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Muscle Specific Differences in the Response of Mitochondrial Proteins to β-GPA Feeding: An Evaluation of Potential Mechanisms

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Abstract

β-GPA feeding leads to reductions in skeletal muscle phosphagen concentrations and has been used as a tool with which to study the effects of energy charge on skeletal muscle metabolism. Supplementing standard rodent diets with β-GPA leads to increases in mitochondrial enzyme content in fast but not slow twitch muscles from male rats. Given this apparent discrepancy between muscle types we used β-GPA feeding as a model to study signaling pathways involved in mitochondrial biogenesis. We hypothesized that β-GPA feeding would result in a preferential activation of p38 MAPK and AMPK signaling and reductions in RIP140 protein content in triceps but not soleus muscle. Despite similar reductions in high energy phosphate concentrations, 6 weeks of β-GPA feeding led to increases in mitochondrial proteins in triceps but not soleus muscles. Differences in the response of mitochondrial proteins to β-GPA feeding did not seem to be related to a differential activation of p38 MAPK and AMPK signaling pathways or discrepancies in the induction of PGC-1α and PGC-1β. The protein content and expression of the nuclear co-repressor RIP140 was reduced in triceps but not soleus muscle. Collectively our results indicate that chronic reductions in high energy phosphates lead to the activation of p38 MAPK and AMPK signaling and increases in the expression of PGC-1α and β in both soleus and triceps muscles. The lack of an effect of β-GPA feeding on mitochondrial proteins in the soleus muscles could be related to a fiber type specific effect of β-GPA on RIP140 protein content.

Key Words: PGC-1, skeletal muscle, rat, kinase, RIP140

Introduction

An increase in skeletal muscle mitochondrial content, i.e. mitochondrial biogenesis, is a well documented adaptation to endurance exercise training. It is thought that the repeated and transient reductions in high energy phosphates that occur within contracting skeletal muscle serves as an initial signal in the induction of this process. Beta guanadinopropionic acid (β-GPA) is a creatine analogue that has been used as a tool with which to study the effects of "energy charge" on skeletal muscle mitochondrial content (3; 8; 21; 22; 34). When supplemented in standard rodent diets β-GPA leads to marked reductions in intramuscular phosphagen levels and increases in mitochondrial enzyme protein content and activity. Interestingly, some (6; 8; 24), but not all (18) investigations, have reported fiber type specific differences in the response to β-GPA feeding with fast twitch muscles displaying a more pronounced increase in mitochondrial enzymes compared to slow twitch oxidative muscle.

The pathway through which decreases in high energy phosphates leads to the induction of mitochondrial biogenesis would appear to involve the activation of the energy sensing enzyme 5'AMP activated protein kinase (AMPK). This enzyme is robustly activated by perturbations in high energy phosphate levels, as occurs with β-GPA feeding (3; 22; 34). Similarly it has been reported that β-GPA induced mitochondrial biogenesis is absent in fast twitch muscle from AMPK dominant negative mice (34). AMPK likely mediates increases in mitochondrial content through PPAR gamma co-activator 1 alpha (PGC-1α), a master regulator of mitochondrial biogenesis (11). While AMPK has been shown to have a direct effect on PGC-1α expression, some have argued that AMPK can also activate p38 MAPK (12; 13), a signaling pathway that has been implicated in the control of PGC-1α and mitochondrial biogenesis (1; 19; 32). Given the fact that mitochondrial enzyme content appears to increase to a much larger extent in fast compared to slow twitch skeletal muscle (6; 8; 24) following β-GPA feeding it would seem reasonable to speculate that a differential activation of p38 MAPK signaling could in part explain these differences. To date, this premise has not been explored.

PGC-1α gain and loss of function studies demonstrate the importance of this molecule in the regulation of skeletal muscle mitochondrial biogenesis (14; 30). However, increasing evidence points towards the involvement of additional factors in the control of this intricate process. For instance RIP140, a co-repressor of nuclear receptors, has been shown to suppress genes involved in the TCA cycle and fatty acid oxidation (23). The deletion of RIP140 results in mitochondrial biogenesis in skeletal muscle while the over-expression of RIP140 leads to reductions in skeletal muscle mitochondrial enzyme expression (23). At this juncture it is not known if decreases in high energy phosphates regulate RIP140 content in skeletal muscle. However, it is interesting to note that RIP140 deletion increases mitochondrial content in fast but not slow twitch skeletal muscle, a phenotype similar to what has been reported in skeletal muscle following β-GPA feeding (7).

The purpose of the present study was to explore in further detail the signaling pathways that are activated in rat triceps and soleus muscle following long term reductions in high energy phosphates. We sought to exploit the apparent divergence of fiber type responses to reductions in phosphagens as a tool to further elucidate the mechanisms underlying mitochondrial biogenesis. Specifically we hypothesized that β-GPA feeding would lead to increases in markers of mitochondrial biogenesis in triceps but not soleus muscle, and these changes would be associated with a differential regulation of AMPK, p38 MAPK and RIP140 in fast versus slow twitch muscle.

Methods

Materials

Reagents, molecular weight marker, and nitrocellulose membranes for SDS-PAGE were purchased from Bio-Rad (Mississauga, ON). ECL Plus was a product of Amersham Pharmacia Biotech (Arlington Heights, IL). Antibodies against COXIV (CAT# A6403), COXI (CAT# A6403) and CORE I (CAT# A21362) were purchased from Molecular Probes (Eugene, OR). δ-Aminolevulinate synthase (ALAS) antibodies were a kind gift from Dr. John Holloszy at Washington University School of Medicine. Antibodies against p38 MAPK (CAT# 9212), phospho-p38 MAPK(CAT# 9211), ACC (CAT# 3662), phospho-ACC (CAT# 3661), phospho ATF-2 (CAT# 9221), AMPKα (CAT# 2532) and p-AMPK (CAT# 2531) were purchased from Cell Signaling (Danvers, MA). Medium chain acyl-CoA dehydrogenase (MCAD) antibodies (CAT#50587) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti α-actin antibodies (CAT# 2172) were a product of Sigma (St. Louis, MO). An antibody against RIP140 (CAT# ab3425) was a product of Abcam (Cambridge, MA). PGC-1 antibodies were obtained from Chemicon (CAT# ab3242). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). RNeasy extraction kits were purchased from Qiagen (Mississauga, ON). SuperScript II Reverse Transcriptase was a product of Invitrogen (Burlington, ON). Taqman Gene Expression Assays for β-actin, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), MCAD and ALAS were from Applied Biosytems (Foster City, CA). Forward and reverse primers for PGC-1α, PGC-1β, RIP140 and COXI were from Integrated DNA technologies (Coraville, IA), while probes for these genes were from Applied Biosytems (Foster City, CA). Citrate synthase assay kits were purchased from Sigma (St. Louis).

Treatment of animals

All protocols followed Canadian Council on Animal Care (CCAC) guidelines and were approved by the Animal Use and Welfare Committee at the University of Alberta. Male Wistar rats (Charles River, Wilmington, MA) weighing ~180 grams were housed 2 per cage with a 12/12-hour light/dark cycle. Rats were fed a powdered, semi-purified diet (15% fat w/w, 27% w/w protein, 43% w/w carbohydrate) supplemented with or without 1% w/w β-GPA for 6 weeks. The control diet was supplemented with an additional 1% (w/w) cellulose in order to maintain equivalent nutrient density. The nutrient composition of the basal diet has been previously described in detail (29). The only difference being that in the present study, the source of fat in the diet was flaxseed oil, soy tallow and sunflower oil providing a P:S ratio of 0.5. In preliminary experiments we found that food intake was reduced in rats fed a β-GPA supplemented diet. Consequently, control animals were pair fed to minimize differences in body weight. Food was weighed every day in the morning between 9-11:00 AM. Control fed rats were given the same amount of food that the β-GPA fed rats consumed the day prior. Average food intake was calculated as the average of the daily food intake during the duration of the 6 week feeding studying, i.e. the average amount of food consumed as recorded on 41 consecutive days. Between ~9:00-11:00 AM animals were anesthesized with sodium pentabaritol (5 mg/100 g body weight). Triceps and soleus muscles were carefully dissected, washed in sterile saline to remove any blood and clamp frozen in aluminum tongs cooled to the temperature of liquid nitrogen. Samples were stored at – 80° C until analysis.

