected insulin-stimulated glucose uptake in the presence of high palmitate concentrations. This was hypothesized and consistent with previous findings from our lab [9]. In the present study, a novel finding was that exercise training prevented the 6w HFD induced loss of UnAG's response. This was demonstrated by UnAG's ability to significantly stimulate FAO, which was associated with the protection of insulin-stimulated glucose uptake during acute lipid oversupply. The major unexpected finding from the current study was the absence of a ghrelin response in muscle from 6w LFD-SED animals. Clearly, this confounds the interpretation of a HFD being the predominant cause of ghrelin resistance. Further investigation is warranted. A graphical representation of the overall study findings is shown in Fig. 8.

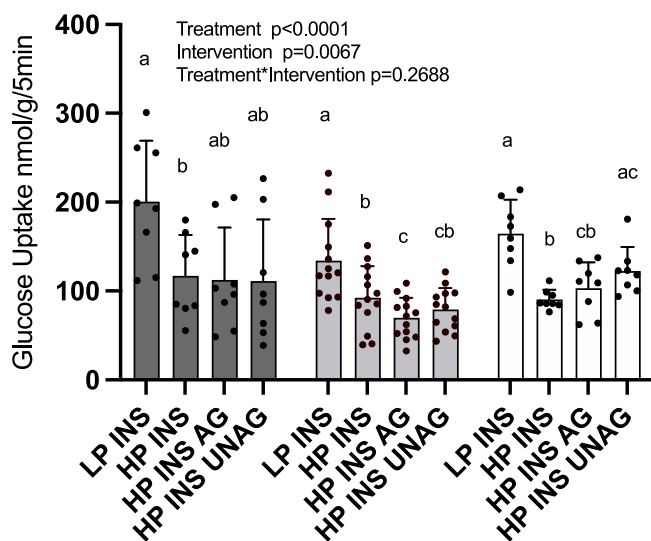


**Fig. 2.** Body weight, caloric intake, and blood glucose measurements following 6w of dietary and exercise interventions. Food intake (Fig. 2A), caloric intake (Fig. 2B), body weights (Fig. 2C), blood glucose concentrations (Fig. 2D) and glucose AUC (Fig. 2E) following LFD-SED, HFD-SED, and HFD-EX interventions. Body weight, food intake, caloric intake ( $n = 17-19$ /group) and blood glucose tolerance data ( $n = 9-11$ /group) were analyzed using a repeated measures two-way ANOVA. Data are expressed as mean  $\pm$  standard deviation. Asterisks denote significant differences between groups: \* indicates a difference between HFD-SED and LFD-SED animals (Fig. 2D), \*\* indicate differences between HFD-SED in comparison to both HFD-EX and LFD-SED animals (Fig. 2B; Fig. 2C) and \*\*\* indicates significant differences between all groups. For AUC (Fig. 2E), data not sharing a letter are statistically significant from each other.



■ LFD-SED  
 ■ HFD-SED  
 □ HFD-EX

**Fig. 3.** Muscle fatty acid oxidation following 6w of dietary and exercise interventions. Rates of palmitate oxidation in isolated soleus muscle from LFD-SED, HFD-SED and HFD-EX animals following low-palmitate (LP; 0.2 mM) or high-palmitate (HP; 2.0 mM) exposure +/- AG/UnAG (150 ng/ml) ghrelin treatment. Data were analyzed using a repeated measures two-way ANOVA ( $n = 9$  for each group) and expressed as individual data points and the mean  $\pm$  standard deviation.  $p < 0.05$  was considered statistically significant. Data sharing a letter are not statistically significant from each other within each diet condition.



