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# Chronic AMP-Activated Protein Kinase Activation and a High-Fat Diet Have an Additive Effect on Mitochondria in Rat Skeletal Muscle

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Chronic AMP-Activated Protein Kinase Activation and a High Fat Diet  
Have an Additive Effect on Mitochondria in Rat Skeletal Muscle

Natasha Fillmore

A thesis submitted to the faculty of Brigham Young University in  
partial fulfillment of the requirements for the degree of

Master of Science

William W. Winder, chair  
Chad R. Hancock  
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Natasha Fillmore

The thesis of Natasha Fillmore is acceptable in its final form including (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory and ready for submission.

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## ABSTRACT

### Chronic AMP-Activated Protein Kinase Activation and a High Fat Diet

#### Have an Additive Effect on Mitochondria in Rat Skeletal Muscle

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Master of Science

Factors that stimulate mitochondrial biogenesis in skeletal muscle include AMPK, calcium, and circulating FFAs. Chronic treatment with either AICAR, a chemical activator of AMPK, or increasing circulating FFAs with a high fat diet increases mitochondria in rat skeletal muscle. The purpose of this study was to determine whether the combination of chronic chemical activation of AMPK and high fat feeding would have an additive effect on skeletal muscle mitochondria levels. We treated Wistar male rats with a high fat diet (HF), AICAR injections (AICAR), or a high fat diet and AICAR injections (HF+AICAR) for six weeks. At the end of the treatment period, markers of mitochondrial content were examined in white quadriceps, red quadriceps, and soleus muscles, predominantly composed of unique muscle-fiber types. In white quadriceps, there was a cumulative effect of treatments on LCAD, cytochrome c, and PGC-1 $\alpha$  protein, as well as on citrate synthase and  $\beta$ -HAD activity. In contrast, no additive effect was noted in the soleus and in the red quadriceps only  $\beta$ -HAD activity increased additively. The additive increase of mitochondrial markers observed in the white quadriceps may be explained by a combined effect of two separate mechanisms: high fat diet-induced post transcriptional increase in PGC-1 $\alpha$  protein and AMPK mediated increase in PGC-1 $\alpha$  protein via a transcriptional mechanism. These data show that chronic chemical activation of AMPK and a high fat diet have a muscle type specific additive effect on markers of fatty acid oxidation, the citric acid cycle, the electron transport chain, and transcriptional regulation.

Key words: AICAR, fiber type, mitochondrial biogenesis, PGC-1 $\alpha$ , PPAR $\delta$

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**Chronic AMP-Activated Protein Kinase Activation and a High Fat Diet Have an  
Additive Effect on Mitochondria in Rat Skeletal Muscle**

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Running Title: Additive Effect of AMPK and FFAs on Muscle Mitochondria

Key words: AICAR, fiber type, mitochondrial biogenesis, PGC-1 $\alpha$ , PPAR $\delta$



**Abstract**

Factors that stimulate mitochondrial biogenesis in skeletal muscle include AMPK, calcium, and circulating FFAs. Chronic treatment with either AICAR, a chemical activator of AMPK, or increasing circulating FFAs with a high fat diet increases mitochondria in rat skeletal muscle. The purpose of this study was to determine whether the combination of chronic chemical activation of AMPK and high fat feeding would have an additive effect on skeletal muscle mitochondria levels. We treated Wistar male rats with a high fat diet (HF), AICAR injections (AICAR), or a high fat diet and AICAR injections (HF+AICAR) for six weeks. At the end of the treatment period, markers of mitochondrial content were examined in white quadriceps, red quadriceps, and soleus muscles, predominantly composed of unique muscle-fiber types. In white quadriceps, there was a cumulative effect of treatments on LCAD, cytochrome c, and PGC-1 $\alpha$  protein, as well as on citrate synthase and  $\beta$ -HAD activity. In contrast, no additive effect was noted in the soleus and in the red quadriceps only  $\beta$ -HAD activity increased additively. The additive increase of mitochondrial markers observed in the white quadriceps may be explained by a combined effect of two separate mechanisms: high fat diet-induced post transcriptional increase in PGC-1 $\alpha$  protein and AMPK mediated increase in PGC-1 $\alpha$  protein via a transcriptional mechanism. These data show that chronic chemical activation of AMPK and a high fat diet have a muscle type specific additive effect on markers of fatty acid oxidation, the citric acid cycle, the electron transport chain, and transcriptional regulation.

## Introduction

Factors known to stimulate mitochondrial biogenesis in skeletal muscle include AMP-activated protein kinase (AMPK) activity and circulating free fatty acids (FFAs). In response to an endurance exercise training bout both AMPK activity in skeletal muscle and circulating FFAs are elevated (43, 61) raising the question as to whether these two factors could work together to induce mitochondrial biogenesis. Rats treated with 5-aminoimidazole-4-carboxamide riboside (AICAR) for four weeks have increased levels of certain mitochondrial markers in skeletal muscle (63). Furthermore, models of reduced AMPK activity have less skeletal muscle mitochondrial proteins compared to controls (36, 57). High fat feeding has also been shown to increase fatty acid oxidative capacity and mitochondrial content in skeletal muscle, likely due to elevated circulating FFAs (26, 40-42, 49, 58). Treating rats with both heparin, which increases circulating FFAs, and a high fat diet results in mitochondrial biogenesis (22). In contrast, treating diabetics for seven days with acipimox, which decreases circulating FFAs, results in decreased messenger RNA (mRNA) levels of a number of mitochondrial proteins and transcription regulators (7). These data show that circulating FFAs play an important role in mitochondrial biogenesis in skeletal muscle (22).

The mechanisms through which AMPK and circulating FFAs induce mitochondrial biogenesis appear to be somewhat distinct. For example, both are believed to induce mitochondrial biogenesis by increasing the ability of peroxisome proliferator-activated receptor gamma coactivator-1  $\alpha$  (PGC-1 $\alpha$ ) to coactivate transcription factors through different mechanisms. AMPK regulates PGC-1 $\alpha$  at both the gene and protein level. Constitutively activating AMPK by mutating a regulatory subunit of AMPK increases both the protein and mRNA levels of PGC-1 $\alpha$  (23). AMPK can increase PGC-1 $\alpha$  mRNA levels by regulating

binding of transcription factors to regions in the PGC-1 $\alpha$  gene promoter (30, 33, 35).

Additionally, it has been reported that AMPK activates PGC-1 $\alpha$  by phosphorylating PGC-1 $\alpha$  (34-35) and via activation of sirtuin 1 (SIRT1) (12, 31). Together these studies show that AMPK increases PGC-1 $\alpha$  abundance and/or binding activity through multiple mechanisms.

High fat feeding is believed to influence mitochondrial gene transcription by regulating PGC-1 $\alpha$  through a different mechanism. Raising circulating FFAs increases peroxisome proliferator-activated receptor  $\delta$  (PPAR $\delta$ ) binding to the muscle carnitine palmitoyltransferase (mCPT) promoter in rat epitrochlearis (22). FFAs activate PPAR $\delta$  by binding tightly inside the ligand binding domain causing movement of the c-terminal helix, a region involved in the binding of PPAR $\delta$  coactivators (19, 21). Interestingly, inducing high levels of PPAR $\delta$  protein in rat skeletal muscle increases PGC-1 $\alpha$  protein but not mRNA abundance (26, 39). A similar pattern is also observed when rats are treated with a high fat diet for five weeks, which elevates PPAR $\delta$  protein (26, 40). Since increasing PPAR $\delta$  protein levels induces similar effects as raising circulating FFAs and FFAs are ligands of PPAR $\delta$ , this suggests that FFAs increase skeletal muscle mitochondria by activating PPAR $\delta$ . In summary, it is believed that high fat feeding can stimulate mitochondrial biogenesis by raising circulating FFAs that activate PPAR $\delta$ , and over a period of a few weeks, leads to a post-transcriptional increase in PGC-1 $\alpha$  protein (26).

The availability of blood FFAs and AMPK protein abundance vary considerably between skeletal muscle fiber types. Muscles predominantly composed of Type IIa and Type I fibers receive greater blood flow, and consequently receive more FFAs than muscles mostly composed of Type IIb fibers (24). The protein abundance of one of the two catalytic subunits of AMPK, AMPK $\alpha$ 1, is greater in muscles predominantly composed of Type I

and/or Type IIa fibers compared to those predominantly composed of Type IIb fibers (48, 62). Conflicting reports exist on the relative abundance of the other catalytic subunit, AMPK  $\alpha 2$  in different muscle fiber types (48, 62). The AMPK  $\gamma 3$  subunit, believed to be the only  $\gamma$  subunit bound to heterotrimeric AMPK complexes in skeletal muscle that is activated by exercise (8), is higher in the red quadriceps and soleus compared to white quadriceps (16). Because blood FFA availability and the expression of AMPK subunits differ between the muscle fiber types, elevation of circulating FFA or AMPK activity would also likely not have the same degree of an effect on mitochondria content.