Western Blotting

Clamp-frozen muscle samples were homogenized in 15 volumes of ice-cold buffer cell lysis buffer supplemented with Protease Inhibitor Cocktail (Sigma) and phenylmethylsulfonyl fluoride. Homogenized samples were sonicated for 5 seconds and centrifuged for 15 min at 2500 X G at 4°C. The supernatant was collected and protein concentration determined using the BCA method. There were no differences in protein concentrations between control and β-GPA fed rats in either the soleus (control

10.52 ± 0.40, β-GPA 10.52 ± 0.34 μg/μl) or triceps (control 5.84 ± 0.54, β-GPA 6.58 ± 0.23 μg/μl). The protein content and/or phosphorylation status of CORE 1, COXIV, COXI, MCAD, ALAS, p38, ACC, AMPK, ATF-2, and RIP140 were determined by Western blot analysis as described previously (32). Briefly, equal amounts of protein were separated on 6.25% (RIP140, ACC, PGC-1), 10% (CORE 1, p38, ATF-2, AMPK, COXI, MCAD, ALAS) or 15% (COXIV) gels. Proteins were wet transferred to nitrocellulose membranes at 200mA/tank. Membranes were blocked in tris buffered saline/0.01% tween 20 (TBST) supplemented with 5% non-fat dry milk for 1 hour at room temperature with gentle agitation. Membranes were incubated in TBST/5% non-fat dry milk supplemented with appropriate primary antibodies overnight at 4°C with gentle agitation. The following morning blots were briefly washed in TBST and then incubated in TBST/1% non-fat dry milk supplemented with HRP conjugated secondary antibodies for 1 hour at room temperature. Bands were visualized using ECL plus and captured using a Typhoon Imaging system (GE Health Care). Imagequant software was used to quantify relative band intensities. Alpha actin was used as an internal control as we found that it was not altered by β-GPA feeding.

Real Time PCR

RNA was isolated from skeletal muscle using a Fibrous RNeasy kit according to the manufacturer's instructions. 1 μg of RNA was used for the synthesis of complementary DNA (cDNA) using SuperScript II Reverse Transcriptase, oligo(dT) and dNTP. Real time PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosytems). Taqman Gene Expression Assays were used to determine the expression of GAPDH, β-actin, MCAD and ALAS. Primers and probes for PGC-1α and β, COXI, and RIP140 were designed using Primer Express 3.0 software (sequences are available upon request). Samples were run in duplicate in a 96 well plate format. For gene expression determined using Taqman expression assays each well (20 μl total volume) contained 1 μl gene expression assay, 1 μl of cDNA template, 10 μl of Taqman Fast Universal PCR Master Mix and 8 μl of RNase free water. For PGC-1α

and β, each 20 µl reaction contained 12.5 µl of PCR Master mix, 0.225 µl each of forward and reverse primers, 0.05 µl of probe and 3.0 ul of RNase free water.

In soleus muscle β-actin was used as an endogenous control as the expression of this gene did not change following β-GPA feeding. In the triceps we used GAPDH as our control gene as β-actin expression was decreased with β-GPA feeding (data not shown). Relative differences in gene expression between control and β-GPA fat fed rats were determined using the $2^{-\Delta\Delta CT}$ method (15). Standard curve assays were performed for GAPDH/β-actin and the genes of interest. The amplification efficiencies of the gene of interest and GAPDH/β-actin were equivalent as determined using the equation $10^{(-1/slope)}$ -1. Likewise, when plotting log cDNA dilution versus delta CT (ΔCT, $CT_{\text{gene of interest}}$-$CT_{\text{beta actin}}$) the slope of this relationship was <0.1, indicating that the genes of interest were amplified with equal efficiency.

Mitochondrial DNA Copy Number

Relative mitochondrial DNA copy number (mtDNA) was determined as described previously (25). Briefly, relative mitochondrial DNA copy number was measured by determining the ratio of a mitochondrial DNA target sequence (mitochondrial D-loop) to the expression of a nuclear target sequence (beta actin for the soleus and GAPDH for the triceps) by real time PCR. These target genes have previously been used to determine relative amounts of mtDNA in rat tissue (4; 16; 25). In preliminary experiments we found that there was a trend towards an increase in beta actin expression in rat triceps muscle from β-GPA fed rats. Therefore GAPDH was used as the nuclear encoded gene in the triceps. Primers and probes for beta actin and the mitochondrial D-loop were designed using Primer Express 1.5 software (sequences are available upon request) while GAPDH was measured using a Taqman Gene Expression Assay. Samples were run in duplicate using a 96 well plate format using a 7900HT Fast Real-Time PCR system (Applied Biosytems). Relative differences in mitochondrial DNA copy number in skeletal muscle between control and β-GPA fed rats was determined using $2^{-\Delta\Delta CT}$ as described previously (25).

Citrate Synthase Activity

Samples were prepared as described above in the methods for Western blotting. Citrate synthase activity was determined using a commercially available kit from Sigma. The formation of 5-thio-2-nitrobenzoic acid was measured spectrophotometrically at 412 nm in 96 well format. The CV of this assay in our laboratory is <10%.

Determination of ATP and Phosphocreatine Concentrations

ATP and phosphocreatine concentrations were determined in freeze dried soleus and triceps samples as described previously (5; 27).

Statistical Analysis

Data are presented as means ± SEM. Comparisons between the means of control and β-GPA fed groups were made using an unpaired Student's t-test. Statistical significance was set at $P < 0.05$

Results

Differences in Body Weight following β-GPA Feeding

There were no differences in body weight between control and β-GPA fed rats at the beginning of the study. Following six weeks of feeding, rats in the β-GPA group weighed significantly less than control rats (Table 1). The average daily food intake during the course of the study did not differ between control (24.3 ± 0.6 grams/day) and β-GPA fed rats (23.8 ± 0.6 grams/day).

β-GPA Feeding Leads to Reductions in High Energy Phosphate Concentrations

Following 6 weeks of dietary β-GPA supplementation there were marked reductions in ATP, PCr, Cr and calculated total creatine in both soleus and triceps muscles (Table 2).

β-GPA Feeding Increases Mitochondrial Proteins in Triceps but not Soleus Muscle

Six weeks of β-GPA feeding led to increases in mitochondrial proteins in rat triceps such as COXIV, COXI, CORE1, MCAD and ALAS (Figure 1). Similarly, citrate synthase activity was higher in triceps from β-GPA fed rats compared to controls, when expressed relative to either tissue (control 19.9 ± 1.8, beta GPA 24.9 ± 1.2 μmol/min/gram tissue $p<0.05$) or protein (control 95.1 ± 3.4, beta GPA 132.1. ± 5.8 μmol/min/gram protein $p<0.05$). In contrast to the triceps, the protein content of mitochondrial marker proteins and the activity of citrate synthase (control 56.4 ± 1.4, beta GPA 59.4 ± 2.7 μmol/min/gram tissue, control 194.2 ± 5.8, beta GPA 205.9 ± 9.5 μmol/min/gram protein) in soleus muscle, was not increased following β-GPA feeding.

To further assess the effects of β-GPA feeding on mitochondrial biogenesis we assessed changes in the mRNA expression of mitochondrial enzymes and relative mtDNA copy number. As seen in figure 2, six weeks of β-GPA feeding resulted in significant increases in ALAS and MCAD in triceps but not soleus muscles. There were no differences in relative mtDNA copy number in either muscle type following β-GPA feeding (soleus 0.85 ± 0.15 fold compared to control, triceps 0.99 ± 0.24 fold compared to control).

