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Original article

CNR2 inverse-agonist AM630 increases citrate synthase activity in the skeletal muscle of diet induced obese rats

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ABSTRACT

Aims: Diet-induced obesity (DIO) results in alterations in skeletal muscle metabolism that affects oxidative capacity and adiponectin signaling. In obesity, agonism of the cannabinoid receptor 2 (CNR2) has been shown to reduce body weight and increase energy expenditure but the effect on skeletal muscle metabolism is unknown. We aimed to investigate the effects of a CNR2 inverse agonist, AM630 and a CNR2 agonist, AM1241, on skeletal muscle.**Methods:** Male Sprague Dawley rats (n = 30) were fed a high fat diet (HFD) (21% fat) for 9 weeks, then received daily intraperitoneal injections with vehicle (n = 10), AM630 (0.3 mg/kg, n = 10) or AM1241 (3 mg/kg, n = 10) for another six weeks. Skeletal muscle, citrate synthase (CS) activity, mRNA and protein expression of markers of the fatty acid oxidative pathways with and without acute *ex vivo* adiponectin (0.1 mg/ml) treatment were measured.**Results:** AM630 increased CS enzyme activity in the red gastrocnemius. In addition, in soleus AM630 increased the ratio of 4EBP1 phosphorylated to total protein, reduced AMPKα phosphorylated to total protein and reduced *PDK4* mRNA. In soleus, AM1241 activated 4EBP1 and reduced *PDK4* mRNA. While, in the EDL both AM630 and AM1241 reduced *PGC1-α* and AM1241 increased *AdipoR1* mRNA. Interestingly, in the EDL, acute *ex vivo* adiponectin exposure reduced 4EBP1 phosphorylated to total protein in control animals, however increased *PGC1-α* in AM630 treated animals.**Conclusions:** Targeting CNR2 in obesity alters mitochondrial content in the skeletal muscle of rodents, yet the relationship appears to be complex and further exploration is required.

1. Introduction

The endocannabinoid system comprises endogenous lipid-derived endocannabinoids, primarily anandamide and 2-arachidonoylglycerol, their G protein-coupled cannabinoid receptors (cannabinoid receptor 1 (CNR1) and cannabinoid receptor 2 (CNR2)), and the biosynthetic and degradative enzymes responsible for their synthesis, transport, and metabolism, which are critical for the maintenance of cellular homeostasis. CNR2 is located in the brain and peripheral tissues including the

immune and nervous tissues, endothelial and smooth muscles, and cardiac, liver, spleen, and kidneys [1]. In adipose tissue, we have shown that pharmacological modulation of CNR2 with either agonist AM1241 or inverse agonist AM630 decreases circulating leptin and alters the thermogenic response by decreasing the *beta-3 adrenergic receptor* and *tumor necrosis factor alpha (TNF-α)* mRNA in epididymal white adipose tissue and *TNF-α* in peri-renal white adipose tissue [1]. Emerging research has identified a role for CNR2 in metabolism and body weight maintenance. For example, we have demonstrated that selective

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agonism of CNR2 in obese mice reduces fat mass and adipocyte size [2]. Currently little is known of the role CNR2 plays in skeletal muscle metabolism in obesity. Obesity decreases fatty acid oxidative capacity and decreases adiponectin, a key adipokine that stimulates fatty acid oxidation [3].

The decrease in adiponectin and the associated reduction in adiponectin signaling may be due to altered interactions with multiple signaling cascades. The adiponectin pathway in skeletal muscle is yet to be fully characterized. It is possible that in obesity there may be changes in adiponectin receptors, adiponectin receptor 1 (ADIPOR1) or adiponectin receptor 2 (ADIPOR2), to alter adiponectin signaling. Adapter protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) and APPL2 are adapter proteins that bind directly to ADIPOR1 and ADIPOR2 to mediate signaling, with APPL1 acting as a positive mediator, potentially via AKT, and APPL2 acting as an inhibitor in adipose-tissue-related metabolic signaling [4]. Similarly, 5'adenosine mono-phosphate-activated protein kinase (AMPK) controls activation of AKT, which under control conditions, promotes anabolic metabolism and cell survival, and maintains oxidative metabolism in the mitochondria. AMPK acts as an energy sensor that boosts fatty acid oxidation, and AKT mediates insulin signaling; with both decreased in obesity, while adiponectin has been shown to reverse this down regulation [5]. Implicated in the crosstalk of adiponectin, skeletal muscle and adipose storage is eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), which increases biogenesis and mitochondrial activity [6]. 4EBP1 and adiponectin activate peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC1- α) which is known as the regulator of intracellular fatty acid metabolism by stimulating mitochondria biogenesis and increasing skeletal muscle oxidative capacity [7]. Moreover, the scavenger receptor fatty acid translocase/Cluster of Differentiation 36 (FAT/CD36), is increased in response to a high fat diet (HFD) [8], which may be due to adiponectin modulating human skeletal muscle intracellular pyruvate dehydrogenase kinase 4 (PDK4) in response to a HFD [9].

Collectively these demonstrate that the skeletal muscle's response to a HFD and obesity is to decrease fatty acid oxidation which may contribute to alterations in adiponectin signaling. Given that CNR2 knockout mice display an increase in circulating adiponectin, this study aimed to investigate, in a DIO rat model, if pharmacological modulation of CNR2 altered skeletal muscle enzyme activity and fatty acid oxidative pathways and if the adaptations in skeletal muscle proteins occur with or without acute adiponectin treatment. We hypothesize that pharmacological modulation of CNR2 in a DIO rat model will increase markers associated with skeletal muscle fatty acid oxidation, which will be further enhanced by acute adiponectin exposure.

2. Materials and methodology

2.1. Animals and experimental protocols

All animal experimental procedures were approved by the Howard Florey Animal Ethics Committee (AEC 11-036), and rats were housed in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purpose, following the ARRIVE Guidelines for Reporting Animal Research [10]. Seven-week-old male Sprague Dawley rats were used (The Animal Resource Center, Canning Vale, WA, Australia). Following a 1–2 week acclimatization period, the rats were individually housed in a plastic tub with stainless steel lid (cage dimensions; width 27.5 × length 41.0 × height 25.5 cm) (R.E. Walters, Sunshine, Victoria, Australia) in an environmentally controlled laboratory (ambient temperature 22–24 °C) with a 12 h light/dark cycle (07:00–19:00). The overall study design is previously published [1]. The Sprague Dawley rats following 9 weeks of HFD were matched according to weight, body composition and blood pressure were maintained on the HFD and randomized for pharmacological treated for six weeks with a daily i.p. injection, with either vehicle (0.9% isotonic saline solution

containing 0.75% Tween 80: $n = 9 - 10$), or 3 mg/kg body weight of AM1241 (Cayman Chemicals, Ann Arbor, MI, USA) or 0.3 mg/kg body weight of AM630 (Cayman Chemicals, Ann Arbor, MI, USA) dissolved in the vehicle solution ($n = 9 - 10$).