The purpose of this study was to determine if chronic AMPK activation in skeletal muscle and elevated FFAs in the blood have an additive effect on mitochondrial content of skeletal muscle. In addition, we examined three different muscles to determine if responses to treatments were muscle type specific. In the future, these findings could be applied to better understanding the mechanisms involved in exercise training-induced mitochondrial biogenesis.

## Materials and Methods

*Animal Care.* All experimental procedures used were approved by the Institutional Animal Care Committee of Brigham Young University. Wistar male rats were kept in a temperature controlled and well ventilated room with a 12:12 hr light-dark cycle. Rats were fed rodent laboratory chow diet, 8604 Harlan Teklad Rodent Diet, and water *ad libitum*.

Treatments lasted six weeks. Rats were treated with either AICAR injections (AICAR) (n=8), a high fat diet (HF) (n=11), AICAR injections and a high fat diet (HF+AICAR) (n=10), or nothing (Control) (n=9). In order to examine the effect of our treatments on different muscle types we measured mitochondrial markers in white quadriceps, red quadriceps, and soleus. Listed below is the fiber type composition of these three muscles.

(Fiber type- Population%): white quadriceps- Rectus Femoris (Type I- 1%, Type IIa- 25%, Type IIb- 74%), Vastus Lateralis (Type I- 0%, Type IIa- 3%, Type IIb- 97%); red quadriceps- Rectus Femoris (Type I- 7%, Type IIa- 53%, Type IIb- 40%), Vastus Lateralis (Type I- 9%, Type IIa- 56%, Type IIb- 35%); soleus (Type I- 87%, Type IIa- 13%, Type IIb- 0%) (4).

*AICAR injections.* AICAR treatment was given by subcutaneous injection at a dose of 0.5 mg AICAR/g body weight (BW) dissolved in 0.9% NaCl each morning of the treatment period.

AICAR was injected into two regions, axillary and between the scapulas. In order to distinguish the acute response from chronic adaptations to the AICAR treatment, half the rats in the AICAR and HF+AICAR groups were injected with AICAR one hour prior to dissection while the other half were not injected on the day of dissections.

In order to equalize the stress associated with the AICAR treatment, rats from Control and HF groups were handled daily at the time the rats treated with AICAR were handled. Further,

rats in the Control and HF groups were injected with comparable volume of saline to the AICAR injection.

*High Fat diet.* Rats were fed the high fat diet *ad libitum* for six weeks. Of the calories in the high fat diet, 60% came from fat with the fat coming from Flax Seed (40% of calories) and Olive Oil (20% of calories), a diet described previously (22). These fats were chosen in order to maximize the potential activation of PPAR $\delta$  through the binding of fatty acids to PPAR $\delta$  because unsaturated FAs are most effective at activating PPAR $\delta$  (19). The composition of the diet was as follows (g/kg of food): 116.3 g olive oil, 232.7 g flax seed oil, 87.2 g sugar, 174.6 g starch, 226.6 g casein, 4.5 g methionine, 30.7 g gelatin, 51.2 g wheat bran, 22.5 g vitamin mix (Harlan Teklad, AIN76A), 52.2 g mineral mix (Harlan Teklad, AIN76), 1.4 g choline chloride.

*Dissections.* Rats were anesthetized with 65mg/kg pentobarbital sodium. Tissue extraction began once rats were fully sedated. Red (approx. 200-300 mg) and white (approx. 130-200 mg) sections of quadriceps, soleus, and triceps were removed quickly and clamp frozen with liquid nitrogen chilled metal tongs then wrapped in aluminum foil and stored at -90°C. Blood was drawn from the abdominal vena cava and placed in heparinized eppendorf tubes, centrifuged for 10 minutes at 3000xg and supernatant was stored at -90°C. Omental, epididymal, and retroperitoneal fat pads were removed and weighed to determine abdominal fat pad weight.

*Homogenization.* Frozen muscle was pulverized in liquid nitrogen, weighed, and homogenized in 19 x homogenization buffer (50 mM Tris-HCl, 250 mM mannitol, 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM  $\beta$ -

glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml soybean trypsin inhibitor pH 7.4). Homogenate was stored at -90°C.

*Citrate Synthase Assay.* Whole raw homogenates, obtained using methods as described above, that had been freeze-thawed three times to disrupt the mitochondria were diluted in 100 mM Tris buffer, pH 8.0 and citrate synthase activity was measured using the method described by Srere (51).

*β-HAD Activity Assay.* β-HAD (β-hydroxyacyl-CoA dehydrogenase) activity was measured as described previously (63) with the exception that supernatant spun at 1200 x g was used instead of whole raw homogenate. Briefly, potassium phosphate buffer pH 7.5, NADH, and supernatant were added to a cuvette. The cuvette was incubated for 10 min at 30°C.

Acetoacetyl-CoA was added to the cuvette and activity was measured at 340 nm.

*NEFA Assay.* Non-esterified free fatty acids (NEFA) were measured using a commercially available assay, NEFA-HR(2) (WAKO Diagnostics, Richmond, VA).

*RT-qPCR.* RNA was isolated by homogenizing frozen ground muscle in Trizol Reagent (Invitrogen, Carlsbad, CA; cat. no. 15596-026) using an Ultra Turrax T10 homogenizer and then using the Qiagen mini kit (Qiagen, Alencia, CA; cat. no. 74104). Isolated RNA was quantified using NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) using 260/280 absorbance ratio. RNA was reverse transcribed using the Invitrogen Superscript III kit (cat. no. 18080-051) according to manufacturer's protocol and cDNA was stored at 4°C. For quantified PCR (qPCR), cDNA, forward and reverse primers, and Syber Green were added to wells of a clear polypropylene plate (BioRad, Hercules, CA; cat. no. MLL9601) in triplicate, sealed with Microseal 'B' Film (BioRad; cat. no. MSB1001), centrifuged to remove air bubbles, and placed in a C1000 Thermal Cycler (BioRad). 8 Control, 6 AICAR, 9 HF,

and 8 HF+AICAR white quadriceps were used to measure PGC-1 $\alpha$  mRNA. Samples were subjected to 2 min at 50°C, 8.5 min at 95°C, and 40 cycles of 15 sec at 95°C and 60°C for 1 min. The melt curve was obtained by changing temperature from 55°C to 90°C at 10 sec 0.5°C increments. BioRad CFX manager software was used. We used the quantification cycle (Cq) values provided by the software to quantify the relative expression of mRNA using the Livak method. Samples from the qPCR plate post qPCR reaction diluted in 6x loading dye solution (Fermentas, Burlington, Ontario, Canada; cat. no. R0611) and Perfect Size 50 bp ladder (5 Prime, Gaithersburg, MD; cat. no. 2500320) were loaded into the wells of a 2% ethidium bromide gel. cDNA bands were visualized with a UV light. Only one amplicon for each primer set, which appeared at their predicted weights, was detected (see Figure S1 in supplemental materials). In order to verify RNA stability we ran the RNA samples diluted in 2x Sample loading buffer (Fisher Scientific, Rockford, IL; cat. no. BP2812500) which contained ethidium bromide and Riboladder 1 kb RNA standard (Fisher BioReagents BP281150) in a 1.5% agarose gel. RNA bands were visualized with a UV light and 18S and 28S bands were checked to verify RNA stability (see Figure S2 in supplemental materials). Primer Sequences (5' to 3'): 18S mRNA (Invitrogen)- forward- GTGCATGGCCGTTCTTAGTTG, reverse- GCCACTTGTCCTCTAAGAAGTTG; PGC-1 $\alpha$  mRNA (Biosynthesis Inc, Lewisville, Texas)- forward- CGATGACCCT CCTCACACCA, reverse- TTGGCTTGAGCATGTTGCG.

