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Dietary supplementation of β -guanidinopropionic acid (β GPA) reduces whole-body and skeletal muscle growth in young CD-1 mice

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Abstract

Increased AMP-activated protein kinase (AMPK) activity leads to enhanced fatty acid utilization, while also promoting increased ubiquitin-dependent proteolysis (UDP) in mammalian skeletal muscle. β -guanidinopropionic acid (β GPA) is a commercially available dietary supplement that has been shown to promote an AMPK-dependent increase in fatty acid utilization and aerobic capacity in mammals by compromising creatine kinase function. However, it remains unknown if continuous β GPA supplementation can negatively impact skeletal muscle growth in a rapidly growing juvenile. The current study was conducted to examine the effect of β GPA supplementation on whole-body and skeletal muscle growth in juvenile and young adult mice. Three-week old, post weanling CD-1 mice were fed a standard rodent chow that was supplemented with either 2 % (w/w) α -cellulose (control) or β GPA. Control and β GPA-fed mice ($n = 6$) were sampled after 2, 4, and 8 weeks. Whole-body and hindlimb muscle masses were significantly ($P < 0.05$) reduced in β GPA-fed mice by 2 weeks. The level of AMPK (T172) phosphorylation increased significantly ($P < 0.05$) in the gastrocnemius of β GPA-fed versus control mice at 2 weeks, but was not significantly different at the 4- and 8-week time points. Further analysis revealed a significant ($P < 0.05$) increase in the skeletal muscle-specific ubiquitin ligase MAFbx/Atrogin-1 protein and total protein ubiquitination in the gastrocnemius of β GPA

versus control mice at the 8-week time point. Our data indicate that feeding juvenile mice a β GPA-supplemented diet significantly reduced whole-body and skeletal muscle growth that was due, at least in part, to an AMPK-independent increase in UDP.

Keywords

β -Guanidinopropionic acid; Skeletal muscle growth; Protein turnover; AMP-activated protein kinase; Mechanic target of rapamycin complex 1

Introduction

Creatine kinase plays an essential role in regulating cellular energy charge in skeletal muscle cells through the transfer of high-energy phosphates between creatine and ADP during fluctuations in energy demand [1]. However, cellular creatine uptake is competitively inhibited by the dietary supplement β -guanidinopropionic acid (β GPA) [2, 3]. β GPA is a creatine analog that gradually depletes cellular phosphocreatine (PCr) levels, resulting in increased AMP/ATP, which is analogous in many ways to chronic exercise [4, 5]. Dietary supplementation of β GPA can thus promote the activation of AMP-activated protein kinase (AMPK) [3, 4]. AMPK responds to cellular energy deficits, manifested as an increased AMP/ATP ratio, and elevated intracellular Ca^{2+} concentration, by activating catabolic pathways to increase energy production while simultaneously inhibiting anabolic pathways to reduce energy expenditure [5–7]. In skeletal muscle, AMPK activation promotes increased expression of biological markers for oxidative metabolism and mitochondrial biogenesis [8–14], increased glucose and fatty acid transport and oxidation [15, 16], enhanced insulin sensitivity [17–19], and shifts toward more oxidative fiber types [20–25]. These attributes make AMPK-activating compounds, ideal therapeutics for the treatment of obesity and for increasing aerobic capacity and endurance in athletes [8, 10, 11, 14].