These compounds and their doses were chosen based on previously published literature [11] and [12]. AM1241 is a potent CNR2 inverse agonist in rodents, with an 80-fold selectivity over CNR1, AM630 is a potent inverse agonist/antagonist of CNR2 with a low infinity for CNR1 with both compounds potentially displaying off target activation of TRPA1 ion channels [13]. Following treatment, rats were anesthetized with 3% isoflurane inhalation (Abbott, Botany, NSW, Australia) with each animal undergoing surgical removal of skeletal muscle, rats were then administered a lethal injection of 100 mg/kg sodium pentobarbital (Virobac, Peakhurst, Australia) and euthanised via cardiac puncture, then all other major organs including fat pads were removed post-mortem, weighed, snap frozen in liquid nitrogen (LN₂) and stored at −80 °C for further analyses.

2.2. Muscle sample preparations

Each muscle was carefully dissected, including the soleus and extensor digitorum longus (EDL), immediately after their removal longitudinal strips of the soleus and EDL were incubated in a 14-lane oxygenated organ bath (37 °C pre-gassed at 95% O₂ –5% CO₂). Each longitudinal strip was placed in an individual chamber of Krebs-Henseleit buffer with either 0.1 µg/ml of adiponectin or the contralateral hind limb muscle exposed to saline for 30 min. The skeletal muscles were taken from opposing limbs from each animal to ensure that alternate muscles were exposed to adiponectin treatment for example: rat one- right then left hind limb muscle; rat two- left and then right limb muscle was treated with the adiponectin or saline and this sequence was then switched for each subsequent rat. Following the treatment skeletal muscle samples were snap frozen and stored in LN₂ for subsequent analysis. The gastrocnemius muscle was dissected into red and white sections and was immediately frozen in LN₂ then subsequently used for CS activity as previously described [3].

2.3. RNA extraction

Skeletal muscle total RNA was isolated from the soleus and EDL muscle by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) as previously described [14]. In short, rodent muscle tissue extracts (approximately 15 mg) were dissociated using 1000 mg of ceramic/silica beads and RNA was extracted in TRIzol and treated with the RQ1 RNase-free DNase kit (Promega Corporations, Madison, WI, USA) following instructions of the manufacturer. First-strand cDNA was then generated from 0.3 µg of template RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) using random hexamers and oligo dTs. cDNA was stored at −20 °C for subsequent analysis.

2.4. 'Real time' polymerase chain reaction

qPCR was conducted using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and the MyiQ™ single color qPCR detection system (Bio-Rad Laboratories, Hercules, CA). qPCR reactions were run for 50 cycles of 95 °C for 15 s and 60 °C for 60 s. Samples of mRNA were amplified using forward and reverse oligonucleotide primer sequences to test skeletal muscle for expression of genes: AdipoR1, AdipoR2, APPL1, APPL2, FAT/CD36, AMPK α 2, PDK4 and PGC1- α (Geneworks, Thebarton, SA, USA). The changes in data were normalized to housekeeping genes, GAPDH, cyclophilin and β -actin (Supplementary Figure 1) and quantified using the 2-delta CT method as reported as arbitrary units.

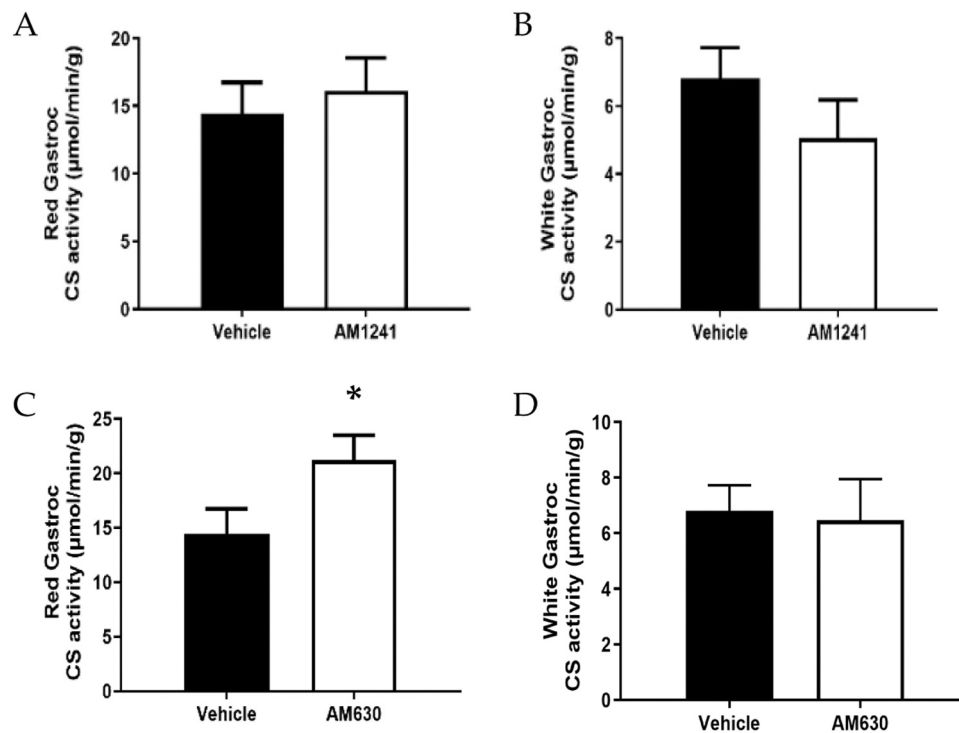


Fig. 1. The effect of AM630 or AM1241 treatment in DIO rats on citrate synthase activity in red and white gastrocnemius skeletal muscle. DIO rats were injected daily with either AM1241 (3 mg/kg of body weight, ip), AM630 (0.3 mg/kg) or vehicle for six weeks. Citrate synthase in AM1241 treated (A) red gastrocnemius ($n = 9$), (B) white gastrocnemius ($n = 9$) and AM630 treated (C) red gastrocnemius ($n = 9$) and (D) white gastrocnemius ($n = 9$). Results are expressed as mean $\pm$ SEM. Significance * $p = 0.05$ compared to vehicle.