β-GPA Feeding Increases PGC-1 Expression in Skeletal Muscle

β-GPA feeding led to increases in the mRNA expression of PGC-1α and the related transcriptional co-activator PGC-1β in both rat triceps and soleus muscle (Figure 3). Likewise, the protein content of PGC-1 was increased in both muscles from β-GPA fed rats.

β-GPA Feeding Increases AMPK Signaling

β-GPA feeding led to increases in the phosphorylation of AMPK and its downstream substrate acetyl coA carboxylase (ACC) in triceps and soleus muscles from β-GPA fed rats (Figure 4). ACC has been shown to be a sensitive marker of AMPK activation (9; 17) and changes in ACC phosphorylation have been used as a marker of AMPK activity following long term treatment with AMPK agonists such as AICAR (2; 20). There were no differences in the total amount of ACC or AMPK following β-GPA feeding in either muscle.

β-GPA Feeding Increases p38 MAPK Signaling

Following six weeks of β-GPA feeding the phosphorylation of p38 MAPK and its downstream substrate ATF-2 were increased in both triceps and soleus muscles (Figure 5).

β-GPA Feeding Reduces RIP140 mRNA Expression and Protein Content in Triceps but not Soleus Muscles

The mRNA expression of RIP140 was decreased ~40% in triceps muscles from β-GPA fed rats. Although reduced, RIP140 mRNA expression in the triceps was still greater than in the soleus by ~50% (data not shown). Similar to mRNA expression, β-GPA feeding led to a ~ 40% reduction in RIP140 protein content. Conversely, neither the expression nor protein content of RIP140 protein content was reduced in soleus muscles following β-GPA feeding (Figure 6).

Discussion

Supplementing rodent diets with the creatine analogue β-GPA is a widely used model with which to study the effects of reduced phosphagen levels on skeletal muscle metabolism and gene expression. It has been demonstrated in skeletal muscle from male rats, that β-GPA feeding leads to increases in mitochondrial enzymes in primarily fast but not slow twitch muscles (6; 8; 24). In an attempt to explain these apparent discrepancies between fiber types, we examined the activation of reputed mediators of mitochondrial biogenesis in rat triceps and soleus, common examples of fast and slow twitch skeletal muscle respectively.

Consistent with previous findings (6; 8; 24) we found that dietary supplementation of β-GPA led to increases in mitochondrial enzymes in triceps but not soleus muscle. These findings were not a result of differences in the degree to which our diet manipulation perturbed phosphagen levels since ATP, PCr and creatine levels were reduced to a similar extent in both muscle types. Previous work from Shulman's laboratory has clearly demonstrated that β-GPA feeding increases AMPK activation (3; 22; 34). Moreover, the over-expression of a dominant negative AMPK mutant blocks the effects of β-GPA on mitochondrial biogenesis in fast twitch mouse skeletal muscle (34). Consistent with these findings we found that the phosphorylation of ACC was increased in triceps muscles from β-GPA fed rats. In soleus muscle, despite the fact that mitochondrial enzyme content was not augmented with β-GPA feeding, we saw increases in AMPK and ACC phosphorylation. These results are in line with a recent report demonstrating that the chronic activation of AMPK with AICAR did not lead to increases in mitochondrial enzyme activity in rat soleus muscle (2). Moreover, several groups have demonstrated a similar disconnect between AMPK activation and glucose transport in rat soleus muscle (7; 33).

In addition to AMPK, mounting evidence has suggested that the p38 MAPK signaling pathway is a central regulator of skeletal muscle mitochondrial biogenesis. For instance Akimoto and colleagues (1), have reported that the over-expression of MKK3, an upstream activator of p38 MAPK, leads to

increases in PGC-1α expression in skeletal muscle cells. This effect was prevented by the expression of a dominant negative mutant of ATF-2, a transcription factor that is a direct substrate of p38 MAPK and that binds to the PGC-1α promoter. In contrast to a previous study which analyzed p38 MAPK phosphorylation in skeletal muscles from creatine kinase deficient mice (28), we found that β-GPA feeding led to increases in p38 MAPK and ATF-2 phosphorylation in both triceps and soleus. Although we do not have any evidence showing a direct effect of AMPK on the activation of the p38 MAPK signaling pathway, our results are consistent with others (10, 11) who have demonstrated cross talk between these pathways. Regardless of the mechanism through which p38 MAPK is activated following β-GPA feeding, differences in the activation of this enzyme do not appear to account for the differential effects of chronic reductions in high energy phosphates on mitochondrial proteins between the triceps and soleus muscles. Moreover, it would appear that the activation of the p38 MAPK-ATF-2 signaling pathway, at least in soleus muscle, is not sufficient to cause increases in mitochondrial enzymes.

The activation of both AMPK (11; 26) and p38 MAPK (1; 19) have been shown to increase the expression of PGC-1α. As would be expected with the chronic activation of these signaling pathways by the dietary administration of β-GPA, we found that PGC-1α and the related transcriptional co-activator, PGC-1β, were increased to a similar extent in both soleus and triceps muscles. Taken in context with our signaling data, it would seem that many of the proximal signals and molecules that serve as positive effectors of mitochondrial biogenesis respond in a similar fashion to chronic reductions in ~P regardless of the fiber type composition of the muscle examined. Within this framework it seems reasonable to assume that differences in the protein content and/or expression of negative regulators of mitochondrial biogenesis could potentially explain, at least in part, the fiber type specific differences of β-GPA on skeletal muscle mitochondrial biogenesis. One likely candidate could be RIP140, a co-repressor of nuclear receptors. The deletion of RIP140 has been shown to lead to increases in mitochondrial enzymes in fast twitch mouse muscle that occur independent of increases in PGC-1α expression (23)

while the over-expression of RIP140 results in reductions in mitochondrial content in slow twitch skeletal muscle (23). It is thought that RIP140 exerts a negative effect on mitochondrial enzyme gene expression by acting as a scaffold protein between nuclear receptors and chromatin remodeling enzymes involved in transcriptional repression (31). Furthermore it has been shown that RIP140 binds to the promoter regions of nuclear receptor target genes and can also interact with, and inhibit the activity of, PGC-1α (10).

In the present study we found that β-GPA feeding led to marked reductions in both the protein content and mRNA expression of RIP140 in fast twitch rat triceps muscles. To the best of our knowledge this is the first investigation demonstrating that chronic reductions in skeletal muscle high energy phosphate levels leads to decreases in RIP140 content. Given the similar effects of β-GPA feeding and exercise training on skeletal muscle mitochondrial biogenesis it would seem likely that exercise could reduce skeletal muscle RIP140 content and that these changes could be involved in the mechanisms through which exercise induces mitochondrial biogenesis. However, arguing against this premise, it has recently been reported that the over-expression of RIP140 does not blunt the exercise induced increase in skeletal muscle oxidative capacity (23). Clearly, the effects of exercise on RIP140 content, and the role of this protein in exercise induced skeletal muscle mitochondrial biogenesis needs to be explored in further detail.

In contrast to the triceps, we did not see any differences in RIP140 protein content or mRNA expression in soleus muscle from control and β-GPA fed rats. It has been suggested that RIP140 and PGC-1α have mutually antagonizing roles in the regulation of skeletal muscle mitochondrial gene expression (23). It has further been suggested that this antagonism may be mediated by the expression of RIP140 relative to PGC-1α . Thus, increases in PGC-1α, independent of reductions in RIP140 may not necessarily lead to increases in mitochondrial enzymes. The findings that certain markers of

mitochondrial biogenesis did not increase in the soleus muscle despite a marked induction of PGC-1α and β would be consistent with this view.