*Western blot.* Muscle homogenates obtained as described above were freeze-thawed three times and then centrifuged for 10 min. at 1000 x g. Muscle homogenates were freeze-thawed three times in order to disrupt mitochondria. Protein concentration was measured on the supernatant fraction using the DC protein assay method (BioRad). An aliquot of the



supernatant fraction was dissolved in 2x Laemmli's buffer and then subjected to SDS-PAGE. Membranes were blocked for 1 hr at room temperature in 5% nonfat dry milk dissolved in TBST, incubated in the appropriate primary antibody overnight, rinsed 4x five min in TBST, incubated for 1 hr at room temperature in the appropriate secondary antibody dissolved in 1% nonfat dry milk dissolved in TBST, and again rinsed 4x five min in TBST. Protein bands were visualized on autoradiographic film (Classic Blue Sensitive, Midwest Scientific, St. Louis, MO) using ECL PLUS (GE Healthcare, Piscataway, NJ) and quantified by densitometry using AlphaEaseFC software (Alpha Innotech, San Leandro, CA). In our lab we have identified the PGC-1 $\alpha$  protein band at 110 kDa by running recombinant PGC-1 $\alpha$  protein (a gift from John O. Holloszy, Washington University School of Medicine, St. Louis, MO) and brown adipose tissue homogenate on a gel along with skeletal muscle homogenates. Brown adipose tissue was loaded because it has a high level of PGC-1 $\alpha$  protein. A representative blot is available in the supplemental section of a recently published paper from our lab (55). In the supplemental section evidence is also provided for the specificity of the Custom PPAR $\delta$  rat-specific antibody (provided by Dong-Ho Han, Washington University School of Medicine, St. Louis, Missouri) that we used (Figure S3 in supplemental materials).

Primary antibodies: Cytochrome C (Santa Cruz, Santa Cruz, CA; cat. no. sc-13156); Hexokinase II (Santa Cruz; cat. no. sc-6521); long-chain acyl-CoA dehydrogenase (LCAD) (a gift from Daniel P. Kelly, Burnham Institute for Medical Research-Lake Nona, Orlando, Florida); PGC-1 $\alpha$  (Calbiochem, La Jolla, CA; cat. no. 516557); PPAR $\delta$  (a gift from Dong-Ho Han, Washington University School of Medicine, St. Louis, Missouri); uncoupling protein 3 (UCP3) (Affinity BioReagents, Golden, CO; cat. no. PA1-055); phospho-AMPK $\alpha$  (pAMPK $\alpha$ ) (Cell Signaling, Beverly, MA; cat. no. 2535L); total AMPK $\alpha$  (Cell Signaling; cat. no. 2532L),

pACC (Upstate, Lake Placid, NY; cat. no. 07-303) ; acetyl-CoA carboxylase (ACC),

Streptavidin Horseradish Peroxidase (GE Healthcare; cat. no. RPN1231V )

Secondary antibodies: Donkey Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories,

West Grove, PA); Donkey Anti-Mouse IgG (Santa Cruz; cat. no. sc-2314); Donkey Anti-goat

IgG (Santa Cruz; cat. no. sc-2020)

*Statistics.* Significant differences between groups were determined using two way ANOVA (High Fat X AICAR) and Bonferonni post hoc test for multiple comparisons. The statistical software SigmaStat (Systat Software Inc, San Jose, CA) was used. Statistical significance is defined as  $p < 0.05$ . Results are presented as means  $\pm$  standard error of mean (SEM).

## Results

*Treatment response to AICAR and high fat feeding.* Acute AMPK activation by AICAR was verified by measuring the phosphorylation of AMPK and ACC in rats from the AICAR and HF+AICAR groups injected with AICAR on the day of dissection. One hour after AICAR injection, there was an increase in pAMPK in the white quadriceps (Figure 1). Further, in the white quadriceps (Figure 1), red quadriceps, and soleus the AICAR treatment significantly elevated pACC levels. Together these data verify that AICAR acutely increased AMPK activation in skeletal muscle. The effectiveness of the chronic activation of AMPK was also confirmed by examining the abundance of Hexokinase II protein. Previously, a similar duration of AICAR treatment as used in our study, albeit at a higher dose (1 mg/g BW) resulted in increased Hexokinase II protein expression (63). It has also been demonstrated that this increase in Hexokinase II in response to AICAR is dependent on the presence of AMPK $\alpha$ 2 (36). Using the lower dose of AICAR (0.5 mg/g BW) that we used in this study also resulted in increased Hexokinase II protein expression (Figure 2). This lower dose was used to limit potential side effects of AICAR on rats.

The high fat diet was effective in elevating circulating FFA levels as there was a significant main effect of high fat feeding on the elevation of circulating levels of FFAs (Table 1). The duration of the high fat diet used in this study was not sufficient to cause a significant increase in BW compared to Control (Table 1). As expected, abdominal fat was significantly increased with high fat feeding (Table 1).

*Mitochondrial enzyme activities in response to chronic AMPK activation and high fat feeding.*

To assess the combined effect of chronic AMPK activation and elevated circulating FFAs in response to high fat feeding on mitochondrial enzyme activities the activity of citrate

synthase, a marker of the Krebs cycle, and  $\beta$ -HAD, a marker of FA metabolism, were measured. As might be expected, changes in markers of mitochondrial content were proportionally smaller and more difficult to discern in muscles with high oxidative capacity in response to chronic AMPK activation and/or high fat feeding. In the white quadricep muscle, predominantly composed of fast twitch glycolytic fibers, AICAR treatment and high fat feeding had an additive effect on citrate synthase and  $\beta$ -HAD activity (Table 2 and 3). Further, an additive effect was also observed for  $\beta$ -HAD activity in red quadriceps (Table 3). While there was a tendency for the treatments to have an additive effect on citrate synthase activity in the red quadriceps, this difference was not consistent enough to reach statistical significance ( $p=0.07$ , observed power=0.66) (Table 2). Interestingly, in the soleus, a predominantly slow twitch muscle with high oxidative capacity, each treatment increased citrate synthase activity, however, an additive effect was not observed (Table 2).  $\beta$ -HAD activity was only increased in response to high fat feeding in the soleus. Thus, only in muscles predominantly composed of fast twitch fibers was a clear combined effect of AICAR and high fat feeding on citrate synthase and  $\beta$ -HAD activity observed.

*Mitochondrial protein expression in response to chronic AMPK activation and high fat feeding.* To determine the combined effect of chronic AMPK activation and elevated circulating FFAs on mitochondrial protein expression we measured the protein abundance of a classic marker of mitochondria content (cytochrome c) and two proteins involved in fatty acid metabolism (LCAD and UCP3). As seen with the changes observed with citrate synthase activity, AICAR injections and a high fat diet had an additive effect on cytochrome c protein in the white quadriceps (Figure 3). In the red quadriceps and soleus muscles, cytochrome c protein was not significantly elevated in any of the treatment groups (Figure 3).

There was an additive effect of treatments on LCAD protein abundance in the white quadriceps (Figure 3). This is consistent with the effect of treatments seen with  $\beta$ -HAD activity. In the red quadriceps LCAD protein levels were only increased by high fat feeding (Figure 3). LCAD protein expression in the soleus was not different between any of the groups (Figure 3). In the white quadriceps, abundance of UCP3 protein was increased with AICAR treatment and high fat feeding, however, no additive effect was observed (Figure 3). UCP3 protein levels in the red quadriceps and soleus were only elevated with high fat feeding (Figure 3).

*PPAR $\delta$  protein expression in response to chronic AMPK activation and high fat feeding.*

PPAR $\delta$  is a transcription factor known to be involved in the transcription of a number of mitochondrial proteins especially those involved in fatty acid oxidation. Since it has been previously shown that high fat feeding elevates PPAR $\delta$  content in skeletal muscle it was no surprise that PPAR $\delta$  protein content was elevated in the white quadriceps in HF (Figure 4). This data suggests that PPAR $\delta$  activity is up with high fat feeding. An interesting observation we made was that the protein content was also elevated in response to AICAR (Figure 4). Further, there was no additive affect of chronically activating AMPK and elevating circulating FFAs on PPAR $\delta$  content (Figure 4). These data suggest that PPAR $\delta$  may be involved in the elevation of some of the mitochondrial markers that we measured.

*PGC-1 $\alpha$  protein and mRNA expression in response to chronic AMPK activation and high fat feeding.*

PGC-1 $\alpha$  is a known coactivator of some of the transcription factors that regulate mitochondria protein expression. Because AMPK and elevated circulating FFAs are known to increase PGC-1 $\alpha$  binding activity and/or abundance we evaluated whether a combined effect would be observed in our model. In the white quadriceps, AICAR treatment and high

fat feeding had an additive effect on PGC-1 $\alpha$  protein expression (Figure 4). In the red quadriceps and soleus, PGC-1 $\alpha$  protein abundance was not elevated in AICAR, HF, or HF+AICAR compared to Control (data not included). Further, a significant increase in PGC-1 $\alpha$  mRNA was only observed in response to AICAR treatment (Figure 4). Together this confirms distinct mechanisms for AMPK and circulating FFA regulation of PGC-1 $\alpha$  protein expression. These data suggest a possible mechanism for how chronic activation of AMPK and elevated circulating FFAs induce additive effects on mitochondrial content.