2.5. Western blots

Protein was isolated as described previously [15]. Protein was isolated from rat skeletal muscle, and aliquots (20–25 µl/mg) of protein lysate were separated on a 7.5–20.0% SDS-PAGE gel and transferred to a nitrocellulose membrane. The background reading was blocked to prevent non-specific binding by 2.5/ 5% skim milk powder or BSA in TBST solution. PGC1- α , AKT, AKT phosphorylation Ser⁴⁷³, 4EBP-1, 4EBP1 phosphorylation Thr^{37/46}, AMPK, and AMPK phosphorylation Thr¹⁷² (Sigma-Aldrich) were detected using Western blot analysis from skeletal muscle lysate using specific primary antibodies with pooled internal standards as a loading control. Secondary anti-mouse and anti-rabbit antibodies were purchased from Sigma Aldrich. Band densitometry was normalized to pooled internal standards, visualized through chemiluminescence, and analyzed using Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA). Phosphorylation detection was recorded at 4EBP1 – 15 kDa, AKT – 60 kDa, AMPK – 63 kDa and PGC1- α – 105 kDa.

2.6. Citrate synthase analysis

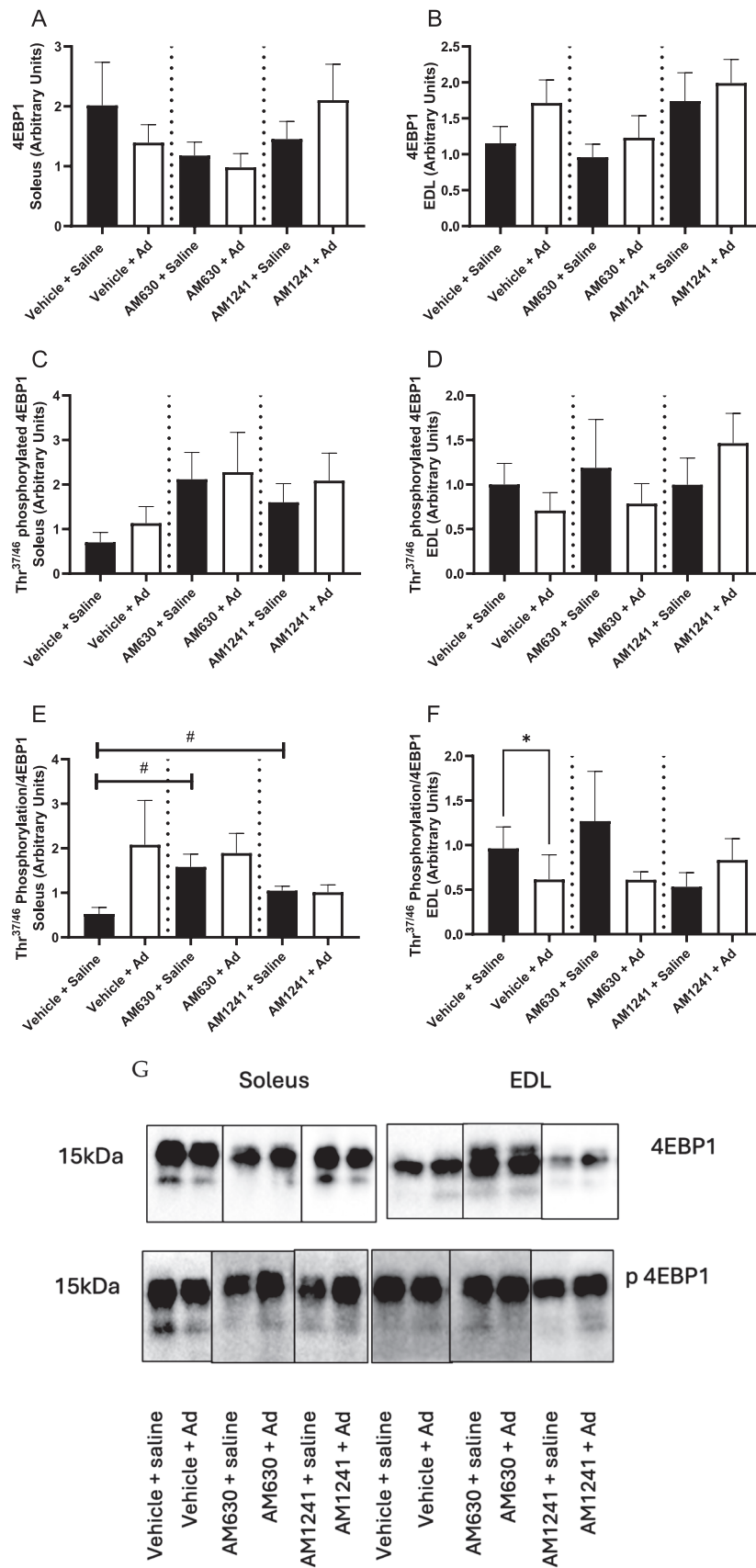
Citrate Synthase (CS) activity in red and white gastrocnemius muscle was determined as previously described [16]. Briefly, after the addition of 5 µL of muscle homogenate (0.175 mM KCl & 2 mM EDTA, pH 7.4), 230 µL of reagent cocktail (3 mM Acetyl CoA (Sigma A-2056), 100 mM Tris buffer (BioRad, California, United States); pH 8.3), 1 mM DTNB (Sigma D-8130) and 15 µL of 10 mM Oxalacetate (Sigma O-4126). CS activity was measured at 412 nm (xMark™ Microplate Spectrophotometer, BioRad). Enzyme activity was expressed relative to wet weight, change in absorbance/min was calculated to measure CS activity (µmol/min/g) [16].

2.7. Statistical analysis

A power calculation was performed to determine the minimum number of animals per group, as described previously [17] using G*power software. A power calculation was performed to determine the minimum number of animals per group based on the parameters of Western blot [18] and real time PCR [1], using the G*power software. All data are presented as mean $\pm$ SEM, and figures produced and analyzed using GraphPad 10.0 Prism software. Differences among treatment groups, following the 6-week treatment with AM630 or AM1241 were compared to vehicle using unpaired t -test or where data was not normally distributed, as determined by a Shapiro-Wilk test, a Mann-Whitney test was undertaken. To compare the effects of the acute treatment with adiponectin, groups were compared using a paired t -test or where data was not normally distributed, as determined by a Shapiro-Wilk test, a Wilcoxon test was undertaken. Outliers were excluded if they varied greater than two standard deviations away from the mean. Significance was set at $P \leq 0.05$.