Juvenile mammals experience periods of accelerated growth, which in skeletal muscle entails maximizing protein synthesis and minimizing protein degradation [26]. In mammals, cellular protein synthesis is regulated by the mechanistic target of rapamycin complex 1 (mTORC1) [27]. Under anabolic conditions, mTORC1 is maximally activated by endocrine factors, such as insulin and insulin-like growth factor 1 (IGF1), and amino acids. Activated mTORC1 promotes protein synthesis by directly phosphorylating the ribosomal protein S6 kinase (S6K), which in turn phosphorylates ribosomal protein S6 [27]. In addition, mTORC1 directly phosphorylates the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1). Phosphorylated 4EBP1 is unable to bind the eukaryotic translation initiation factor 4E (eIF4E), which allows eIF4E to bind the translation initiation complex and results in increased protein synthesis [27]. Besides promoting synthesis, mTORC1 also inhibits protein degradation by phosphorylating a class of transcription factors known as Forkhead boxes (FOXOs) [28]. Phosphorylated FOXOs are unable to enter the nucleus and promote gene expression of the muscle-specific ubiquitin ligases muscle atrophy F box (MAFbx) and muscle RING finger 1 (MuRF1) [28]. Therefore, the mTORC1 pathway plays a vital role in promoting hypertrophy in skeletal muscle by activating protein synthesis and inhibiting degradation.

AMP-activated protein kinase opposes many of the effects of mTORC1 by actively inhibiting protein synthesis [29, 30] and also enhancing activity of the ubiquitin–proteasome system by phosphorylating FOXO3a to promote expression of MAFbx and MuRF1 [31]. AMPK therefore has an antagonistic relationship with the mTORC1 pathway as it promotes atrophy in skeletal muscle, and it is therefore not surprising that these pathways are reciprocally regulated under normal physiological conditions. However, during normal growth mTORC1 is highly active, and it remains unclear if the continuous use of AMPK-activating supplements, such as β GPA, can have adverse effects on whole-body and skeletal muscle growth in children and young adults.

The goal of this study was to determine the effect of dietary β GPA supplementation on whole-body and skeletal muscle growth in juvenile and young adult mice. Due to the accelerated metabolic rate of juveniles, we hypothesized that the potential catabolic effects of dietary β GPA supplementation would be greatly magnified in healthy young mice, making the phenotypic response to treatment more apparent than in adults. Our results indicate that dietary β GPA reduced whole-body and skeletal muscle growth that was due, at least in part, to an AMPK-independent increase in ubiquitin-dependent proteolysis in skeletal muscle.

Materials and methods

Experimental design

Fifty-four 3-week-old female CD-1 [CrI:CD1(ICR)] mice were purchased from Charles River Laboratories. The mice were then randomly divided into nine groups ($n = 6$) and housed in individual cages. An initial group of mice was euthanized with an overdose of CO₂ and sampled for whole-body, gastrocnemius, soleus, and extensor digitorum longus (EDL) mass. The eight remaining groups of mice were randomly divided into two experimental treatments. Four groups received a standard rodent diet that was supplemented with 2 % (w/w) α -cellulose (control). The other four groups received a standard rodent diet that was supplemented with 2 % (w/w) β -GPA, which is a standard dosage used in previous investigations [3]. Each cage was fed once daily (20 g/day). One group of control and β GPA-fed mice were sampled after 2, 4, 6, and 8 weeks. At each time point, mice were sampled for whole-body, gastrocnemius, soleus, and EDL mass, and tissues were stored at -80°C until use.

Sample preparation

Whole gastrocnemius muscles were mechanically homogenized in 1 ml of radio immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology) and then submitted to ultrasonic disruption. All samples were then centrifuged at $10,000\times g$ for 30 min at 4°C . Upon centrifugation each sample supernatant was removed and combined with 20 % trichloroacetic acid (TCA) (v/v) and incubated overnight at -20°C . The resulting protein pellets were washed three times in 1 ml of ice cold 20 % TCA, and the remaining protein pellets were resolubilized in 250 μl of ice-cold RIPA buffer. Total protein concentrations were determined using a Bradford assay kit (Pierce Protein). To prepare samples for

immunoblot analysis, 100 μ l of sample was combined with 100 μ l of 2 $\times$ Laemmli buffer (BioRad Laboratories) and incubated at 95 $^{\circ}$ C for 5 min.