## Discussion

The purpose of this study was to determine the combined effect of chronic AMPK activation and elevated circulating FFAs on mitochondrial content of skeletal muscle. Rats were treated for six weeks with AICAR injections, a high fat diet, or both AICAR injections and a high fat diet. The effect of the combined treatments on mitochondria content was examined in three muscles predominantly composed of unique fiber types (white quadriceps (Type IIb), red quadriceps (Type IIa), and soleus (Type I)) (4). Particularly in muscles with low oxidative capacity we report an additive increase in mitochondrial markers in response to chronic AMPK activation and high fat feeding. These data show that chronically activating AMPK and elevating circulating FFAs with a high fat diet has muscle type specific additive effects on markers of FA metabolism, the citric acid cycle, the electron transport chain, and transcriptional regulation.

Under physiological conditions, such as exercise, in which AMPK activity and circulating FFAs are elevated, both AMPK and FFAs may have varying degrees of effect on mitochondria depending on the muscle type being examined. As previously reported, a four week AICAR treatment increases mitochondrial markers to a much greater degree in the white quadriceps compared to red quadriceps (63). In agreement with these results, we observed that the response of mitochondrial markers to AICAR was smaller in red quadriceps and soleus compared to white quadriceps. This may be due to a higher average twenty four hour level of AMPK activity in the red quadriceps and soleus compared to the white quadriceps in the absence of any treatment. Thus, greater increases in AMPK activity are likely required to stimulate mitochondrial biogenesis in the red quadriceps and soleus muscles. Elevating circulating FFAs also have muscle type specific effects on mitochondria

content. This could be due to the difference in availability of blood FFAs between skeletal muscle fiber types. Muscles predominantly composed of Type IIa and/or Type I fibers receive greater blood flow, and consequently would receive more FFAs than those predominantly composed of Type IIb fibers (24). The higher FFA availability to the red quadriceps and soleus would likely result in lower sensitivity to the effects of elevated circulating FFAs on mitochondria content in these muscles compared to muscles that receive less blood flow and have more limited capacity for fatty acid oxidation such as the white quadriceps muscle. In addition, it may be difficult to discern changes in mitochondrial proteins in muscles that are already rich in mitochondria. The mitochondrial markers we measured are mostly consistent with this predicted pattern with the exception of UCP3 protein abundance.

UCP3 protein expression follows a unique pattern compared to the other measurements made. The role of UCP3 is not well understood, but some recent evidence points to UCP3 inhibiting reactive oxygen species (ROS) production or playing a role in the regulation fatty acid oxidation (5). UCP3 expression is regulated by the PPAR transcription factors, predominantly PPAR $\delta$  in skeletal muscle, and PGC-1 $\alpha$ , which coactivates PPAR $\delta$  (38, 50). The 5' flanking region of the human UCP3 gene contain peroxisome proliferator response elements present, which PPAR $\delta$  likely binds to regulate UCP3 gene expression (1). Knocking out PPAR $\delta$  in cardiac muscle results in reduced levels of both UCP3 mRNA and protein content in mouse hearts (14). Further, in primary myotubes PPAR $\delta$  shRNA completely blocks the GW501516, a chemical activator of PPAR $\delta$ , induced increase in UCP3 mRNA strongly supporting the idea that PPAR $\delta$  directly regulates UCP3 gene expression (38). As would be expected since FFAs directly activate PPAR $\delta$ , UCP3 mRNA level in



skeletal muscle is also increased in response to elevated circulating FFAs (50, 60).

Therefore, the rise in UCP3 protein content that we observed when circulating FFAs were elevated strongly suggests that PPAR $\delta$  activity was increased in the muscles in rats fed the high fat diet. PGC-1 $\alpha$  protein abundance could also help explain the interesting pattern of UCP3 protein expression. Since PGC-1 $\alpha$  coactivates PPAR $\delta$ , the elevation of PGC-1 $\alpha$  protein in the white quadriceps in response to either AICAR injections or a high fat diet could explain why UCP3 protein content is only increased in response to both treatments in the white quadriceps. These data suggest that in muscles with a large proportion of oxidative fibers, circulating FFAs may have a much larger role in elevating UCP3 protein abundance than AMPK activation in conditions that elevate both factors such as exercise.

We then asked what mechanism(s) may be responsible for the additive increase in mitochondrial content observed with chronic activation of AMPK and elevating circulating FFAs. To do this we measured PGC-1 $\alpha$  protein, a transcription factor coactivator that can induce mitochondrial biogenesis. We observed an additive effect of chronic AMPK activation and high fat feeding on PGC-1 $\alpha$  protein abundance in the white quadriceps. We also noted that PGC-1 $\alpha$  mRNA was elevated in response to chronic AMPK activation but was not elevated by high fat feeding consistent with a previous report using the same dietary treatment (26). Together, these data support our hypothesis that the additive increase in PGC-1 $\alpha$  protein abundance was a combined effect of high fat feeding induced post transcriptional and AMPK dependent transcriptional increases in PGC-1 $\alpha$  protein expression.

Exercise training also increases PGC-1 $\alpha$  protein and mRNA expression (6, 25, 32, 46, 54). AMPK dependent increases in PGC-1 $\alpha$  mRNA and protein are thought to be important in the exercise induced elevation in mitochondrial content. Exercise increases the binding

activity of PGC-1 $\alpha$  in skeletal muscle by initially activating PGC-1 $\alpha$  protein and later increasing PGC-1 $\alpha$  protein expression (64). The MEF and CRE binding sites on the PGC-1 gene promoter are essential for contraction-induced PGC-1 $\alpha$  gene transcription (2-3). Recently, it was discovered that AMPK activates members of the CREB family (56), which regulate CRE promoter regions, and this may result in increased binding to the PGC-1 $\alpha$  gene promoter CRE sites (56). AMPK is known to increase PGC-1 $\alpha$  mRNA levels by regulating the binding of transcription factors to the MEF and CRE sites in the PGC-1 $\alpha$  gene promoter (30, 33, 35), which are the same sites regulated by muscle contraction. Further, AMPK increases PGC-1 $\alpha$  binding activity via phosphorylation (34-35) and less direct mechanisms such as increasing the activity of SIRT1 resulting in deacetylation of PGC-1 $\alpha$  (12).

Exercise training and high fat feeding both regulate PPAR $\delta$ . The protein abundance of PPAR $\delta$ , a transcription factor coactivated by PGC-1 $\alpha$ , is increased in skeletal muscle after three weeks of exercise (39). Exercise training and high fat feeding increase expression of proteins regulated by PPAR $\delta$ , such as PDK4 (45). High fat feeding also increases PPAR $\delta$  protein abundance in skeletal muscle (26). We also measured an increase in PPAR $\delta$  protein content in the white quadriceps of rats fed a high fat diet. These similarities are not surprising since both exercise and high fat feeding increase circulating levels of FFAs, which are ligands for PPAR $\delta$ .

Conditions that raise FFAs, such as a high fat diet or lipid infusion cause insulin resistance (13, 26, 53) which has been associated with reduced mitochondrial content in skeletal muscle (9, 37, 44). If mitochondrial content and insulin resistance are causally related, this could be a confounding factor in our study. However, experimental models where insulin resistance would be expected or was measured have not confirmed this

relationship (22, 26, 58). As demonstrated previously, feeding rats the high fat diet used in this study causes insulin resistance (26). While high fat feeding is known to induce insulin resistance, AMPK activation has been linked to increased insulin sensitivity (18, 20, 47, 52). If insulin resistance caused by high fat feeding and the expected insulin sensitivity from AICAR treatment were confounding factors in our study, we might expect to see a reduction in skeletal muscle mitochondrial levels in the HF group compared to the Control group. Also, the values for the high fat fed group that was chronically treated with AICAR would likely be somewhere between the values of the HF and AICAR groups. In contrast, we observed an additive effect of AICAR and high fat feeding on mitochondrial marker expression in a number of instances. Further, we did not observe HF to be less than Control in any of the measurements. Therefore, any negative effect that insulin resistance may be having on mitochondria content does not appear to be confounding our results.