In summary, this is the first investigation to examine fiber type specific differences in the regulation of AMPK, p38 MAPK and RIP140 by β-GPA feeding. We found that chronic reductions in phosphagens led to the activation of p38 MAPK and AMPK signaling and reductions in RIP140 in the triceps and these changes were associated with increases in mitochondrial enzyme content. On the other hand, the activation of p38 MAPK and AMPK and the induction of PGC-1 mRNA did not appear to be sufficient to lead to increases in mitochondrial enzymes in the soleus. Although clearly a complex process involving multiple signaling pathways and transcriptional regulators, our results suggest that the differences in the response to long term reductions in phosphagens between fast twitch muscles like the triceps and slow twitch muscles like the soleus, may be accounted for, at least in part, by a differential effect of β-GPA feeding on RIP140.

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Figure Legends

Figure 1. β-GPA feeding increases a) CORE1, COXIV, COXI, ALAS and MCAD protein content in rat triceps but not soleus (B) muscle. Data are presented as means + SEM for 5-12 samples per group. * $P<0.05$ compared to the corresponding muscle from control fed rats. Representative Western blots for the proteins of interest and actin are shown beside the quantified data.

Figure 2. β-GPA feeding increases the expression of mitochondrial enzyme mRNA expression in rat A) triceps but not B) soleus muscles. Data are presented as means + SEM for 7-9 samples per group and are expressed as fold differences relative to control. * $P<0.05$ compared to the corresponding control value in the same muscle from control fed rats.

Figure 3. β-GPA feeding increases the mRNA expression of PGC-1α, PGC-1β and PGC-1 protein content in rat triceps (A & B) and soleus (C & D) muscles. Data are presented as means + SEM for 8-12 samples per group. * $P<0.05$ compared to the corresponding control value in the same muscle from control fed rats. Representative Western blots for PGC-1 and actin are shown above the quantified data.

Figure 4. β-GPA feeding increases the phosphorylation of ACC in rat triceps (A) and soleus (B) muscles. Data are presented as means + SEM for 11-12 samples per group. * $P<0.05$ compared to the corresponding muscle from control fed rats. Representative Western blots for p-ACC and t-ACC are shown above the quantified data.

Figure 5. β-GPA feeding increases the phosphorylation of p38 MAPK and ATF-2 in rat triceps (A) and soleus (B) muscles. Data are presented as means + SEM for 11-12 samples per group. * $P<0.05$ compared to the corresponding muscle from control fed rats. Representative Western blots for p-p38 MAPK, p38 MAPK, p-ATF-2, and actin are shown above the quantified data.

Figure 6. β-GPA feeding decreases the protein content and mRNA expression of RIP140 in triceps but not soleus muscles. Data are presented as means + SEM for 11-12 samples per group. * $P<0.05$ compared to the corresponding muscle from control fed rats. Representative Western blots for RIP140 and actin are shown above the quantified data.

Table 1. The effects of β-GPA feeding on body weight and weight gain in male Wistar rats. Data are presented as means ± SEM for 12 rats/group. * $p<0.05$ versus control.

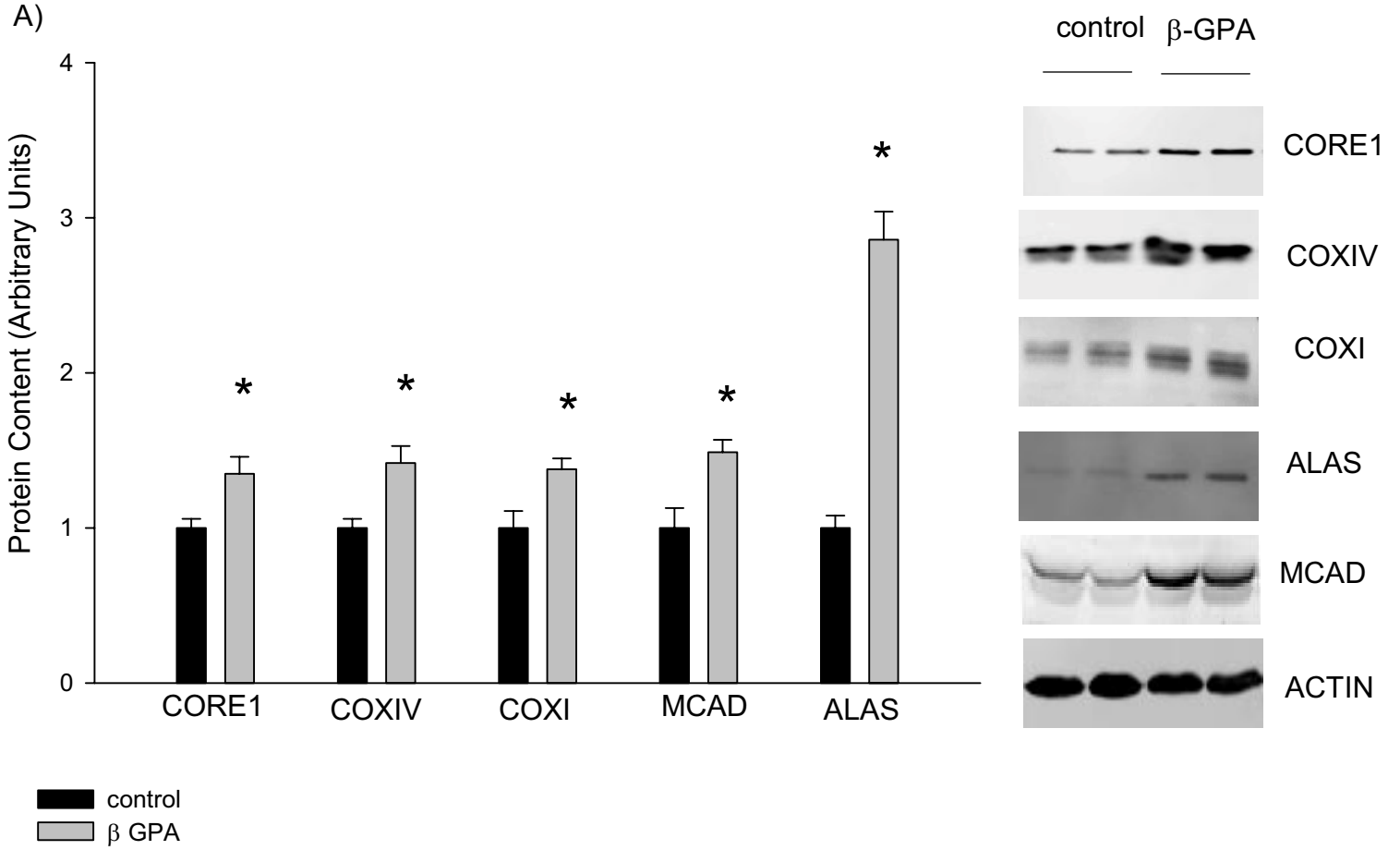
	Control	β-GPA
Initial Body Weight (grams)	172.8 ± 2.7	172.6 ± 1.6
Final Body Weight (grams)	425.4 ± 5.3	391.7 ± 10.7*
Weight Gain (grams)	253.5 ± 3.7	219.9 ± 9.7*

Table 2. The effects of β-GPA feeding on muscle phosphagens (ATP, phosphocreatine (PCr), and total creatine (TCr)) in soleus and triceps muscles of control and β-GPA fed rats. Values are means ± SEM for 3-6 muscles/group and are expressed as mmol/kg dry mass. * $p<0.05$ than corresponding control muscle.