3. Results

Other tissues obtained from the current DIO model have been explored previously, AM1241 (0.3 mg/kg), [1] AM630 (3 mg/kg) [18]. In summary the metabolic responses to 6 weeks of treatment did not alter body weight, food intake, or liver weight, and it had no effect on numerous circulating cytokines or peri-renal fat pad mass and decreased plasma leptin levels. Moreover, both treatments decreased adipose tissue biomarkers. Individual treatment with AM1241 resulted in a decreased heart weight and brown adipose tissue weight, significantly reduced systolic blood pressure, peri-renal adipose accumulation, plasma leptin, urinary protein, urinary albumin, urinary sodium excretion and the fibrotic markers transforming growth factor-beta 1



(caption on next page)

Fig. 2. 4EBP1 total protein, Thr^{37/46} phosphorylated 4EBP1 and ratio (Thr^{37/46} phosphorylated 4EBP1/4EBP1) in skeletal muscle of diet induced obese (DIO) rats treated with vehicle, AM1241 or AM630 in the presence or absence adiponectin. A) Soleus 4EBP1 total protein, B) EDL 4EBP1 total protein, C) Soleus Thr^{37/46} phosphorylated 4EBP1, D) EDL Thr^{37/46} phosphorylated 4EBP1, E) Soleus Thr^{37/46} phosphorylated 4EBP1/4EBP1 ratio, F) EDL Thr^{37/46} phosphorylated 4EBP1/4EBP1 ratio (n = 6–10). G) Representative Western blots from groups. All data are presented as a mean $\pm$ SEM in arbitrary units. Significance # $p < 0.05$ compared to vehicle plus saline, * $p < 0.05$ compared to saline control.

(TGF- β 1), collagen IV and vascular endothelial growth factor in kidney lysate. AM630 treatment resulted in decreased plasma ghrelin and glucagon-like peptide 1 levels and increased interleukin 6 levels, significantly reduced creatinine clearance and increased glomerular area and kidney weight and significantly reduced diastolic blood pressure, glucose tolerance, insulin sensitivity, plasma creatinine and plasma TGF- β 1.

In the current study there was no change in CS activity in either red (Fig. 1A) or white gastrocnemius skeletal muscle (Fig. 1B) in DIO rats treated with AM1241, compared to the vehicle control treated DIO rats. In the red gastrocnemius muscle (Fig. 1C) obtained from rats treated with AM630, there was a significant increase in CS activity (Fig. 1C; $p = 0.05$), however, there was no change in CS activity in the white gastrocnemius muscle treated with AM630 (Fig. 1D).

4EBP1 and Thr^{37/46} phosphorylated 4EBP1 were measured in soleus and EDL muscle from DIO rats treated with AM1241 or AM630 and following acute exposure to adiponectin. Total 4EBP1 protein in soleus (Fig. 2A) and EDL (Fig. 2B) was unaltered. Thr^{37/46} phosphorylated 4EBP1 in soleus and EDL (Fig. 2C, 2D) was unaltered. The skeletal muscle phosphorylated to total 4EBP1 protein ratio in soleus was significantly increased in AM630 treated muscle and AM1241 treated muscle compared to control (Fig. 2E, $p = 0.014$ and $p = 0.011$). In EDL adiponectin treatment significantly reduced the phosphorylated to total ratio of 4EBP1 in controls (Fig. 2F, $p = 0.031$). Representative blots for 4EBP1 are shown in Fig. 2G.

We measured AKT and Ser⁴⁷³ phosphorylated AKT in soleus and EDL muscle from DIO rats treated with AM1241 or AM630 and adiponectin. There were no changes in total AKT protein in the soleus (Fig. 3A), or EDL (Fig. 3B). Ser⁴⁷³ phosphorylated AKT in soleus significantly decreased in AM1241 treated animals compared to control (Fig. 3C, $p = 0.001$) and in EDL, there was a significant decrease in AM630 treated animals compared to control (Fig. 3D, $p = 0.049$). Despite this, the ratio of Ser⁴⁷³ phosphorylated to total AKT in soleus (Fig. 3E) or EDL (Fig. 3F) was unaltered. Representative blots for AKT are shown in Fig. 3G.

We measured AMPK and Thr¹⁷² phosphorylated AMPK in soleus and EDL muscle from DIO rats treated with vehicle, AM1241 or AM630 and following acute exposure to adiponectin. There was a significant increase compared to vehicle control in total AMPK protein detected in the soleus (Fig. 4A, $p = 0.001$) and EDL (Fig. 4B, $p = 0.007$) following AM630 treatment. For Thr¹⁷² phosphorylated AMPK, in soleus there was a significant increase in AM630 treated animals following acute adiponectin exposure compared to the acute saline control (Fig. 4C, $p = 0.037$). There were no significant changes in Thr¹⁷² phosphorylated AMPK in EDL (Fig. 4D). There was a significant decrease in the total to Thr¹⁷² phosphorylated ratio in AMPK in the soleus treated with AM630 (Fig. 4E, $p = 0.005$), but no significant differences in EDL (Fig. 4F). Representative blots for AMPK are shown in Fig. 4G.

We measured PGC1- α in soleus and EDL muscle from DIO rats treated with vehicle, AM1241 or AM630 for six weeks and following acute exposure to adiponectin. There were no changes in total protein detected in the soleus muscle for PGC1- α (Fig. 5A). In EDL, there was a significant decrease in PGC1- α in AM630 (Fig. 5B, $p = 0.005$) and AM1241 treated animals (Fig. 5B, $p = 0.026$). In AM630 group when exposed to acute adiponectin exposure there was a significant increase in PGC1- α compared to AM630 saline treated controls (Fig. 5B, $p = 0.02$). Representative blots for PGC1- α are shown in Fig. 5C.

Expression of *ADIPOR1*, *ADIPOR2*, *APPL1*, *APPL2*, *FAT/CD36*, *AMPK*, *PKD4* and *PGC1- α* mRNA were measured in soleus and EDL

muscle from DIO rats treated with AM1241 or AM630 and acute exposure to adiponectin (Table 1). Treatment with AM1241 for 6 weeks increased *ADIPOR1* mRNA in the EDL compared to vehicle (Table 1, $p = 0.044$). While treatment with AM630 or AM1241 for 6 weeks decreased *PKD4* mRNA in the soleus compared to vehicle (Table 1, $p = 0.02$ and $p = 0.006$ respectively). Acute exposure to adiponectin did not significantly alter mRNA expression in any of the target genes (Table 1).

4. Discussion

In the DIO rat model, this study aimed to investigate whether pharmacological modulation of CNR2 altered CS enzyme activity and fatty acid oxidative pathways with or without acute *ex vivo* adiponectin treatment. Inverse agonist AM630 increased CS activity, a key marker of mitochondrial content, in the red gastrocnemius skeletal muscle. In addition, in soleus AM630 increased activation of 4EBP1 (indicated by an increased ratio of Thr^{37/46} phosphorylated to total protein), reduced AMPK α activation and reduced *PKD4* mRNA. In soleus, AM1241 activated 4EBP1 and reduced *PKD4* mRNA. While, in the EDL AM630 or AM1241 reduced PGC1- α and AM1241 increased *AdipoR1* mRNA. Interestingly, *ex vivo* adiponectin exposure reduced 4EBP1 activation in the EDL in vehicle treated animals only. Finally, in EDL, AM630 in the presence of adiponectin increased PGC1- α total protein. Interestingly, increased CS activity suggests enhanced mitochondrial oxidative capacity, however the reduced AMPK phosphorylation and PGC1- α expression indicate suppression of the canonical energy stress-induced mitochondrial biogenesis pathway. This pattern may reflect a state in which mitochondrial function has already adapted or improved, reducing cellular energy stress and therefore lowering the need for continued AMPK/PGC-1 α signaling. Despite this, these data suggest that the link between CNR2 and adiponectin varies dependant on muscle type.