It is well known that circulating FFAs are elevated during and/or after prolonged exercise bouts (43). Our findings suggest that this elevation in circulating FFAs may contribute to exercise training-induced mitochondrial biogenesis. Furthermore, it is reasonable to consider that increases in skeletal muscle mitochondria capacity could be enhanced if training were performed under conditions that further elevated the levels of circulating FFAs. This idea is not new. A number of studies have examined the effect of a high fat diet combined with training on endurance capacity. Feeding rats a high fat diet has been reported to enhance endurance exercise capacity and increase mitochondrial markers in skeletal muscle (41, 49). In contrast, some human studies have failed to demonstrate a beneficial effect on endurance capacity that might be expected when training is combined with a high fat diet for either four or eight weeks (28-29). Further, a seven week high fat diet

combined with exercise training induces a comparable increase in citrate synthase activity compared to those trained but fed a carbohydrate rich diet (27). The difference in results may be due to the fat composition of the rat control diet being about half that of the human control diets. It should be noted that the control diet used in our study and the rat training studies just mentioned consisted of 10% of the calories from fat, while the typical American diet consists of 33-34% fat (59). It is possible that since the fat content of American diets is already high, further elevating dietary fat would be less likely to enhance exercise training-induced mitochondrial biogenesis than if the fat composition of the regular diet was closer to the control diet in our study. Regardless, consuming a high fat diet is not suggested since it has numerous deleterious health effects including impaired cardiovascular system function, insulin resistance, and inflammation (see these papers for a review (10-11, 15))

In conclusion, chronically activating AMPK and elevating circulating FFAs for six weeks has a muscle type specific additive effect on markers of fatty acid metabolism, the citric acid cycle, the electron transport chain, and transcriptional regulation. The additive effect on mitochondrial content was most prominent in the white quadriceps, predominantly composed of Type IIb fibers. These data support our hypothesis that chronically activating AMPK activity in skeletal muscle and increasing circulating FFAs has an additive effect on mitochondria levels in skeletal muscle. They also suggest that both the exercise induced increase in AMPK activity in skeletal muscle and elevation in circulating FFAs could be simultaneously contributing to exercise training-induced mitochondrial biogenesis in skeletal muscle. Future work needs to be done to determine whether this is in fact occurring.

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*Table 1. Average body weight, liver weight, and abdominal fat pad weight at end of treatment*

	Control	AICAR	HF	HF+AICAR
Body weight (g)	345±8 a	310±3 b	348±6 a‡	341±10 a‡
Liver weight (g)	14.9±0.3 a	15.2±0.6 a	14.6±0.4 a	15.8±0.7 a
Abdominal Fat pads (fat pads weight g/ g body wt)	0.028±0.002 a	0.028±.002 a	0.049±0.003 b‡	0.044±.004 b‡
NEFA in plasma (mmol/L)	0.254±0.02 a	0.274±0.05 a	0.444±0.05 b‡	0.336±0.03 ab‡

Values expressed are mean ± SEM (n=8-11). Two way ANOVA was used to determine significance. Groups with different letters are significantly different from each other (p<0.05). AICAR and HF were not compared. ‡There was a main effect of High fat feeding (p<0.05).

*Table 2. Citrate Synthase activity*

	Control	AICAR	HF	HF+AICAR
White quadriceps	18.1±0.8 a	28.1±1.7 b*	23.6±0.8 b‡	38.0±1.6 c*‡
Red quadriceps	72.5±2.7 a	78.8±2.7 a*	85.8±1.7 b‡	91.9±2.3 b*‡
Soleus	42.1±1.1 a	47.1±1.6 a	49.0±2.0 b‡	50.9±1.7 b‡

Values are expressed as  $\mu\text{mol/g/min} \pm \text{SEM}$  (n = 7-11). Two way ANOVA was used to determine significance. Groups with different letters are significantly different from each other ( $p < 0.05$ ). AICAR and HF were not compared. \*There was a main effect of the AICAR treatment ( $p < 0.05$ ). ‡There was a main effect of High fat feeding ( $p < 0.05$ ).

Table 3.  $\beta$ -HAD activity

	Control	AICAR	HF	HF+AICAR
White quadriceps	4.4±0.17 a	5.7±0.35 b*	6.5±0.28 b‡	8.6±0.38 c*‡
Red quadriceps	28.8±1.68 a	30.3±0.78 a*	39.0±1.59 b‡	43.8±0.86 c*‡
Soleus	22.6±0.91 a	24.6±1.06 a	29.9±1.36 b‡	30.6±1.03 b‡

Values are expressed as  $\mu\text{mol/g/min} \pm \text{SEM}$  (n = 7-10). Two way ANOVA was used to determine significance. Groups with different letters are significantly different from each other ( $p < 0.05$ ). AICAR and HF were not compared. \*There was a main effect of the AICAR ( $p < 0.05$ ). ‡There was a main effect of High fat feeding ( $p < 0.05$ ).

### Figure Legend

Figure 1. Acute AMPK Activation. AMPK activity increased with acute AICAR treatment. Muscles from AICAR treated rats were removed 1 hr after the daily AICAR injection. A. pAMPK/ total AMPK protein level in white quadriceps (n=3-4). B. pACC/ total ACC protein level in white quadriceps (n=4-5). \*Main treatment effect ( $p<0.05$ ).

Figure 2. Chronic AMPK Activation. Hexokinase II protein levels increased with chronic AICAR treatment in skeletal muscle (n=6-10). Letters are used to represent significance, same letter means no significant difference ( $p<0.05$ ). AICAR and HF were not compared. \*Main treatment effect ( $p<0.05$ ).

Figure 3. Mitochondrial Protein Abundance. LCAD, and Cytochrome c protein levels greater in animals fed a high fat diet and given AICAR than either individual treatment in the white quadriceps. A. Cytochrome c protein levels in skeletal muscle (n=7-10). B. LCAD protein levels in skeletal muscle (n=7-11). C. UCP3 protein levels in skeletal muscle (n=6-10). D. Representative western blots. Letters are used to represent significance, same letter means no significant difference ( $p<0.05$ ). AICAR and HF were not compared. \*Main treatment effect ( $p<0.05$ ).

Figure 4. Transcriptional Regulation. PGC-1  $\alpha$  protein is greater in HF+AICAR than either individual treatment and AICAR treatment elevates PGC-1 $\alpha$  mRNA in the white quadriceps. A. PGC-1 $\alpha$  protein levels in white quadriceps (n=8-10). B. PGC-1 $\alpha$  mRNA fold difference in white quadriceps (n=6-9). C. PPAR $\delta$  protein levels in white quadriceps (n=4-6). Letters are used to represent significance, same letter means no significant difference ( $p<0.05$ ). AICAR and HF were not compared. \*Main treatment effect ( $p<0.05$ ). #Greater than AICAR ( $p=0.05$ ).