	Control Triceps	β-GPA Triceps	Control Soleus	β-GPA Soleus
ATP	26.8 ± 2.0	17.4 ± 0.4*	17.9 ± 2.6	5.4 ± 1.3*
PCr	58.1 ± 15.9	4.4 ± 1.2*	42.5 ± 7.5	0.5 ± .2*
Cr	81.3 ± 15.3	14.8 ± 0.9*	55.9 ± 10.9	8.5 ± 1.2*
TCr	139.4 ± 1.5	19.6 ± 0.5*	77.4 ± 6.1	9.0 ± 1.2*

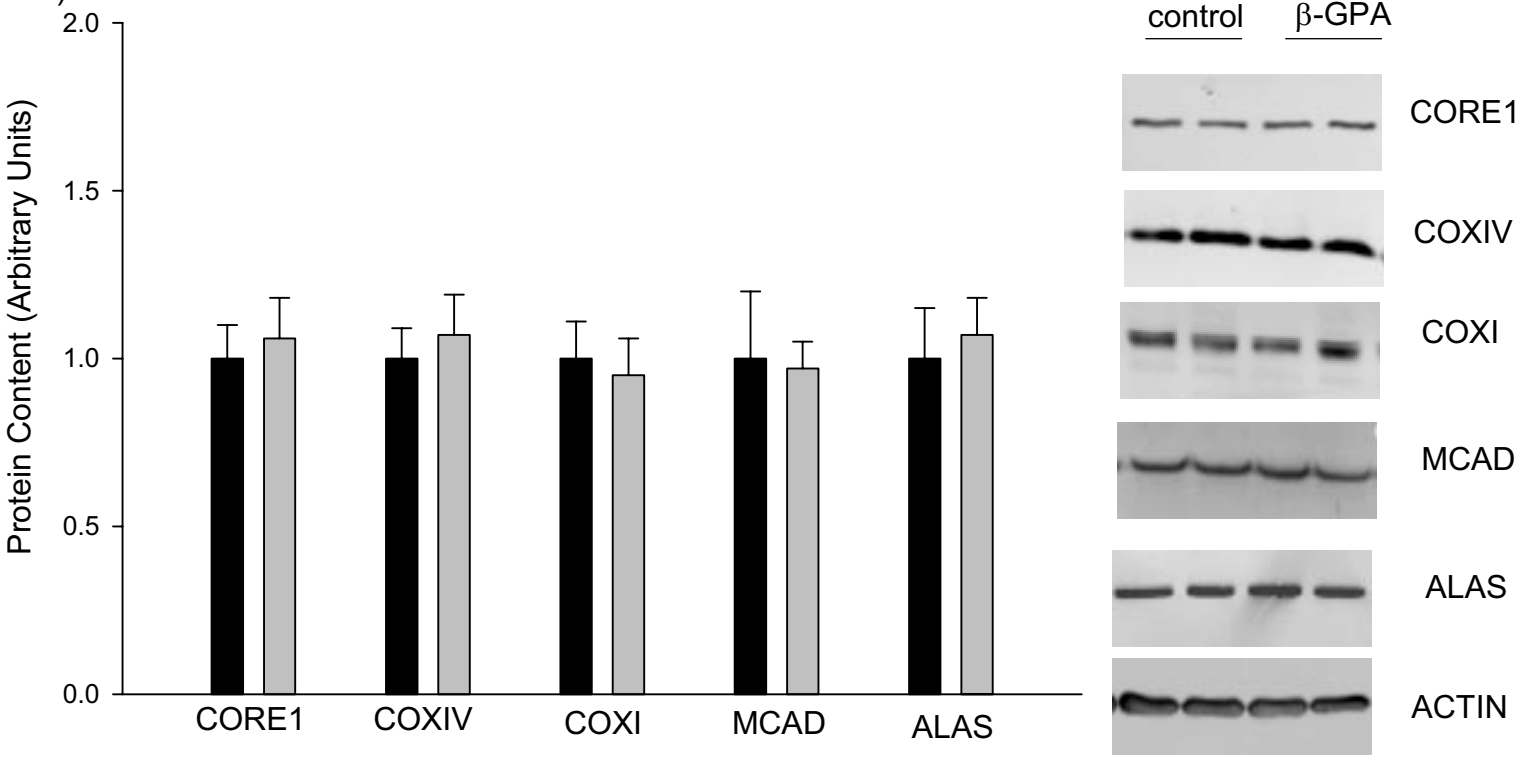
Triceps

A)

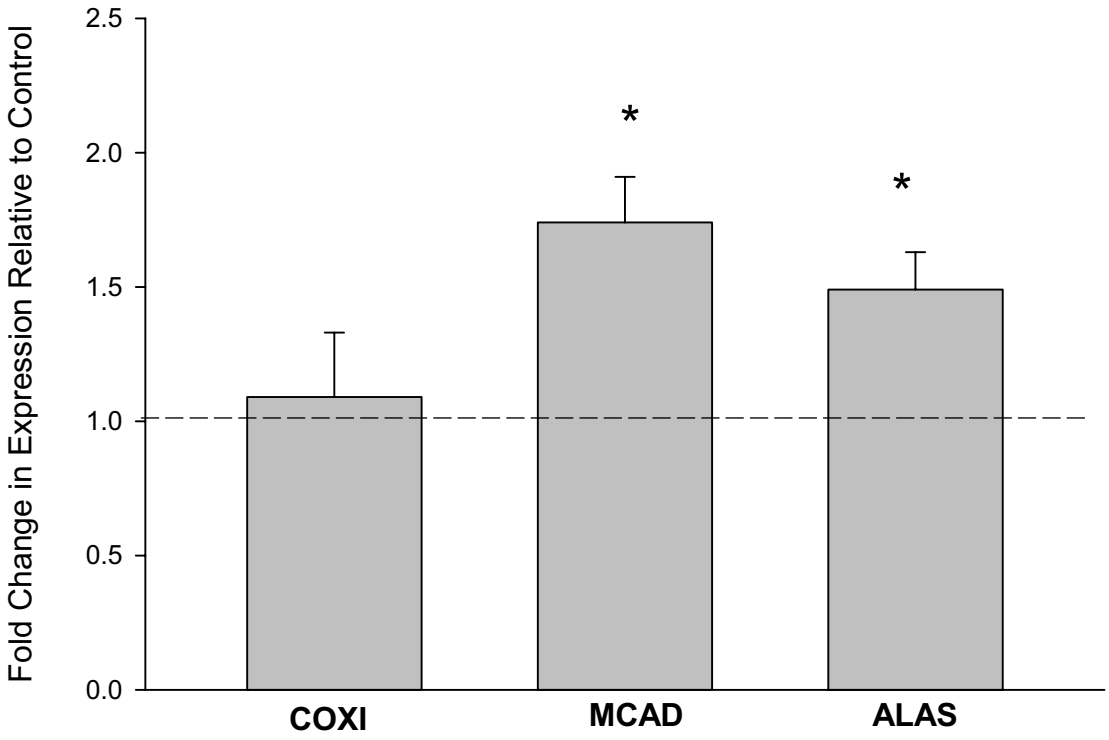


Soleus

B)