■ LFD-SED  
 ■ HFD-SED  
 □ HFD-EX

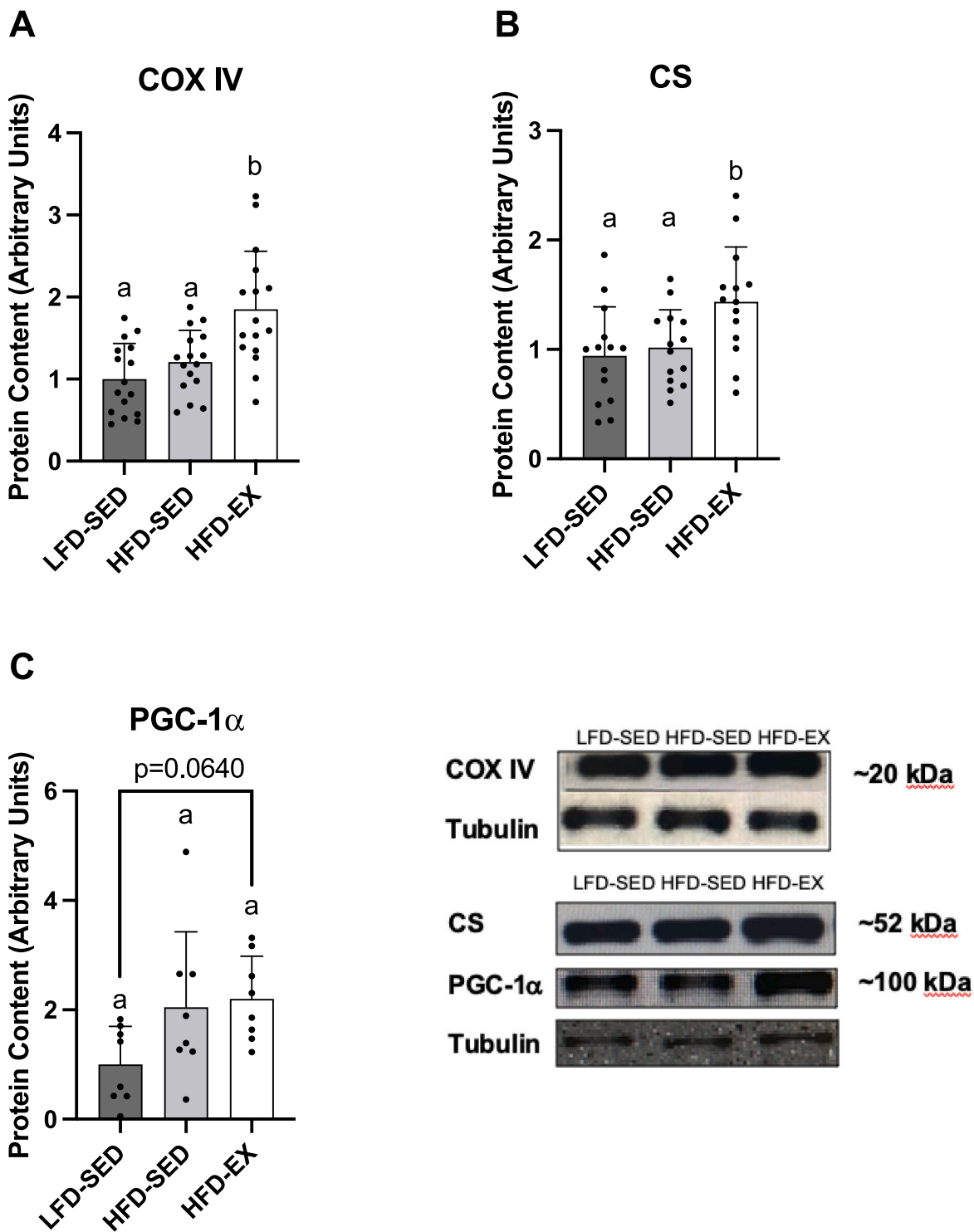
**Fig. 4.** Muscle glucose uptake following 6w of dietary and exercise interventions. Rates of insulin (INS; 10mU/ml) stimulated glucose uptake in isolated soleus muscle from LFD-SED, HFD-SED and HFD-EX animals following low palmitate (LP; 0.2 mM) or high palmitate (HP; 2.0 mM) exposure +/- AG/UnAG (150 ng/ml) ghrelin treatment. Data were analyzed using a repeated measures two-way ANOVA ( $n = 8$  for LFD-SED and HFD-EX;  $n = 13$  for HFD-SED) and expressed as individual data points and the mean  $\pm$  standard deviation.  $p < 0.05$  was considered statistically significant. Data sharing a letter are not statistically significant from each other within each diet condition.