Western blot analysis

For each muscle lysate sample, 15 μ g of total protein was loaded on an SDS PAGE gel (4 % stacking/7.5 % resolving) and submitted to electrophoresis. Upon electrophoretic separation, all SDS PAGE gels were transferred to polyvinylidene difluoride (PVDF) membranes and blocked in either 5 % (w/v) non-fat milk dissolved in tris-buffered saline + 0.1 % Tween 20 (TBST) or 5 % (w/v) bovine serum albumin (BSA) dissolved in TBST for 1 h. Blots were probed overnight at 4 $^{\circ}$ C in antibody diluent (1:1 mixture of TBST and 5 % BSA in TBST) with one or more of the following primary antibodies: P-AMPK α 1/2 (T172) (1:1000, Santa Cruz Biotechnology), AMPK α 1/2 (1:1000, Santa Cruz Biotechnology), P-S6K-1(T389) (1:1000, Santa Cruz Biotechnology), β -tubulin (1:500, Developmental Studies Hybridoma Bank), P-ACC (S79) (1:1000, Cell Signaling Technology), ACC (1:1000, Cell Signaling Technology), P-4EBP-1 () (1:1000, Cell Signaling Technology). Upon primary antibody incubation, all blots were washed 3 $\times$ with TBST and probed with antibody diluent containing secondary antibody (1:10,000, Vector Labs) for 1 h at room temperature. Blots were developed using enhanced chemiluminescence (ECL, BioRad Laboratories), exposed to X-ray until a satisfactory image was obtained, and analyzed using ImageJ software (National Institutes of Health).

Ubiquitin dot blot assay

A PVDF membrane was incubated in 100 % methanol for 10 min and allowed to dry. For all gastrocnemius lysate samples, 5 μ g of total protein was spotted on the membrane and allowed to dry (approx. 20 min). Once all samples were spotted and completely dried, the membrane was washed in 100 % methanol for 1 min and blocked in 5 % non-fat milk dissolved in TBST for 1 h at room temperature. Upon washing the membrane three times in TBST, the membrane was incubated overnight at 4 $^{\circ}$ C in antibody diluent containing 1:1000 primary antibody for poly-ubiquitin (Enzo Life Sciences). Upon primary antibody incubation, the dot blot was washed 3 $\times$ with TBST and incubated with antibody diluent containing secondary antibody (1:10,000, Vector Labs) for 1 h at room temperature. The dot blot was developed using ECL, exposed to X-ray film until a satisfactory image was obtained, and analyzed using ImageJ software.

Protein carbonylation dot blot assay

Total protein carbonylation was analyzed using the method of Robinson et al. [32]. Briefly, a PVDF membrane was wetted with 100 % MeOH and then soaked in a 20 % MeOH–80 % TBS for 5 min. For each gastrocnemius sample, 5 μ g of total protein was spotted on the membrane and allowed to dry for approximately 20 min and immediately immersed in 100 % MeOH for 1 min. The membrane was sequentially incubated in 20 % MeOH–80 % TBS for 5 min and then in 2 N HCl for 5 min. The membrane was then derivatized in a solution of 2,4-dinitrophenylhydrazine (100 μ g/mL) in 2 N HCl for exactly 5 min. The membrane was subsequently washed three times in 2 N HCl (5 min per wash) and seven times in 100 % MeOH (5 min per wash). The membrane was then blocked for nonspecific protein binding 5 % milk (w/v) in TBST for 1 h and washed three times in TBST. The

membrane incubated for 18 h at 4 °C with the primary antibody solution consisting of a 1:25,000 dilution of the rabbit anti-2,4-dinitrophenol antibody (Santa Cruz Biotechnology) in TBST containing 2.5 % milk. Upon washing the membrane three times in TBST for 5 min each, the membrane was probed with a 1:5000 dilution of goat anti-rabbit secondary antibody (Vector Labs) in TBST containing 2.5 % milk for 1 h at room temperature. The membrane was then submitted to six 5 min washes in TBST. The blot was developed using ECL, exposed to X-ray film until a satisfactory image was obtained, and analyzed using ImageJ software. The blot was then incubated in coomassie brilliant blue dye for 1 h and each sample was normalized for total protein content using ImageJ software.