A) Triceps



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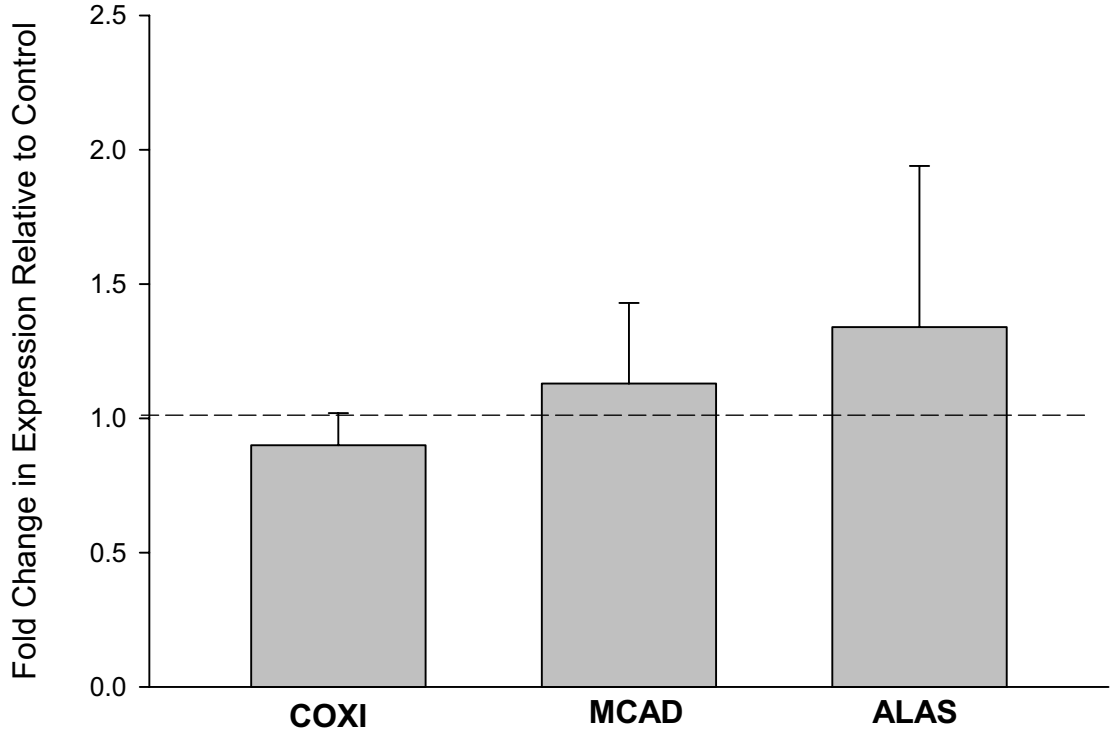
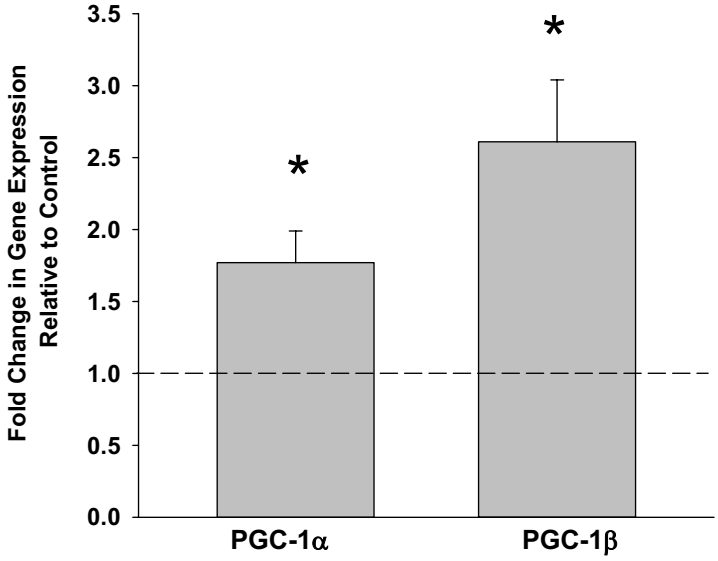


Triceps

A)



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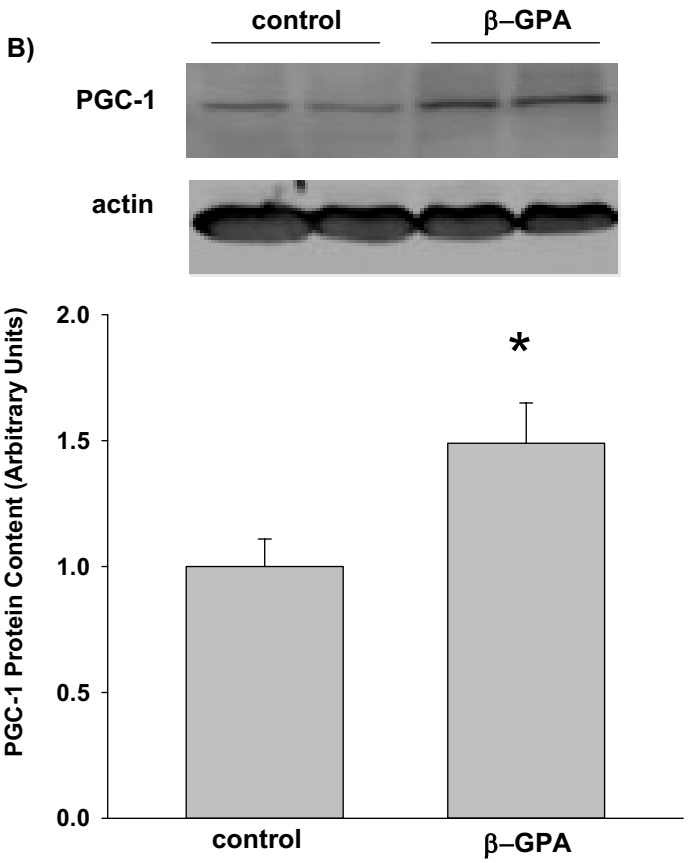
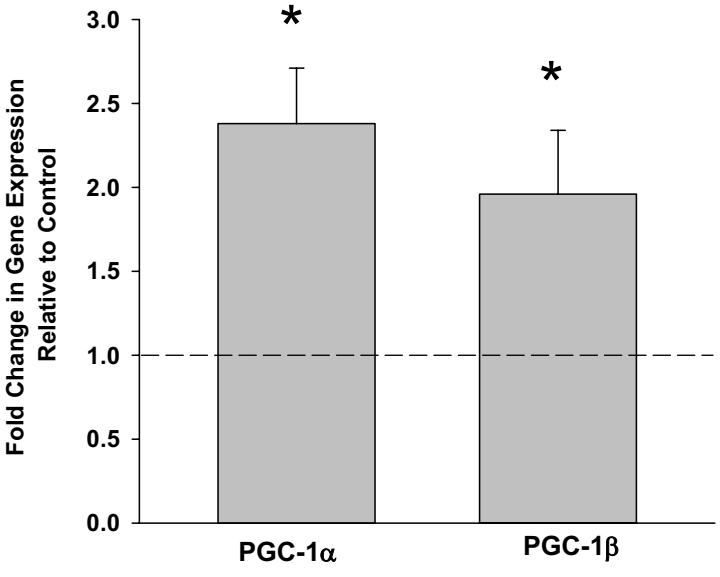
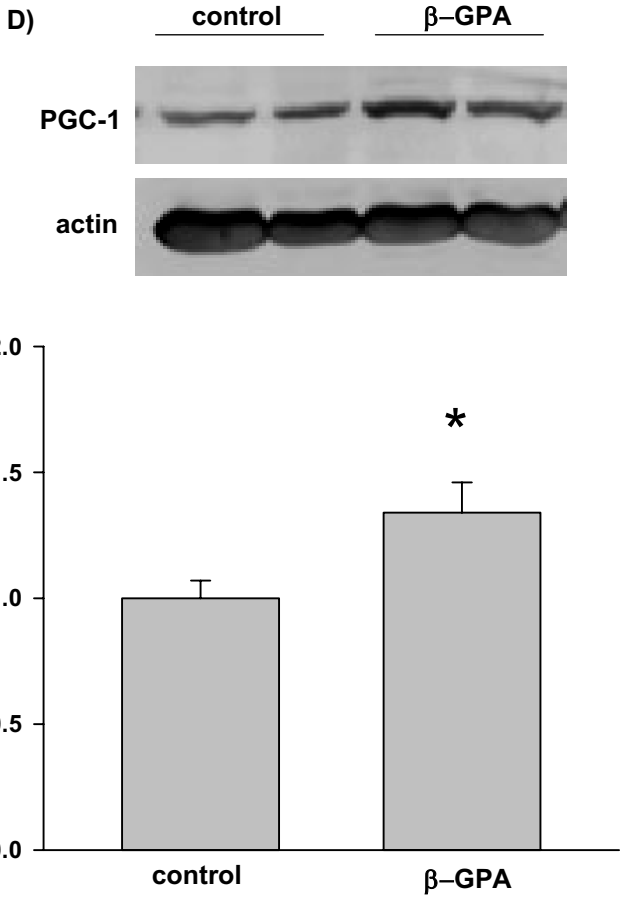