#### 4.2. Ghrelin resistance in skeletal muscle

High-fat feeding rapidly (i.e., within days to weeks), induces skeletal muscle resistance to a number of hormones including leptin, adiponectin, and insulin [14,15,23,24]. Previous work from our lab has shown that ghrelin resistance occurs in white adipose tissue (WAT) in as early as 5d of HFD consumption in rats [22]. This was evidenced by the loss of ghrelin's ability to blunt beta-adrenergic stimulated lipolysis [22]. Furthermore, central ghrelin resistance can manifest in as little as 12 h of HFD feeding [25]. Given these findings, we were surprised to find that ghrelin response in skeletal muscle remained intact following 5d of HFD feeding. This suggests that the initial etiology of resistance to these hormones is different. HFD-induced insulin resistance is generally attributed to the accumulation of the development of LCFA-CoA intermediates, diacylglycerol, and ceramides [26,27]. The underlying causes of leptin resistance are poorly understood but likely involve negative feedback by suppressor of cytokine signaling (SOCS)-3 [28]. However, the mechanisms underlying ghrelin resistance in peripheral tissues have not yet been examined, as this is a newly discovered phenomenon.

We recently reported that WAT becomes unresponsive to ghrelin following only 5d of a HFD compared to a LFD. However, by 6w, WAT was unresponsive to ghrelin regardless of the dietary fat content [22]. Thus, it seems that dietary fat may be responsible for the initial

development of ghrelin resistance in WAT, but other factors must be contributing to the later development. In the current study, we did not examine any time points between 5d and 6 w; therefore, we cannot exclude the possibility that ghrelin resistance in muscle may have manifested earlier with the HFD vs. LFD. Overall, further work is needed to determine whether dietary fat is a contributor to the development of muscle ghrelin resistance. It is our opinion that a significant factor is the sedentary behavior of the rats, particularly considering that they were housed in cages in groups of 3–4. Insulin resistance, for example, has been shown to manifest in mice with running wheel lock in as little as 10d [29]. In the current study, the inclusion of a moderately intense training protocol prevented the loss of UnAG response observed in the 6w HFD. However, the amount of physical activity or exercise training that is required to prevent ghrelin resistance remains to be determined. We would anticipate that exercise training would also have protected the muscle from ghrelin resistance in the LFD group. Clearly, we did not anticipate finding muscle ghrelin resistance in LFD rats; due to the large number of animals already required, we did not include a group of 6w LFD rats that also received exercise training. Future studies should include this to better establish the role of sedentary behavior vs. dietary fat in the development of muscle ghrelin resistance.