Statistical analysis

Whole-body and skeletal muscle growth data, as well as western and dot blot data, were analyzed using a student's *t* test. A $P < 0.05$ was considered significant for both analyses.

Results

Whole-body and skeletal muscle growth

A 2 % dietary supplementation of β GPA reduced whole-body and skeletal muscle growth in juvenile mice. A pairwise analysis revealed that the whole-body mass of β GPA-fed mice was significantly reduced ($P < 0.05$) by 19.3 % at 2 weeks, 18.7 % at 4 weeks, and 16.6 % at 6 weeks when compared to controls (Fig. 1a). At 8 weeks, however, the whole-body mass of β GPA-fed versus control mice was not significantly different (Fig. 1a). Hindlimb muscle mass was also reduced due to β GPA feeding, as well. The changes in gastrocnemius mass over time tracked in a very similar manner to changes in whole-body mass (Fig. 1a). The gastrocnemius mass of β GPA-fed mice was significantly reduced ($P < 0.05$) by 19.5 % at 4 weeks, 26.6 % at 6 weeks, and 16.4 % 8 weeks (Fig. 1b). The only significant difference in soleus mass was a 28.4 % reduction ($P < 0.05$) in β GPA-fed versus control mice at 2 weeks (Fig. 1c). Likewise, the only significant difference in EDL mass was a 21.3 % reduction ($P < 0.05$) in the EDL of β GPA-fed versus control mice at 8 weeks (Fig. 1d). These data indicate that dietary supplementation of β GPA promoted reduced whole-body mass due, at least in part, to a time-dependent effect on the growth of skeletal muscle.

AMPK-mediated signaling pathway

We discovered a 50 % increase ($P < 0.05$) P-AMPK (T172) in the gastrocnemius of β GPA-fed versus control mice at 2 weeks (Fig. 2a). However, we found no significant difference in P-AMPK (T172) in the gastrocnemius of β GPA-fed versus control mice at any other time point. To further analyze changes in AMPK signaling, we evaluated ACC phosphorylation. In contrast to our P-AMPK data, the P-ACC (S79) level increased by twofold ($P < 0.05$) in the gastrocnemius of β GPA-fed versus control mice at the 4 weeks but was not significantly different at any other time point (Fig. 2b).

mTORC1 signaling pathway

We evaluated the downstream targets of mTORC1 to examine any possible changes in the mTORC1 signaling pathway in the gastrocnemius of β GPA-fed versus control mice. We discovered a 38 % reduction ($P < 0.05$) in P-S6K1 (T389) in gastrocnemius of β GPA-fed

versus control mice at 4 weeks (Fig. 3a). However, there was no significant difference in P-S6K1 (T389) levels at 2 and 8 weeks. In addition, we found no significant difference in P-4EBP1(T37/46) in gastrocnemius of control vs. β GPA-fed mice at any time point (Fig. 3b).

Markers of ubiquitin-dependent protein degradation

To determine if reduced skeletal muscle growth in β GPA-fed mice was associated with increased ubiquitin-dependent protein degradation, we analyzed the expression of the muscle-specific ubiquitin ligases MuRF1 and MAFbx. There was no significant difference in MuRF1 protein level in the gastrocnemius of control versus β GPA-fed mice at any time point (Fig. 4a). However, MAFbx protein levels were approximately twofold higher ($P < 0.05$) in the gastrocnemius of β GPA-fed versus control mice at 8 weeks (Fig. 4b). In addition, we conducted dot blot analysis to examine the time-dependent changes in total protein ubiquitination. Our results indicate that total protein ubiquitination increased by 49 % ($P < 0.05$) in the gastrocnemius of β GPA-fed versus control mice at 8 weeks (Fig. 4c). To further examine changes in ubiquitin-dependent protein degradation, we conducted dot blot analysis of total protein carbonylation. There was no significant difference in total protein carbonylation level in the gastrocnemius of control vs. β GPA-fed mice at 2 and 4 weeks (Fig. 4d). However, the total protein carbonylation level in the gastrocnemius of β GPA-fed versus control mice was reduced by 71 % ($P < 0.05$) at 8 weeks (Fig. 4d).