Soleus

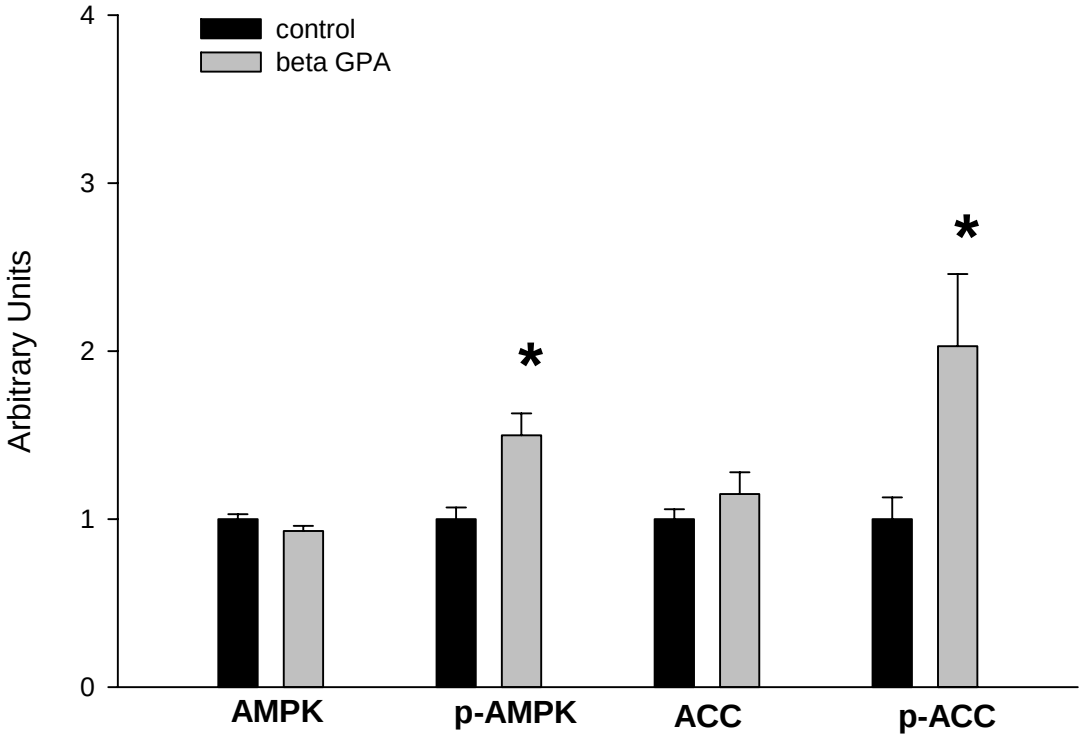
C)

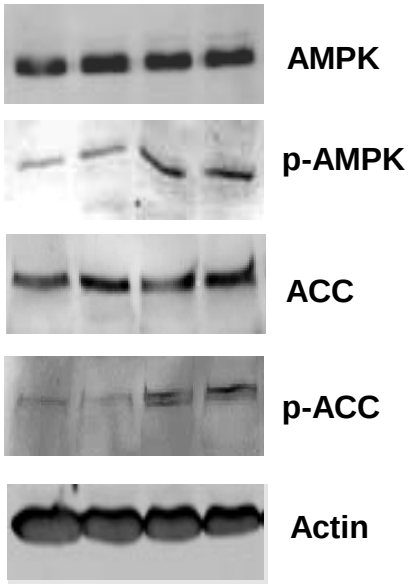


D)