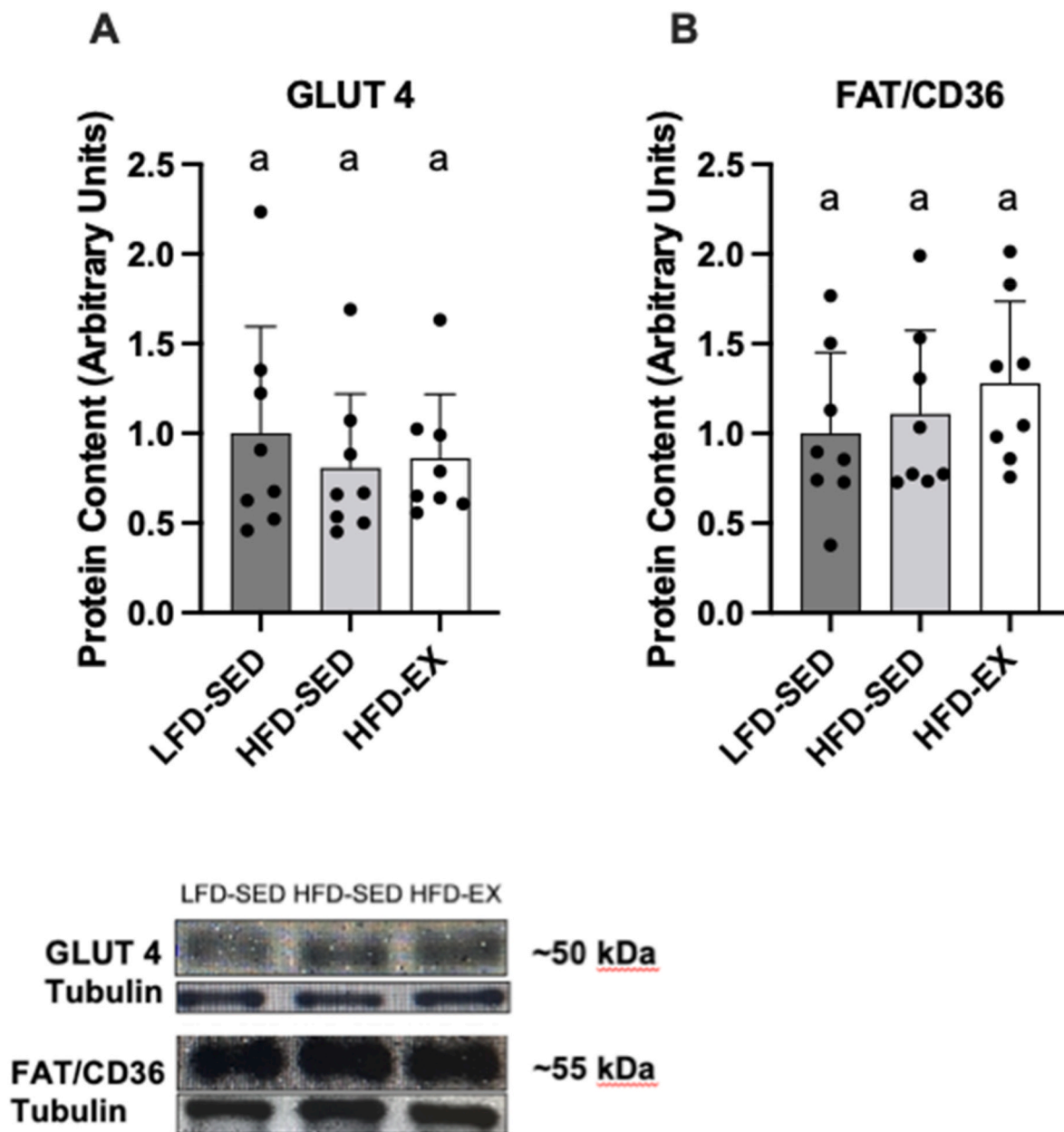
**Fig. 5.** Training adaptations following 6w of dietary and exercise interventions. Cytochrome c oxidase subunit 4 (COX IV), Citrate synthase (CS), and peroxisome proliferator-activated receptor coactivator 1-alpha (PGC-1α) protein expression in isolated soleus of LFD-SED, HFD-SED, and HFD-EX animals. Data were analyzed using an ordinary one-way ANOVA (n = 8–16 for each) and expressed as individual data points and the mean ± standard deviation. p < 0.05 was considered statistically significant. Data sharing a letter are not statistically significant from each other.

### 4.3. Ghrelin signaling in skeletal muscle

Although ghrelin’s orexigenic effects are attributed to AG signaling through GHS-R1a in the hypothalamus and anterior pituitary gland [6, 30], GHS-R1a mRNA has also been detected in other tissues [31]. Our lab has also detected GHS-R1a protein content in muscle [9] and WAT [22]. In the current study, there were no changes in GHS-R1a protein

content in any of the groups. Both AG and UnAG may exert their effects in muscle via the corticotropin releasing factor family receptor (CRF-2R). Through the use of a selective CRF-2R antagonist, Gershon et al. demonstrated in C2C12 cells that the AG-induced increase in glucose uptake [12] and the shift towards fat utilization induced by UnAG [13], were dependent on the CRF-2R receptor.

Previous work in our lab demonstrated a significant reduction in



**Fig. 6.** Effects of 6w dietary and exercise interventions on muscle substrate transporters. GLUT 4 and FAT/CD36 protein expression in isolated soleus of LFD-SED, HFD-SED, and HFD-EX animals. Data were analyzed using an ordinary one-way ANOVA ( $n = 8/\text{group}$ ) and expressed as individual data points and the mean  $\pm$  standard deviation.  $p < 0.05$  was considered statistically significant. Data sharing a letter are not statistically significant from each other.