To investigate if CNR2 ligands altered mitochondrial content in obesity we measured changes in CS in red and white gastrocnemius muscle to elucidate adaptations in different skeletal muscle fiber types, with red being highly oxidative and white being low in oxidative capacity. CS is a quantitative marker of the content of intact mitochondria [19]. We identified no change in skeletal muscle CS activity in the white gastrocnemius for either obese treatment group. However, the inverse agonist, AM630, but not the agonist, AM1241, significantly increased CS in red gastrocnemius from the DIO rodents. This suggests that ablating CNR2 signaling increases mitochondrial content that may increase capacity to oxidize fatty acids in the soleus muscle in the obese state. In skeletal muscle following exercise training there is an increased in CS in sedentary humans [20]. Further, in CNR2 knockout mice fed a HFD which displayed increased food intake and were prone to obesity, however, had markers of improved insulin stimulated glucose uptake in the skeletal muscle [21]. Despite the improvements in CS activity in the DIO rats treated with AM630, these animals did not increase food intake [1] and circulating leptin was reduced [17]. Increasing CS and decreasing leptin have previously resulted in improvement in adults and aging rat skeletal muscle following 7–14 days of continuous treadmill use [19]. This suggests that targeting CNR2 in obesity may improve muscle metabolic function.

AMPK modulates cellular fatty acids and influences cellular metabolism [22]. Others have shown that the activation of AMPK can occur via stimulation by adiponectin [23]. However, we observed an increase in total AMPK α following AM630 treatment in the soleus and EDL of the

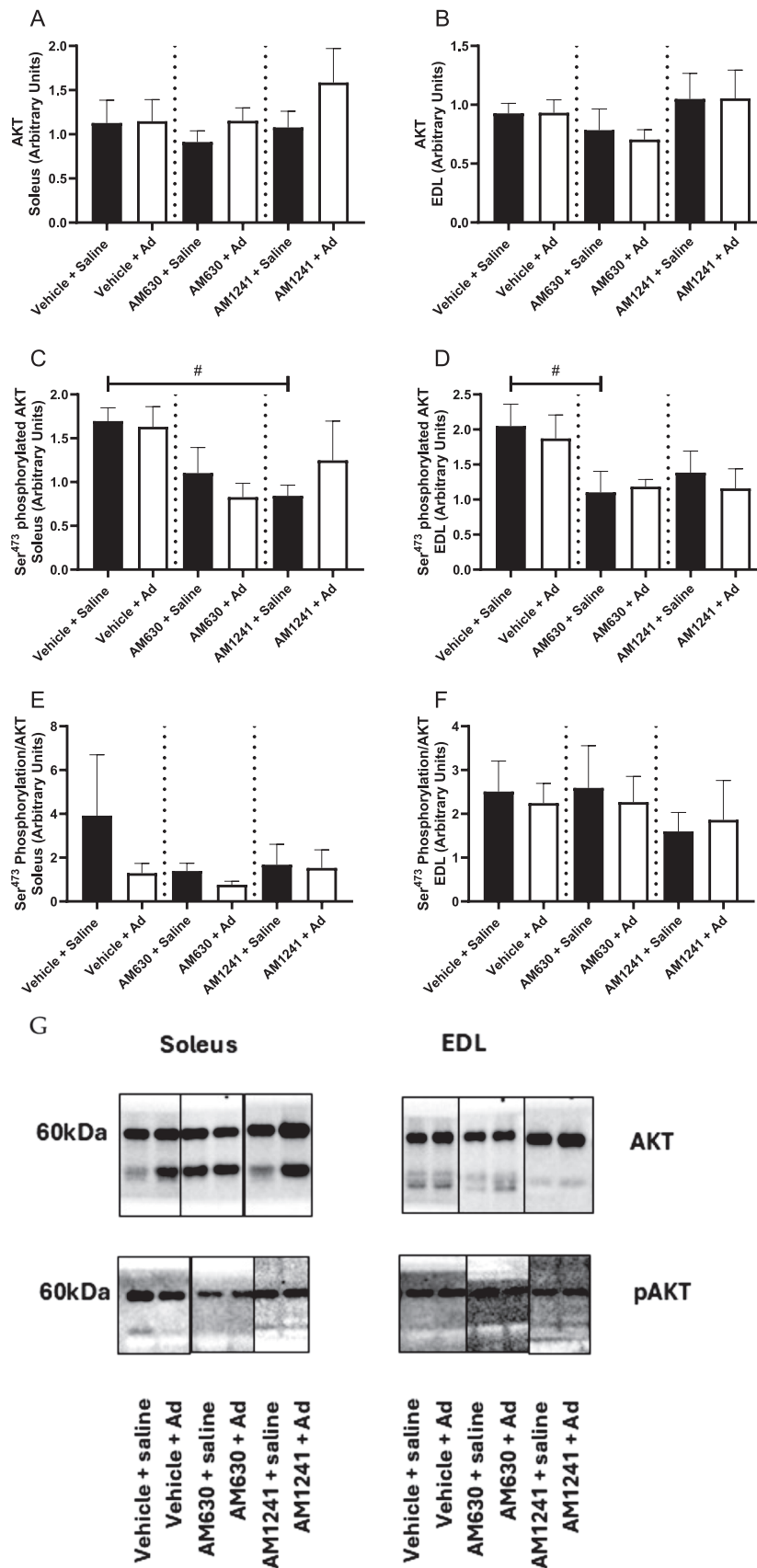


Fig. 3. AKT total protein, Ser⁴⁷³ phosphorylated AKT and ratio (Ser⁴⁷³ phosphorylated AKT/AKT) in skeletal muscle of diet induced obese (DIO) rats treated with vehicle, AM1241 or AM630 in the presence or absence of adiponectin. A) Soleus AKT total protein, B) EDL AKT total protein, C) Soleus Ser⁴⁷³ phosphorylated AKT, D) EDL Ser⁴⁷³ phosphorylated AKT, E) Soleus Ser⁴⁷³ phosphorylated AKT/AKT ratio, F) EDL Ser⁴⁷³ phosphorylated AKT/AKT ratio (n = 5–10). G) Representative Western blots from groups. All data are presented as a mean $\pm$ SEM in arbitrary units. Significance # $p < 0.05$ compared to vehicle plus saline.