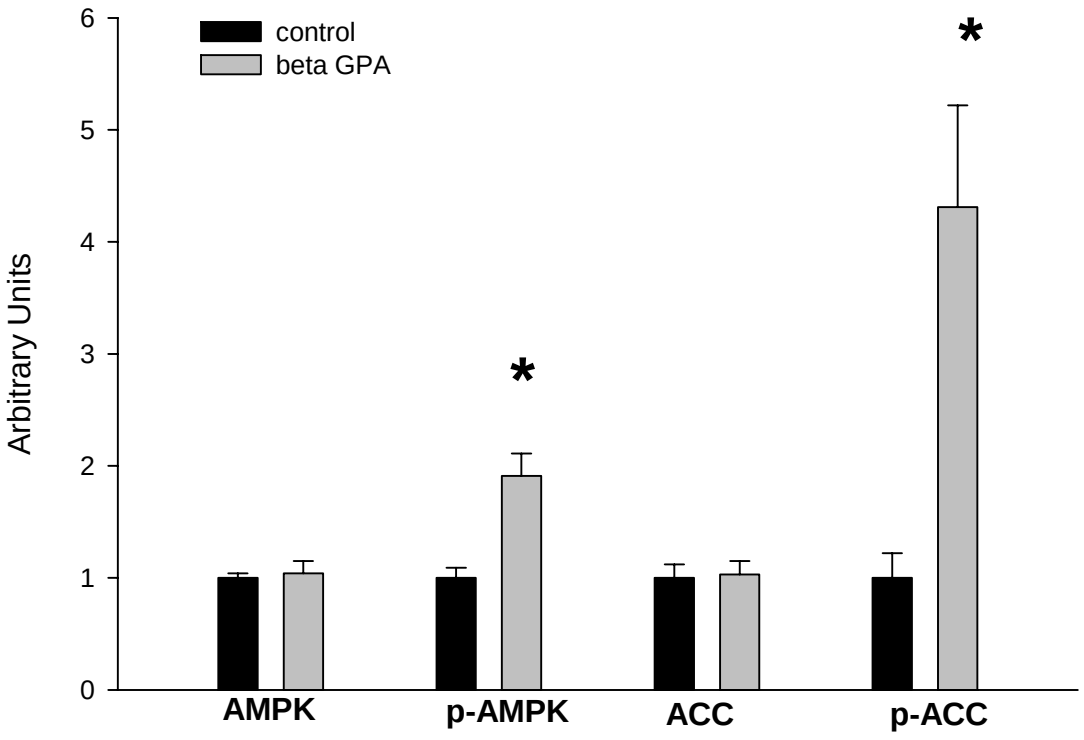


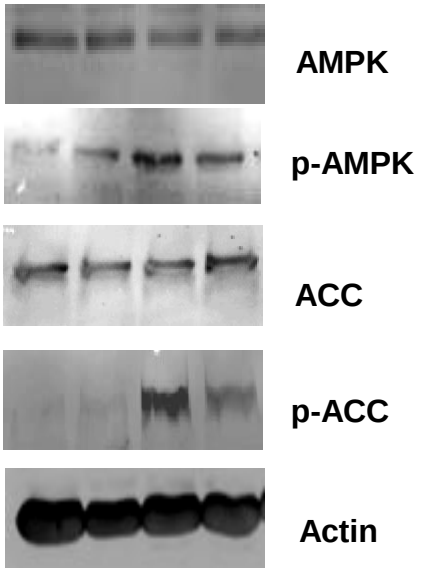
A. Triceps



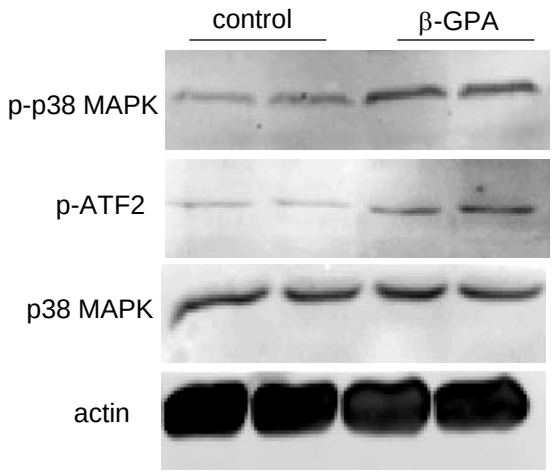


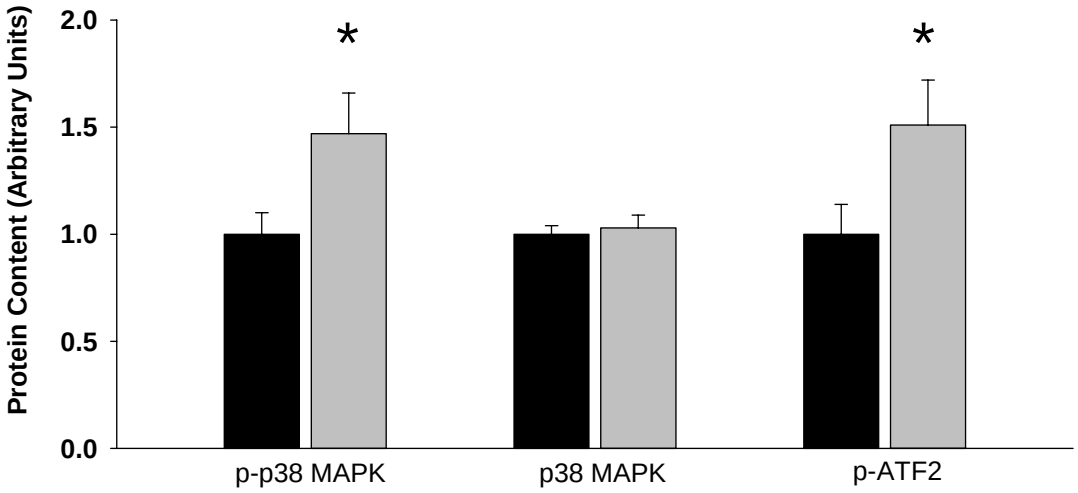
B. Soleus





A) Triceps





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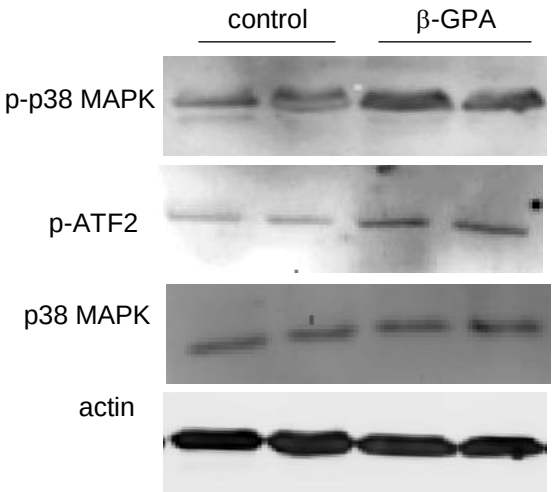
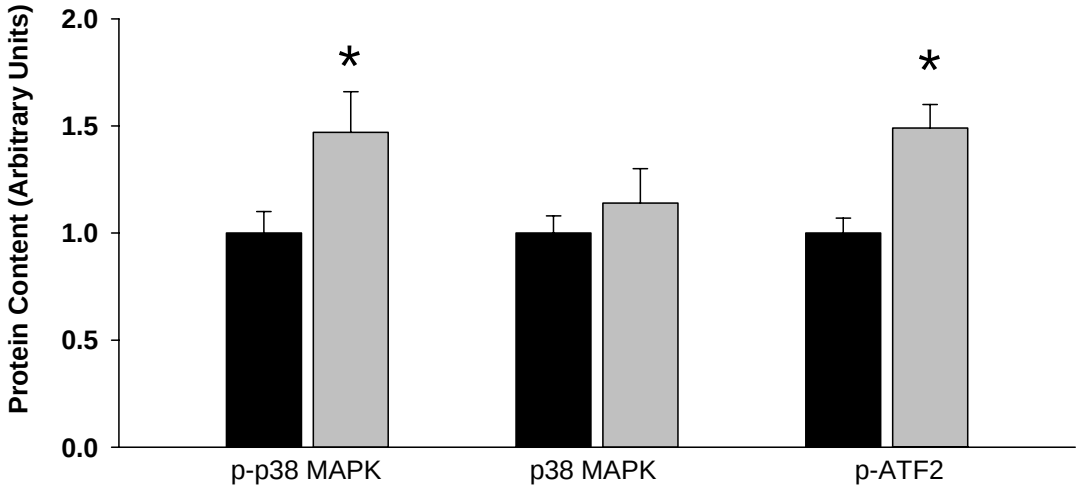
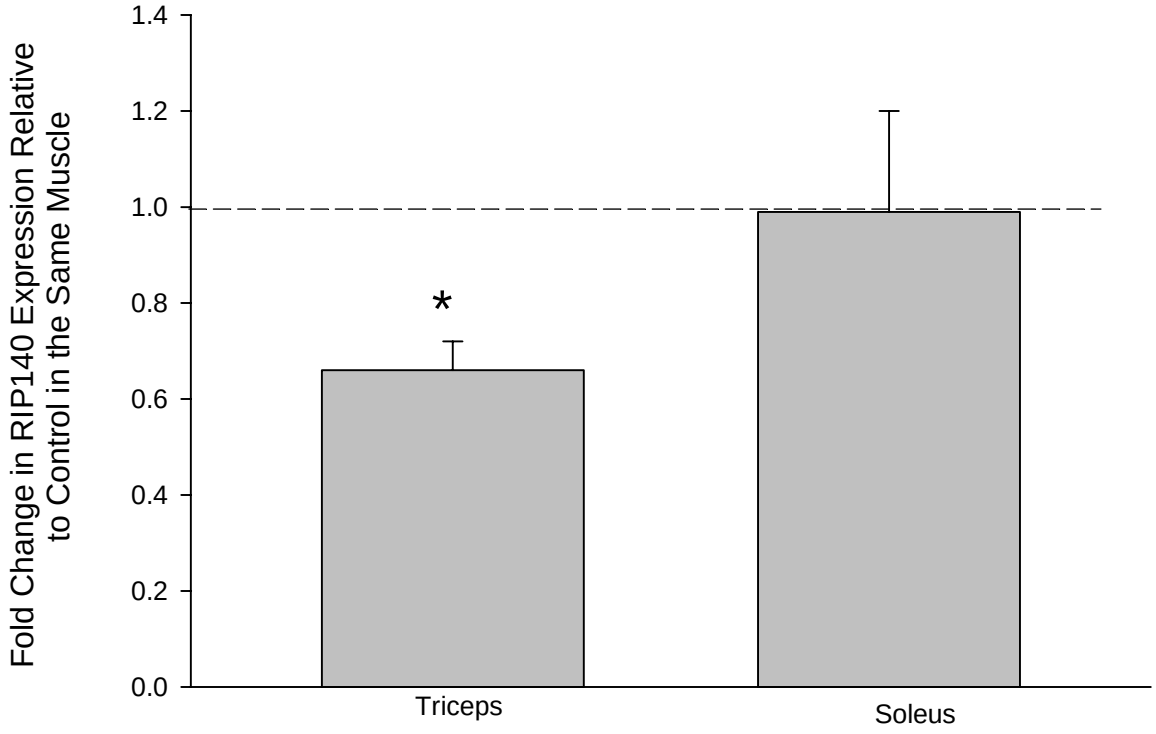
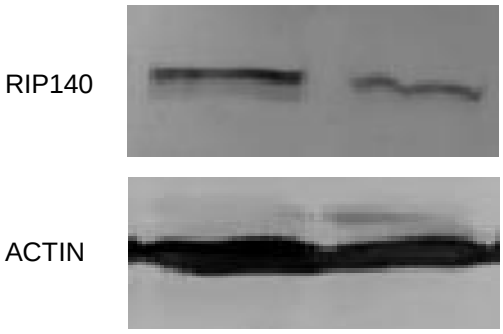




A)



B) Triceps



C) Soleus