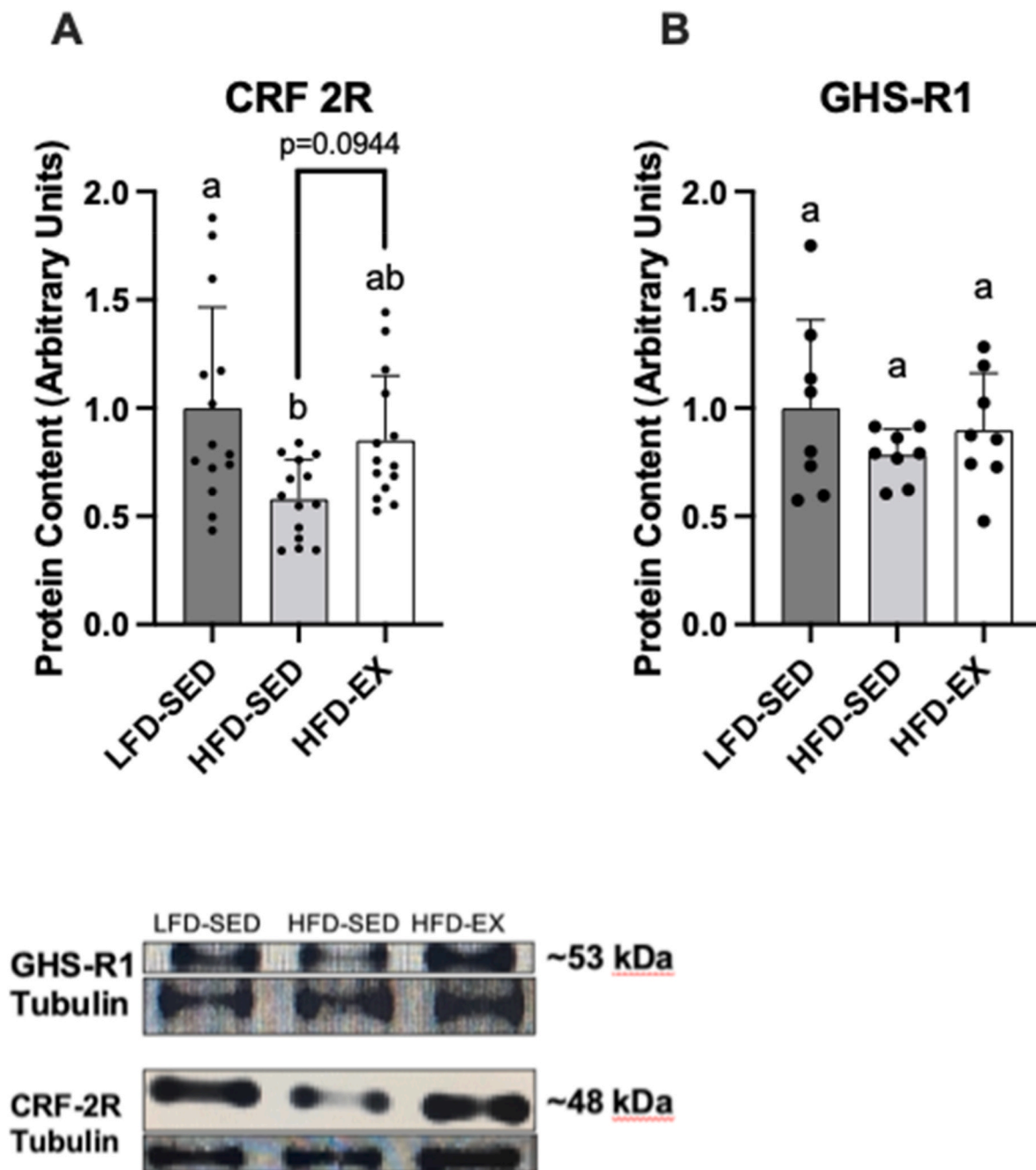
CRF-2R content in muscle following a HFD that was associated with the loss of ghrelin response [9]. In the current study, the soleus content of CRF-2R protein was significantly reduced by the chronic HFD and tended to be increased ( $p = 0.0944$ ) with exercise training. While this data fits with our finding that HFD-EX animals displayed an intact UnAG response compared to HFD-SED rats, it does not explain why the LFD-SED rats also showed no UnAG response. Finally, it should be acknowledged that changes in protein content may not entirely explain changes in the functioning of the CRF-2R receptor, which was beyond the scope of this study.