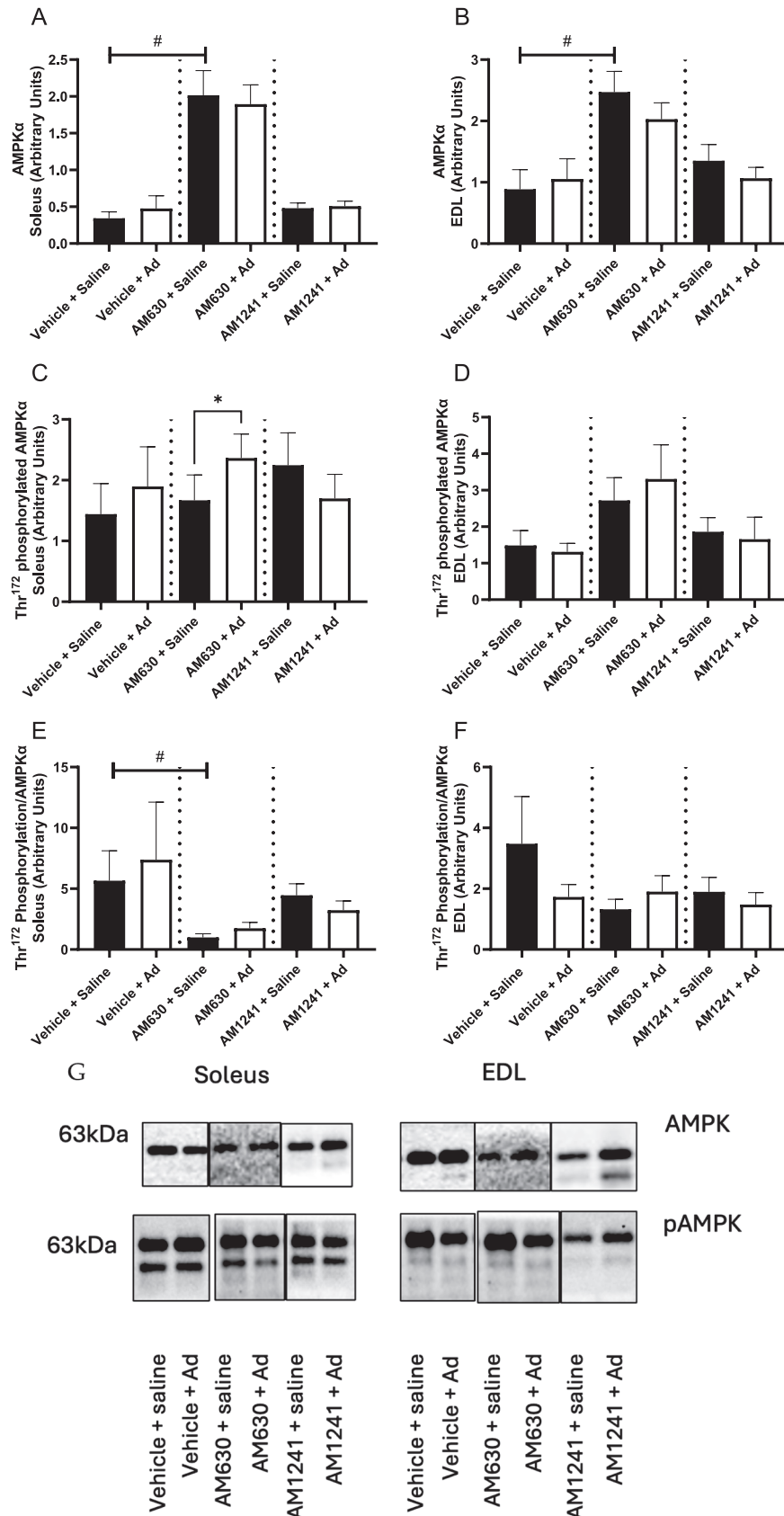


Fig. 4. AMPK total protein, Thr¹⁷² phosphorylated AMPK and ratio (Thr¹⁷² phosphorylated AMPK/AMPK) in skeletal muscle of diet induced obese (DIO) rats treated with vehicle, AM1241 or AM630 in the presence or absence adiponectin. A) Soleus AMPK total protein, B) EDL AMPK total protein, C) Soleus Thr¹⁷² phosphorylated AMPK, D) EDL Thr¹⁷² phosphorylated AMPK, E) Soleus Thr¹⁷² phosphorylated AMPK/AMPK ratio, F) EDL Thr¹⁷² phosphorylated AMPK/AMPK ratio (n = 6–10). G) Representative Western blots from groups. All data are presented as a mean ± SEM in arbitrary units. Significance # p < 0.05 compared to vehicle plus saline, * p < 0.05 compared to saline control.