Figure 1. Acute AMPK Activation

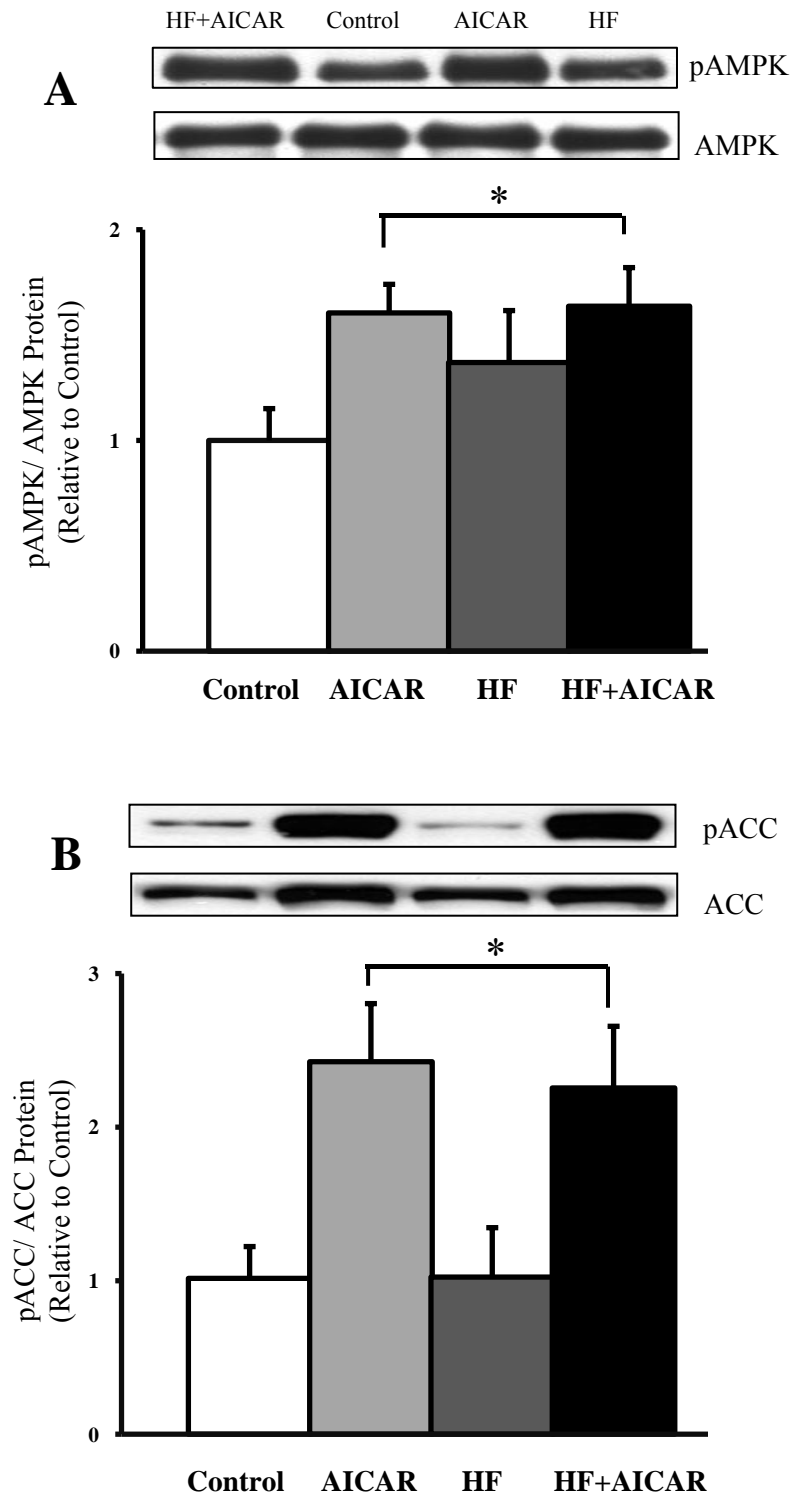


Figure 2. Chronic AMPK Activation

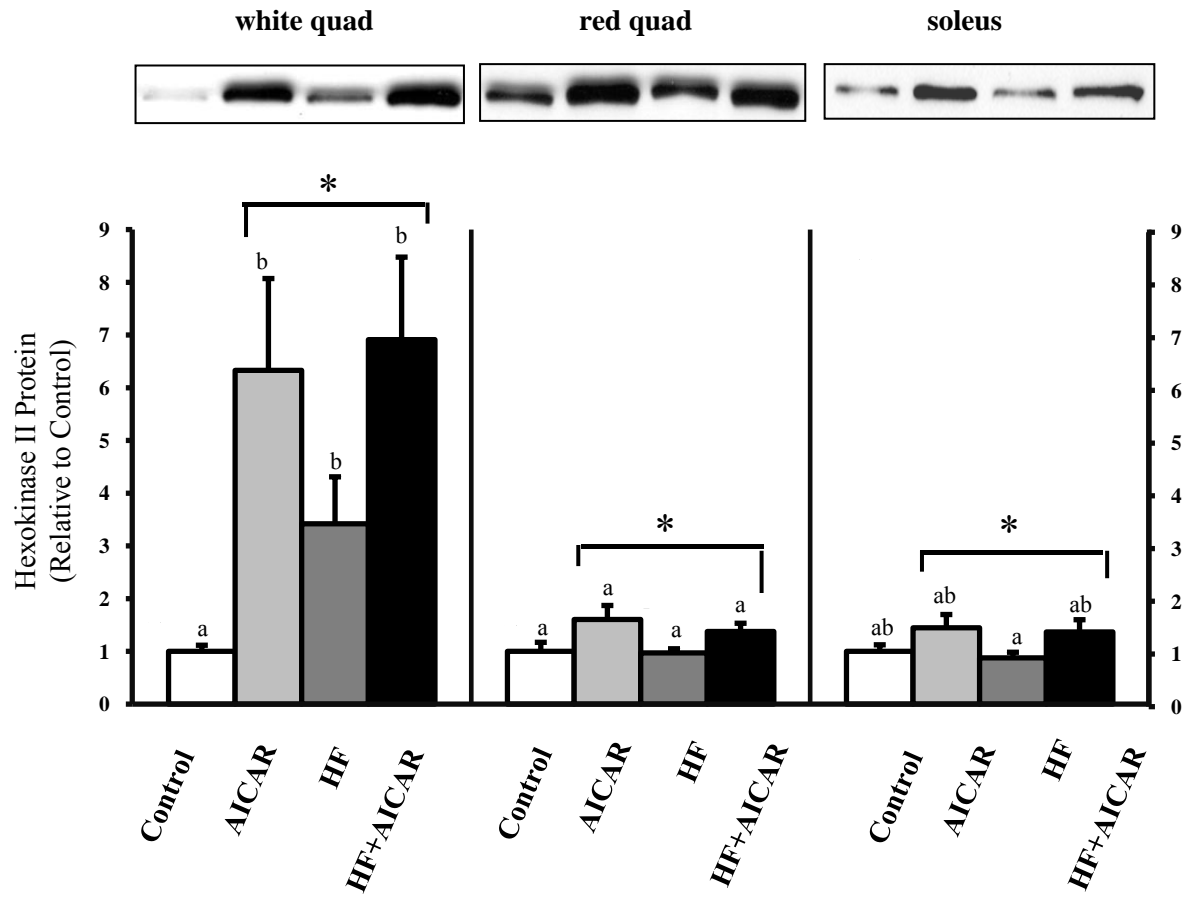
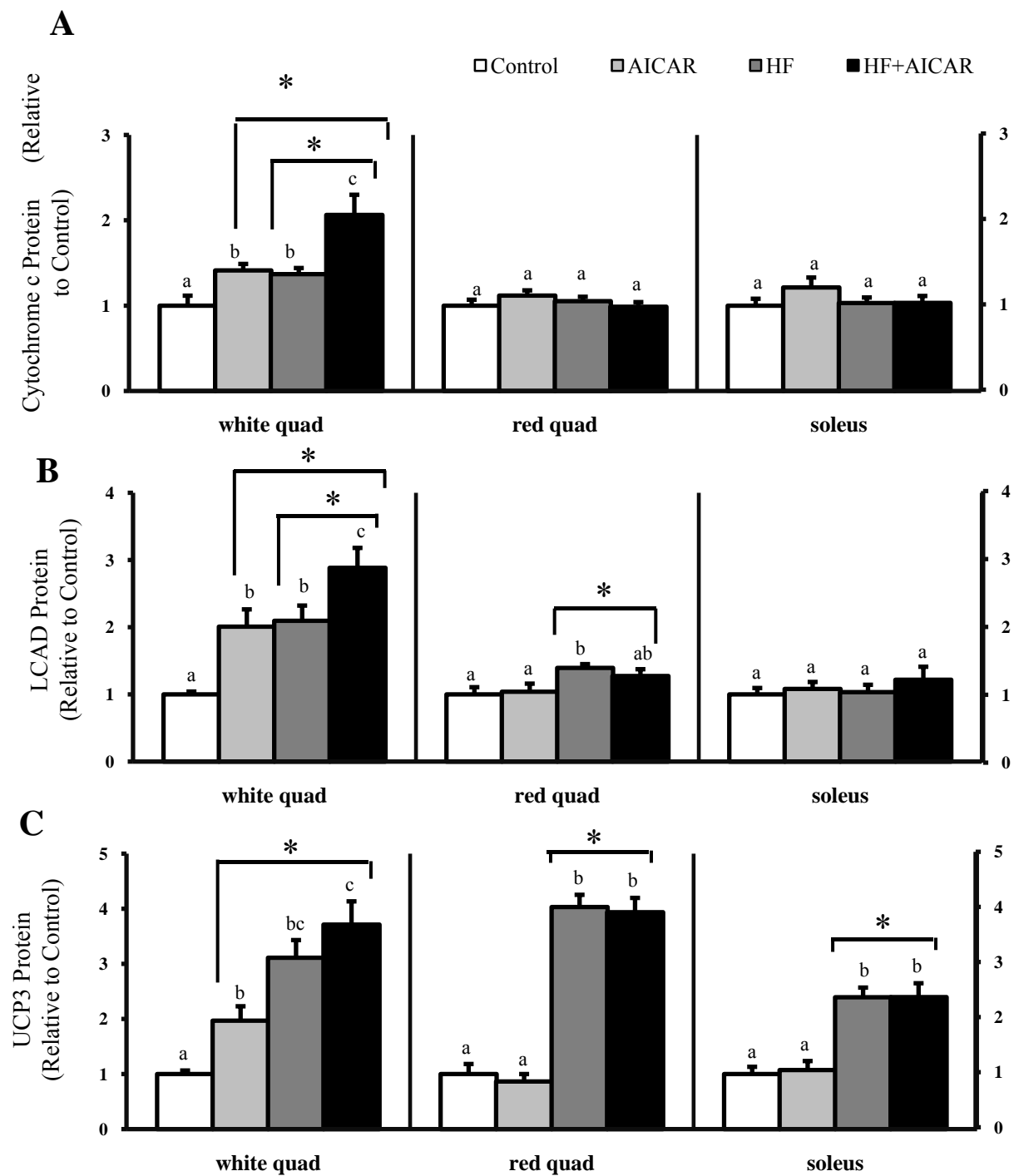