##### 5. Limitations and considerations

In the current study, an acute high-palmitate exposure (lipid oversupply) was utilized to induce impaired insulin stimulated glucose uptake in skeletal muscle. However, this is not representative of the

presence of various fatty acids in the circulation. Isolated incubations permit for the testing of direct effects but cannot replicate *in vivo* substrate and hormonal concentrations. Due to tissue limitations, there was no treatment assessing the combined effects of AG and UnAG. The primary focus of this study was the chronic effect of diet and exercise training on muscle ghrelin response. Therefore, while we included a short-term 5d dietary treatment to determine whether muscle ghrelin resistance could be rapidly induced, we did not also include an exercise training condition for this brief period. Further, muscle measurements after 5d were not performed as there was no observed impairment in ghrelin response. No cellular measurements were made to directly confirm the ghrelin resistance observed in functional outcomes of FAO and glucose transport, as the exact signaling transducers for ghrelin's effects in skeletal muscle remain uncertain. We did not anticipate the loss of muscle ghrelin response following the chronic LFD. Although the reason for this finding is unknown, we suspect it may be caused by





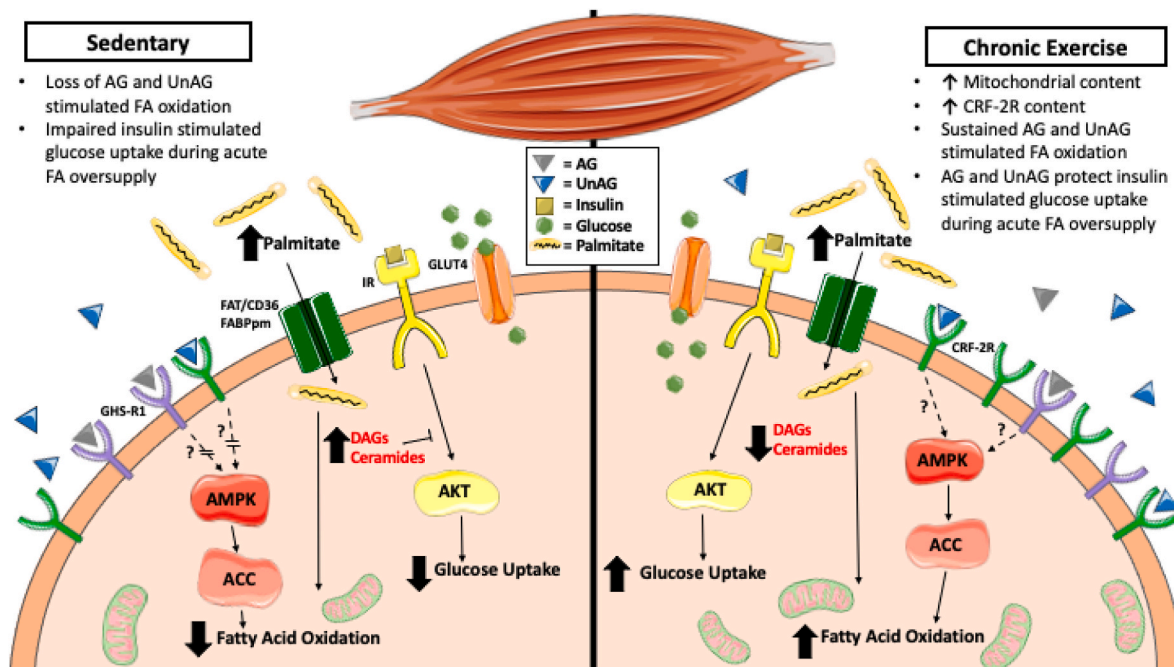
**Fig. 7.** Effects of 6w dietary and exercise interventions on ghrelin receptors. Growth hormone secretagogue receptor type 1 (GHS-R1) and Corticotropin releasing factor receptor 2 (CRF-2R) protein expression in isolated soleus of LFD-SED, HFD-SED, and HFD-EX animals. Data were analyzed using an ordinary one-way ANOVA ( $n = 8-14$  per group) and expressed as individual data points and the mean  $\pm$  standard deviation.  $p < 0.05$  was considered statistically significant. Data sharing a letter are not statistically significant from each other.

sedentary behaviour in these animals. As this was an unexpected finding, there was no exercise training condition combined with the LFD. Future work is required to better understand the mechanisms by which ghrelin resistance occurs.