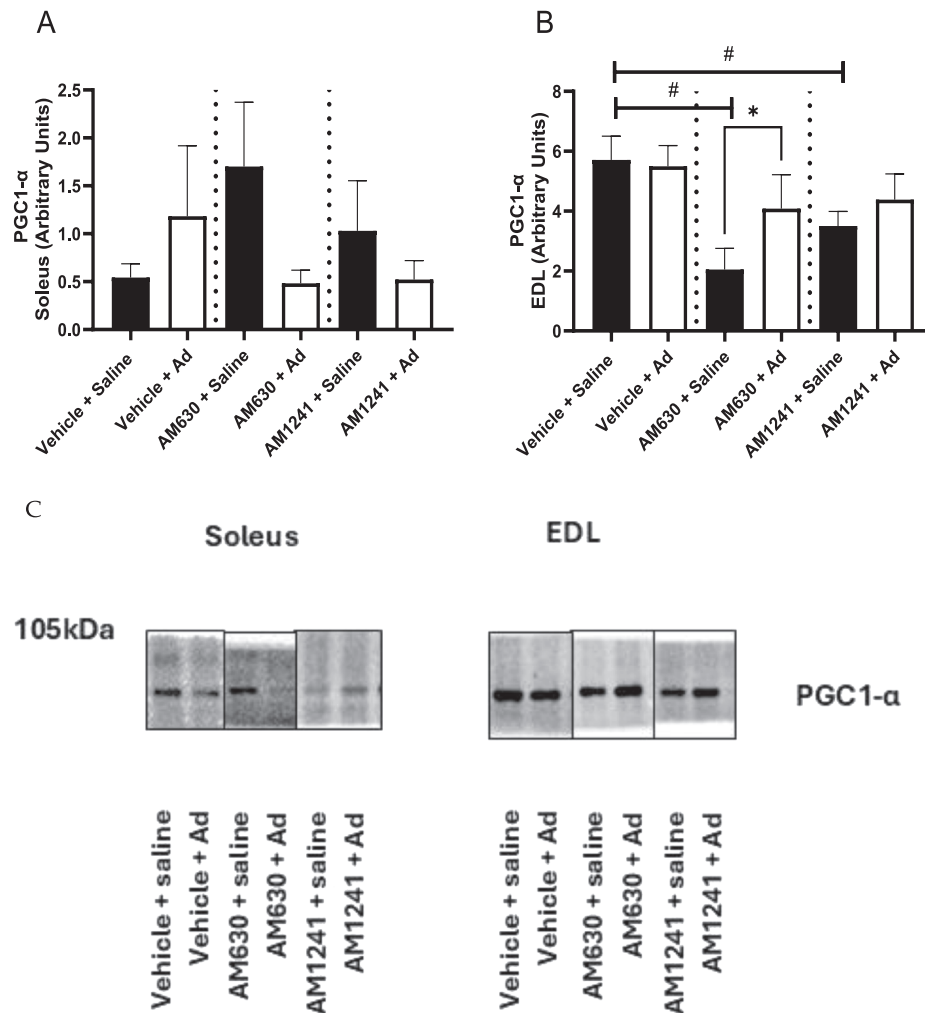


Fig. 5. PGC1- α protein from the skeletal muscle of diet induced obese (DIO) rats treated with vehicle, AM1241 or AM630 in the presence or absence of adiponectin. A) Soleus PGC1- α protein, B) EDL PGC1- α protein, C) Representative Western blots from groups (n = 6–10). All data are presented as a mean $\pm$ SEM in arbitrary units. Significance # $p < 0.05$ compared to vehicle plus saline, * $p < 0.05$ compared to saline control.

DIO rats. Despite this, AMPK α activity was decreased in soleus muscle. AMPK plays a key role in increasing fatty acid oxidation and skeletal muscle functionality in type 2 diabetes [24]. In the skeletal muscle of humans with metabolic syndrome, the mRNA expression of AMPK α 1 and AMPK α 2 was found to be downregulated [25]. However, in this current study, the changes in total AMPK and AMPK phosphorylation in response to targeting CNR2 suggests that CNR2 contributes to oxidative capacity in skeletal muscle in obesity. Previous research suggests a relationship between CNR2 and AMPK. In rat cortical neurons/glia cells *in vitro*, agonism of CNR2 with, transcarboxylase, ameliorated ischemic injury, potentially through the modulation of AMPK/CREB signaling pathways [26].

Changes were also observed in 4EBP1 activation in our study, with AM630 and AM1241 treatment in the soleus from DIO rodents, significantly increasing 4EBP1 activation. In EDL, acute adiponectin treatment significantly reduces 4EBP1 activation in control animals only. Previous research has demonstrated that 4EBP1 prevents excess build-up of lipids in skeletal muscle cells in mice [27]. Therefore, an increase in activation of 4EBP1 following AM630 and AM1241 treatment may indicate the attempt of the muscle to prevent excess build-up of fatty acids following the exposure to DIO, however without further experimentation such as oil red O staining this is speculative. In skeletal muscle, activation of 4EBP1 in obese mice has also resulted in improvements in glucose

sensitivity and an overall increased metabolic rate [28]. However, in the EDL from DIO controls 4EBP1 activation in the presence of adiponectin was reduced which suggests in this tissue the response to adiponectin may differ from the soleus. 4EBP1 is downstream of several identified pathways such as mechanistic target of rapamycin complex (mTORC) and phosphoinositide 3-kinase (PI3K)/AKT and is disrupted in pathological conditions such as obesity and insulin resistance [29]. Limited studies have investigated 4EBP1 and adiponectin in skeletal muscle, however, in osteoclasts adiponectin inhibits differentiation by increasing AdipoR1 expression which phosphorylated 4EBP1 in multiple myeloma sufferers [30]. Further studies investigating the role of 4EBP1 in skeletal muscle, with investigations into downstream pathways such as mTORC and PI3K/AKT, are warranted.

Despite a reduction in AKT Ser⁴⁷³ phosphorylation in the soleus following AM1241 treatment and EDL following AM630 treatment in the obese state, there was no change in activation of AKT in the presence or absence of adiponectin. Phosphorylated AKT is decreased in rodents consuming a HFD [31], potentially via G-protein coupled receptors. Our results differ from a previous study which demonstrated that in obese mice, skeletal muscle AKT increases the growth of glycolytic EDL fibers [32]. There was a significant decrease in PGC1- α , in EDL from DIO rats when treated with either AM630 or AM1241. PGC1- α plays a major role in mitochondria biogenesis and is decreased in the obese state. In our

Table 1

The effect of AM1241, AM630 and acute adiponectin treatment on skeletal muscle gene expression in DIO rats. Soleus and EDL were obtained from DIO rats treated with vehicle, AM630 or AM1241 for six weeks and exposed *ex vivo* to 0.1 µg/ml of adiponectin (n = 5 – 9 and 5 – 9 respectively) or vehicle (n = 6 – 9 and n = 4 – 9 respectively) for 30 min. All data are presented as a mean ± SEM in arbitrary units (normalized to housekeeping genes, *GAPDH*, *cyclophilin* and *β-actin*). Significance # p < 0.05 compared to vehicle plus saline.