Figure 3. Mitochondrial Protein Abundance





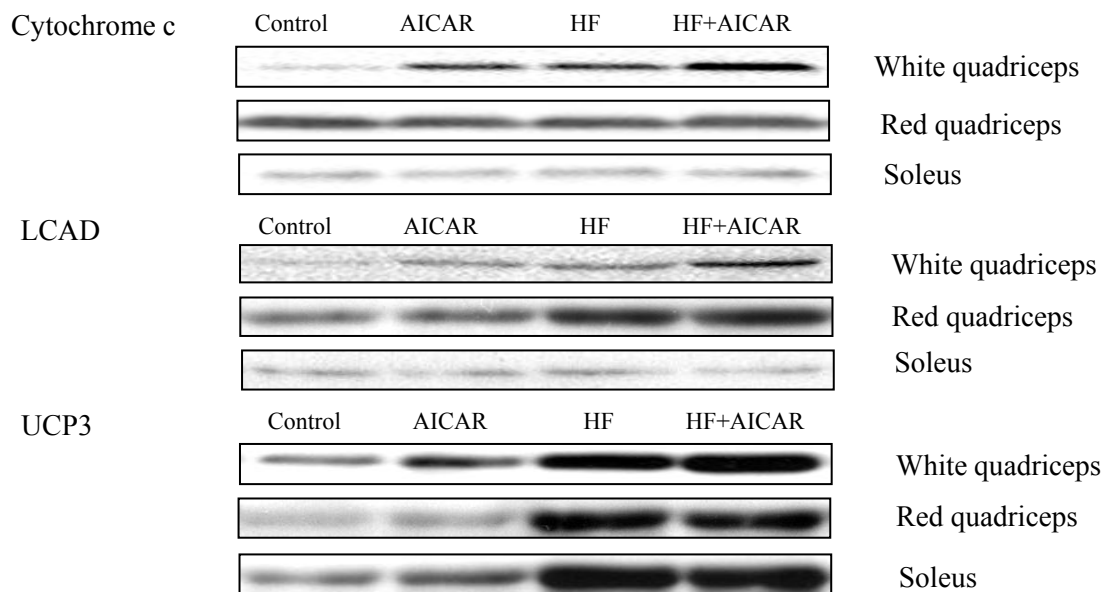
**D**

Figure 4. Transcriptional Regulation

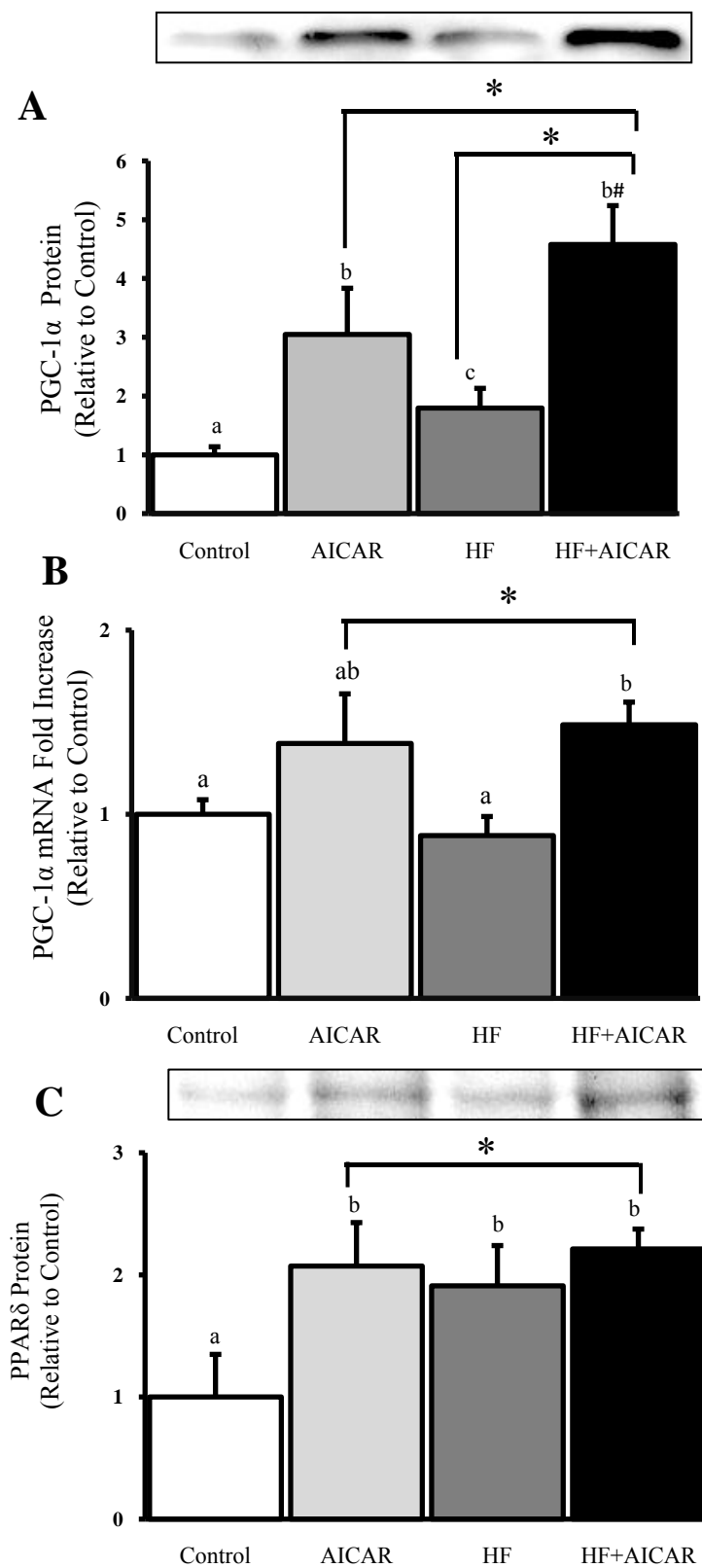


Figure S1. Specificity of 18S and PGC-1 $\alpha$  primers

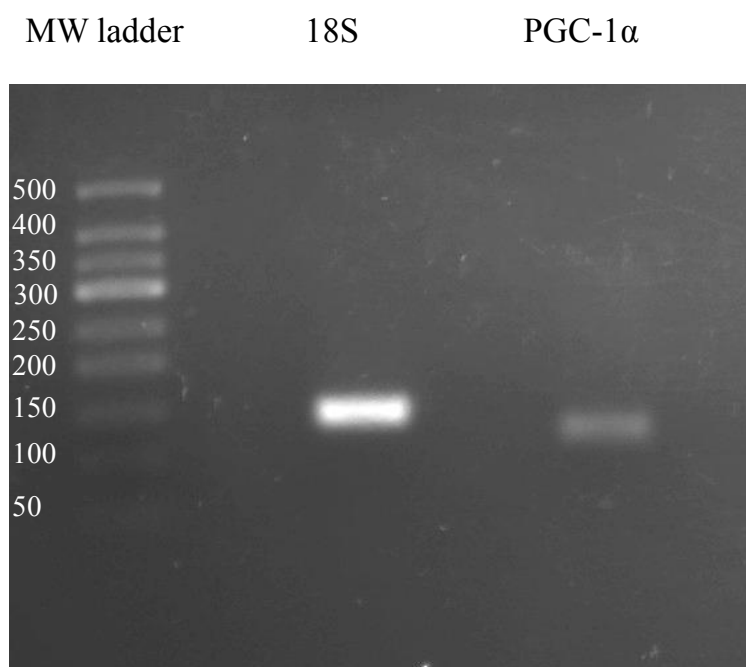
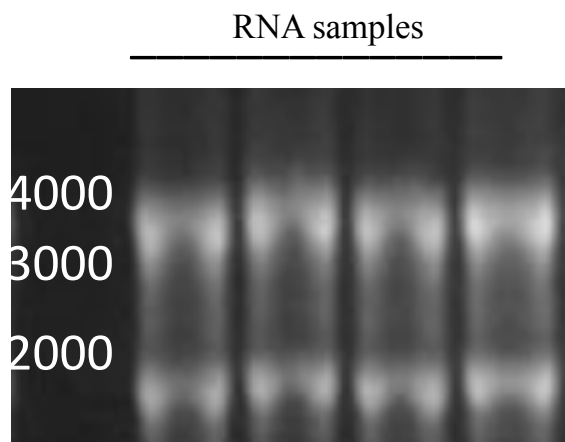