## 6. Conclusions

The results from the current study demonstrate that a short-term 5d diet high in saturated fat does not cause ghrelin resistance, as we previously observed in WAT. Unexpectedly, after the consumption of either a low or high-fat diet fed chronically for 6w, neither AG nor UnAG were able to stimulate FAO in isolated skeletal muscle or prevent the

palmitate-induced impairment in glucose uptake. The reason for the loss of muscle ghrelin response in LFD-SED rats is currently unknown but may be caused by chronic sedentary behavior. 4w of exercise training was able to prevent the development of this resistance. To our knowledge, this is the first study to examine if ghrelin response in skeletal muscle can be altered by chronic exercise training. The mechanisms underlying the development of ghrelin resistance remain unclear and future research in our lab will aim to elucidate this phenomenon. The practical implications to humans, at this point, are not known. However, these findings suggest that muscle might be quite susceptible to becoming ghrelin resistant, with the consequence of losing some protection against the damaging effects of lipid oversupply. Exercise



**Fig. 8.** Graphical representation of acylated (AG) and unacylated (UnAG) ghrelin signalling in isolated rat skeletal muscle during acute lipid oversupply, and the effects on insulin stimulated glucose uptake and palmitate oxidation. This figure serves as a representative schematic; thus, not everything included in the figure was assessed in the current study. Schematic represents sedentary animals and chronic treadmill trained animals on a high-fat diet (HFD). Sedentary animals on a low-fat diet shared a similar metabolic profile to sedentary animals on a HFD and were not included in this figure. Dashed lines and question mark represent unassessed mechanisms. Upward arrow indicates increase. Downward arrow indicates inhibition. Broken arrow indicates loss of signaling pathway. GHS-R1; Growth Hormone Secretagogue Receptor 1, CRF-2R; Corticotropin-Releasing Factor 2 Receptor, FAT/CD36; Fatty Acid Translocase, FABPpm; Fatty Acid Binding Protein plasma membrane, IR; Insulin Receptor, GLUT4; Glucose Transporter 4, DAG; diacylglycerol. This figure was created with use of free images from smart.servier.com.

appears to be an effective strategy to prevent muscle ghrelin resistance from developing. These results provide yet another potential reason for individuals to be regularly physically active.

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## CRedit authorship contribution statement

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

## Declaration of competing interest

No conflicts of interest declared.

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AJL and DJD contributed to the conception of the study and designed the experiments. AJL, EMH and DTC supervised animal maintenance (feeding, training). All authors assisted with measurements. AJL and DJD analyzed and interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