Gene	Skeletal muscle	Vehicle	Vehicle + acute adiponectin treatment	AM630	AM630 + acute adiponectin treatment	AM1241	AM1241 + acute adiponectin treatment
<i>ADIPOR1</i>	(a) Soleus	0.156 ± 0.119	0.095 ± 0.074	0.009 ± 0.003	0.021 ± 0.004	0.659 ± 0.260	0.324 ± 0.109
	(b) EDL	0.055 ± 0.014	0.037 ± 0.011	0.028 ± 0.009	0.036 ± 0.011	0.195 ± 0.070 [#]	0.084 ± 0.030
<i>ADIPOR2</i>	(a) Soleus	0.062 ± 0.018	0.061 ± 0.013	0.054 ± 0.018	0.033 ± 0.007	0.064 ± 0.017	0.060 ± 0.019
	(b) EDL	0.085 ± 0.037	0.086 ± 0.017	0.048 ± 0.018	0.053 ± 0.014	0.062 ± 0.023	0.024 ± 0.008
<i>APPL1</i>	(a) Soleus	0.033 ± 0.011	0.095 ± 0.048	0.071 ± 0.032	0.020 ± 0.010	0.125 ± 0.051	0.222 ± 0.098
	(b) EDL	0.054 ± 0.024	0.044 ± 0.009	0.042 ± 0.013	0.054 ± 0.011	0.028 ± 0.012	0.046 ± 0.021
<i>APPL2</i>	(a) Soleus	0.0054 ± 0.0013	0.0062 ± 0.0018	0.0051 ± 0.0007	0.0026 ± 0.0009	0.0136 ± 0.0041	0.0014 ± 0.0006
	(b) EDL	0.013 ± 0.007	0.026 ± 0.016	0.002 ± 0.0004	0.003 ± 0.001	0.006 ± 0.002	0.003 ± 0.001
<i>FAT/CD36</i>	(a) Soleus	0.698 ± 0.344	1.033 ± 0.330	0.384 ± 0.120	0.405 ± 0.102	2.823 ± 0.839	2.174 ± 1.118
	(b) EDL	0.260 ± 0.083	0.369 ± 0.104	0.484 ± 0.135	0.682 ± 0.278	0.300 ± 0.082	0.225 ± 0.110
<i>AMPK</i>	(a) Soleus	0.037 ± 0.012	0.134 ± 0.047	0.087 ± 0.035	0.138 ± 0.056	0.087 ± 0.029	0.052 ± 0.029
	(b) EDL	0.570 ± 0.237	0.871 ± 0.325	1.927 ± 0.976	1.367 ± 0.672	0.275 ± 0.070	0.049 ± 0.018
<i>PDK4</i>	(a) Soleus	0.663 ± 0.261	0.372 ± 0.121	0.079 ± 0.047 [#]	0.203 ± 0.048	0.053 ± 0.024 [#]	0.279 ± 0.117
	(b) EDL	0.141 ± 0.076	0.592 ± 0.461	0.194 ± 0.091	0.082 ± 0.037	0.011 ± 0.004	0.009 ± 0.003
<i>PGC1-α</i>	(a) Soleus	0.573 ± 0.284	0.248 ± 0.132	0.091 ± 0.058	0.022 ± 0.01	0.047 ± 0.018	0.398 ± 0.241
	(b) EDL	0.111 ± 0.067	0.094 ± 0.053	0.068 ± 0.031	0.057 ± 0.016	0.060 ± 0.031	0.019 ± 0.008

AdipoR1: Adiponectin receptor 1; **AdipoR2:** Adiponectin receptor 2, **APPL1:** adapter protein, phosphotyrosine interacting with PH domain and leucine zipper 1; **APPL2:** adapter protein, phosphotyrosine interacting with PH domain and leucine zipper 2, **FAT/CD36:** fatty acid translocase/Cluster of Differentiation 36; **AMPKα2:** 5'adenosine mono-phosphate-activated protein kinase α-2, **PDK4:** pyruvate dehydrogenase kinase 4, **PGC1-α:** peroxisome proliferator-activated receptor gamma co-activator 1 alpha, **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase, **β-Actin:** Beta-actin.

study, it was a surprising result that in the obese state CNR2 modulation decreased PGC1-α in the EDL but had no effect in the soleus muscle. Interestingly, acute adiponectin exposure in AM630 treated animals, increased PGC1-α. In cultured mouse skeletal muscle *in vitro*, adiponectin increases the expression of PGC1-α [33]. Adiponectin has also been shown to increase PGC1-α in mice skeletal muscle *in vivo*, however, these changes decreased when the mice were exposed to a high fat diet [36]. This suggests that obesity impacts PGC1-α in skeletal muscle. It is interesting that pharmacological modulation of CNR2 appeared to have differing effects on the different muscle fiber types. It has previously been proposed that obesity increases the conversion of red muscle (type 1 fiber type) to white (type 2 fiber type) [34]. However, in female Wistar rats fed a high fat diet for 21 days found no change in the size, shape, fiber types or distribution of skeletal muscle but recorded a significant increase in intramuscular lipid cell accumulation resulting in an overall decrease in muscle area [35]. Given the variability of our results, we acknowledge that pharmacological modulation of CNR2 may have differing effects on different muscle fiber types, the composition of the fiber types and/or lipid accumulation within the muscle fibers.

In summary, pharmacological modulation of CNR2 in a DIO rat model, coupled with adiponectin treatment, altered intracellular targets differently in soleus and EDL muscle. These findings may support a role for CNR2 in skeletal muscle and adiponectin signaling; however, further research is required to fully understand the role of CNR2 in skeletal

muscle and the impact of obesity on skeletal muscle metabolism. Interestingly, increased CS activity and 4EBP1 activity, coupled with a decrease in leptin in DIO rats treated with AM630 [1], suggests improvements in the skeletal muscle oxidative profile. However, these observations were not consistent across all proteins and mRNA measured. At this time the specific pathways involved are unknown, however based on this study there are likely to be differences between soleus and EDL. There were limitations to this study in that we had small sample sizes which may have contributed to the variation within the data, future explorations would require a larger sample. Additionally, this study aimed to compare the individual effects of AM630 or AM1241, or the acute effects of adiponectin treatment against the appropriate controls and thus employed unpaired or paired *t*-tests (or their non-parametric equivalent), rather than an ANOVA comparing across the various groups, thus potentially limiting the interpretation of the results. Moreover, the current study investigated the pharmacological modulations of CNR2 within the skeletal muscle in a DIO model only, and not whether this pharmacological modulation resulted in alterations in CNR2 receptor expression or activity. Future research should investigate muscle metabolism via mitochondrial respiration/oxygen consumption [33], ATP production, fatty acid and glucose oxidation rates, lactate production, glycogen utilization and insulin-stimulated glucose uptake to identify metabolic endpoints.

5. Conclusions

In DIO, pharmacological activation and inhibition of CNR2 in the presence of adiponectin altered different intracellular pathways in skeletal muscle, suggesting there is complexity in the capacity of CNR2 to control skeletal muscle oxidative capacity in obesity. Thus further exploration of the specific targets involved will inform future drug development and research exploring the capacity of the endocannabinoid system as a therapeutic in obesity.

CRedit authorship contribution statement

Lannie O'Keefe: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Anna C. Simcocks:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Kayte A. Jenkin:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Amirabbas Monazzami:** Writing – review & editing, Investigation, Formal analysis. **Shavin Chandrasiri:** Writing – review & editing, Investigation, Formal analysis. **Michael L. Mathai:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Deanne H. Hryciw:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Andrew J. McAinch:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Institutional Review Board Statement

The animal study protocol was approved by the Howard Florey Animal Ethics Committee (AEC 11–036).

Informed Consent Statement

Not applicable.

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Declaration of Competing Interest

The author declares no conflict of interest

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.orcp.2026.09.005](https://doi.org/10.1016/j.orcp.2026.09.005).

Data availability

The data presented in this study are available on request from the corresponding author.

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