Figure S1. Specificity of 18S and PGC-1 $\alpha$  primers. Each primer set amplified only one detectable amplicon which appeared at the predicted length when ran on an ethidium bromide gel.

*Figure S2. Verification of RNA integrity.*



**Figure S2. Verification of RNA integrity. The top band is 28S RNA and the bottom band is 18S RNA.**

Figure S3. Verification of PPAR $\delta$  antibody specificity



Figure S3. Verification of PPAR $\delta$  antibody specificity. A. Endogenous PPAR $\delta$  in skeletal muscle. In lanes labeled epo, samples were from muscles electroporated with a whole sequence PPAR $\delta$  construct as described in reference 25. Importantly, band intensity is higher in those loaded with sample from muscles electroporated with PPAR $\delta$ . B. C2C12 cells expressing a PPAR $\delta$  construct with a V5 tag and immunoblotted with an anti-V5 antibody. Since the V5 tagged band is at a similar molecular weight as endogenous rat skeletal muscle PPAR $\delta$  protein this strongly supports our conclusion that the PPAR $\delta$  antibody is detecting rat PPAR $\delta$  at approximately 55 kDa. Description of methods for electroporation and the V5 tag have been previously published (25).

## CURRICULUM VITAE

### Natasha Fillmore

#### Contact Information:

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#### Education:

**M.S. in Physiology and Developmental Biology** Anticipated August 2010  
 Brigham Young University, Provo, Utah. Fall 2008- present.  
 Advisor: William W. Winder  
 Thesis: Chronic AMP-Activated Protein Kinase Activation and a High Fat Diet Have an Additive Effect on Mitochondria in Rat Skeletal Muscle

**B.S. in Physiology and Developmental Biology**  
 Brigham Young University, Provo, Utah. Fall 2005-August 2008.

#### Research Experience:

**Research Assistant- Master's Student**, Brigham Young University  
 Fall 2008- present. Department of Physiology and Developmental Biology.  
 Work in Dr. Winder's laboratory studying exercise-induced mitochondrial biogenesis for thesis. Other work involves characterizing AMPK in skeletal muscle. Teach undergraduates research techniques and assays.

**Research Assistant**, Brigham Young University  
 Fall 2005- August 30, 2008. Department of Physiology and Developmental Biology.  
 Worked in Dr. Winder's laboratory characterizing AMPK in skeletal muscle. Taught new students research techniques and assays.

#### Publications:

**Natasha Fillmore**, Daniel L. Jacobs, David B. Mills, William W. Winder, Chad R. Hancock. "Chronic AMP-Activated Protein Kinase Activation and a High Fat Diet Have an Additive Effect on Mitochondria in Rat Skeletal Muscle." 2010 June (Epub ahead of print)

David M. Thomson, Chad R. Hancock, Bradley G. Evanson, Steven G. Kenney, Brandon B. Malan, Anthony D. Mongillo, Jacob D. Brown, Squire Hepworth, **Natasha Fillmore**, Allen C. Parcell, David L. Kooyman, and William W. Winder. "Skeletal muscle dysfunction in muscle-specific LKB1 knockout mice." J Appl. Physiol. 108 (2010): 1775-1785.

- Nakken GN, Jacobs DL, Thomson DM, **Fillmore N**, and Winder WW. "Effects of Excess Corticosterone on LKB1 and AMPK Signaling in Rat Skeletal Muscle." J Appl Physiol. 108 (2010):298-305.
- Thomson DM, Brown JD, **Fillmore N**, Ellsworth SK, Jacobs DL, Winder WW, Fick CA, Gordon SE. "AMP- activated protein kinase response to contractions and treatment with the AMPK activator AICAR in young adult and old skeletal muscle." J Physiol. 587 (2009): 2077-2086.
- Branvold DJ, Allred DR, Beckstead DJ, Kim HJ, **Fillmore N**, Condon BM, Brown JD, Sudweeks SN, Thomson DM, Winder WW. "Thyroid hormone effects on LKB1, MO25, phospho-AMPK, phospho-CREB, and PGC-1alpha in rat muscle." J. Appl. Physiol. 105 (2008): 1218-1227.
- Thomson DM, Herway ST, **Fillmore N**, Kim H, Brown JD, Barrow JR, Winder WW. "AMP-activated protein kinase phosphorylates transcription factors of the CREB family." J Appl Physiol. 104 (2008): 429-438.
- Thomson DM, Brown JD, **Fillmore N**, Condon BM, Kim HJ, Barrow JR, and Winder WW. "LKB1 and the regulation of malonyl-CoA and fatty acid oxidation in muscle." Am J Physiol Endocrinol Metab. 293 (2007): E1572-9.

### Invited Presentations:

- 4/13/10 "Additive Effect of AMPK and a High Fat diet on Muscle Mitochondria". Heritage Medical Research Center, University of Alberta, Edmonton, Alberta.
- 3/23/10 "Chronic AMP-Activated Protein Kinase Activation and a High Fat Diet Have an Additive Effect on Mitochondria in Rat Skeletal Muscle". Department of Rehabilitation Sciences, UTMB, Galveston, TX.

### Professional Presentations/Abstracts:

- Fillmore N**, Jacobs DL, Mills D, Winder WW, and Hancock CR. "Effect of high fat diet and chronic chemical activation of AMPK on skeletal muscle mitochondria." Biochemistry of Exercise Conference, 2009. (Poster)
- Thomson DM, **Fillmore N**, Ellsworth SK, Brown JD, Fick CA, Winder WW, and Gordon SE. "Elevated contraction-induced AMPK and ACC phosphorylation in aged skeletal muscle." AMPK Conference, 2008. (Poster)
- Branvold DJ, Allred DR, Beckstead DJ, Thomson DM, Kim HJ, **Fillmore N**, Condon BM, Brown JD, Winder WW. "Regulation of LKB1-STRAD-MO25 complex expression and activation of AMPK in skeletal muscle by thyroid hormone." American Diabetes Association Conference, 2007 (published in *Diabetes* 56 (Suppl 1): A282). (Poster)
- Thomson DM, Brown JD, Kim HJ, Chesser DG, **Fillmore N**, Porter BB, Tall JH, Barrow JR, Winder WW. "LKB is required for AICAR-induced elevations in hexokinase II content in skeletal muscle." American Diabetes Association Conference, 2007 (published in *Diabetes* 56 (Suppl 1): A63). (Oral presentation)

Thomson DM, Porter BB, Tall JH, Kim H-J, Brown JD, Thompson B, Anderson J, Condon BM, **Fillmore N**, Barrow JR, and Winder WW. "Muscle Specific LKB1 Knockout Results in Decreased Voluntary Running and Mitochondrial Enzyme Expression." FASEB Summer Research Conference, 2006. (Poster)

### **Skills:**

#### Assays and Experimental Techniques

Cell Culture, Co-Immunoprecipitation, ELISA, Enzyme Activity Assays, Homogenization, Kinase Activity Assays, NEFA Assay, Palmitate Oxidation, Protein Concentration Assay, Quantitative RTPCR, Recombinant Protein Generation, Western Blotting

#### Certifications

Laboratory Mouse Handling, Laboratory Rat Handling, Radiation Safety

### **Awards/ Funding:**

*Research Conference Travel Award 2009* BYU Graduate Student Society

*Graduate Research Assistant 2008-2010* funding from NIH grant (W.W. Winder)