## References

- [1] Cummings DE, et al. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001;50(8):1714–9.
- [2] Wang Q, et al. Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. *Mol Metabol* 2014;3(1):64–72.
- [3] Barazzoni R, et al. Ghrelin regulates mitochondrial-lipid metabolism gene expression and tissue fat distribution in liver and skeletal muscle. *Am J Physiol Endocrinol Metab* 2005;288(1):E228–35.
- [4] Barazzoni R, et al. Acylated ghrelin treatment normalizes skeletal muscle mitochondrial oxidative capacity and AKT phosphorylation in rat chronic heart failure. *J Cachexia Sarcopenia Muscle* 2017;8(6):991–8.
- [5] Barazzoni R, et al. Acylated ghrelin limits fat accumulation and improves redox state and inflammation markers in the liver of high-fat-fed rats. *Obesity* 2014;22(1):170–7.
- [6] Kojima M, et al. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999;402(6762):656–60.
- [7] DeFronzo RA, et al. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* 1981;30(12):1000–7.
- [8] Ibrahim A, et al. Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. *J Biol Chem* 1999;274(38):26761–6.
- [9] Cervone DT, et al. 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. *Metabol Open* 2020;5:100026.
- [10] Han L, et al. Effects of ghrelin on triglyceride accumulation and glucose uptake in primary cultured rat myoblasts under palmitic acid-induced high fat conditions. *Internat J Endocrinol* 2015;2015:635863.
- [11] Kraft EN, Cervone DT, Dyck DJ. Ghrelin stimulates fatty acid oxidation and inhibits lipolysis in isolated muscle from male rats. *Phys Rep* 2019;7(7):e14028.
- [12] Gershon E, Vale WW. CRF type 2 receptors mediate the metabolic effects of ghrelin in C2C12 cells. *Obesity* 2014;22(2):380–9.
- [13] Elbaz M, Gershon E. Ghrelin, via corticotropin-releasing factor receptors, reduces glucose uptake and increases lipid content in mouse myoblasts cells. *Phys Rep* 2021;9(2):e14654.
- [14] Mullen KL, et al. Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats. *Am J Physiol Regul Integr Comp Physiol* 2009;296(2):R243–51.
- [15] Ritchie IR, et al. Restoration of skeletal muscle leptin response does not precede the exercise-induced recovery of insulin-stimulated glucose uptake in high-fat-fed rats. *Am J Physiol Regul Integr Comp Physiol* 2011;300(2):R492–500.

- [16] Ritchie IR, et al. Adiponectin is sufficient, but not required, for exercise-induced increases in the expression of skeletal muscle mitochondrial enzymes. *J Physiol* 2014;592(12):2653–65.
- [17] Cervone DT, Dyck DJ. Acylated and unacylated ghrelin do not directly stimulate glucose transport in isolated rodent skeletal muscle. *Phys Rep* 2017;5(13).
- [18] Thrush AB, et al. A single prior bout of exercise protects against palmitate-induced insulin resistance despite an increase in total ceramide content. *Am J Physiol Regul Integr Comp Physiol* 2011;300(5):R1200–8.
- [19] Smith PK, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150(1):76–85.
- [20] Baragli A, et al. Acylated and unacylated ghrelin attenuate isoproterenol-induced lipolysis in isolated rat visceral adipocytes through activation of phosphoinositide 3-kinase gamma and phosphodiesterase 3B. *Biochim Biophys Acta* 2011;1811(6):386–96.
- [21] Cervone DT, Lovell AJ, Dyck DJ. Regulation of adipose tissue and skeletal muscle substrate metabolism by the stomach-derived hormone, ghrelin. *Curr Opin Pharmacol* 2020;52:25–32.
- [22] Hucik B, et al. Regulation of adipose tissue lipolysis by ghrelin is impaired with high-fat diet feeding and is not restored with exercise. *Adipocyte* 2021;10(1):338–49.
- [23] Mullen KL, et al. Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats. *Am J Physiol Endocrinol Metab* 2007;293(1):E83–90.
- [24] Krssak M, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* 1999;42(1):113–6.
- [25] Perez-Tilve D, et al. Ghrelin-induced adiposity is independent of orexigenic effects. *Faseb J* 2011;25(8):2814–22.
- [26] Ellis BA, et al. Long-chain acyl-CoA esters as indicators of lipid metabolism and insulin sensitivity in rat and human muscle. *Am J Physiol Endocrinol Metab* 2000;279(3):E554–60.
- [27] Straczkowski M, et al. Increased skeletal muscle ceramide level in men at risk of developing type 2 diabetes. *Diabetologia* 2007;50(11):2366–73.
- [28] Steinberg GR, et al. The suppressor of cytokine signaling 3 inhibits leptin activation of AMP-kinase in cultured skeletal muscle of obese humans. *J Clin Endocrinol Metab* 2006;91(9):3592–7.
- [29] Appriou Z, et al. Skeletal muscle ceramides do not contribute to physical-inactivity-induced insulin resistance. *Appl Physiol Nutr Metabol* 2019;44(11):1180–8.
- [30] Nakazato M, et al. A role for ghrelin in the central regulation of feeding. *Nature* 2001;409(6817):194–8.
- [31] Ueberberg B, et al. Expression of ghrelin and its receptor in human tissues. *Horm Metab Res* 2009;41(11):814–21.