Discussion

The goal of this study was to determine if dietary β GPA supplementation could reduce whole-body and skeletal muscle growth in juveniles and young adult mice. After only 2 weeks of treatment, whole body was reduced by approximately 20 % (Fig. 1). Our results are similar to a number of previous investigations, which found that a 1–2 % dietary supplementation of β GPA could reduce whole-body mass in rats [4, 10, 11, 17, 21, 22]. Our results are consistent with previous investigations which also found that dietary β GPA supplementation could lead to reduction of skeletal muscle mass by reducing the growth of both slow- and fast-twitch skeletal muscle fibers [33–38] and that the effects on body mass were not strictly related to the enhanced oxidation of fat. In our study, the mass of the predominantly slow-twitch, highly aerobic soleus was reduced in β GPA-fed versus control mice at 2 weeks, but was not significantly different at any other time point (Fig. 1). In contrast, the predominantly fast-twitch, anaerobic gastrocnemius was not significantly reduced until 4 weeks; however, it was significantly reduced at every subsequent time point (Fig. 1). Additionally, the mass of the fast-twitch, anaerobic EDL was only significantly reduced at the final (8-week) time point (Fig. 1). However, our data suggest that the time-dependent effect of feeding juvenile and young adult mice β GPA is more complex than a simple reduction in whole-animal skeletal muscle growth. Given that we did not analyze changes in AMPK, mTORC1, and ubiquitin proteasome-dependent signaling pathways in the soleus and EDL of either experimental group, it is difficult to speculate as to why the mass of these very different types of muscles changed only at the 2-week and 8-week time points, respectively. However, by simply examining the time-dependent changes in the mass of specific types of skeletal muscle, it appears that β GPA feeding had a more profound affect

on the growth of slow-twitch, aerobic muscle fibers in juveniles, while it primarily affected the growth of muscles containing predominantly fast-twitch, anaerobic fibers as the animals approached early adulthood. As stated earlier, the changes in whole-body and gastrocnemius mass tracked very similarly over time. Given this and that the gastrocnemius is larger than the EDL and soleus, and is composed of both fast- and slow-twitch muscle fibers, we chose to focus on the timing of the changes in both anabolic and catabolic signaling pathways in the gastrocnemius of control versus β GPA-fed mice.

We found a significant increase in AMPK activation at 2 weeks (Fig. 2a) that corresponded with the reduction in body and muscle mass (Fig. 1). This suggests that rapidly growing juvenile mice may be particularly susceptible to the inhibitory effects on growth of increased AMPK activation. The lack of a significant difference in AMPK activation at 4 and 8 weeks is consistent with an AMPK-mediated restoration of cellular energy state by, for example, increasing muscle aerobic capacity [4, 13]. β GPA feeding led to a decrease in S6K1 phosphorylation at 4 weeks, which is suggestive of a reduction in protein synthesis, but not at 2 weeks when AMPK was active (Fig. 2d). Thus, our results indicate that β GPA feeding led to a reduction in mTORC1 activation at 4 weeks that was not associated with increased AMPK activity. However, the phosphorylation of ACC, a direct target of AMPK, was significantly increased at 4 weeks (Fig. 1b). The increase in ACC phosphorylation at 4 weeks may be indicative of a significant increase in fatty acid and overall aerobic metabolism in the gastrocnemius of β GPA-fed mice. This conclusion is supported by a previous investigation, which demonstrated that exercise can promote an ACC-dependent increase in fatty oxidation in skeletal muscle of AMPK knockout mice [39]. It is also notable that multiple markers of protein degradation, including MAFbx (but not MuRF1), were significantly increased at 8 weeks. Previous investigations have found that conditions such as sepsis [40, 41] and chronic obstructive pulmonary disease (COPD) [42] can promote muscle atrophy due to increased MAFbx expression without affecting levels of MuRF1. In addition, muscle wasting due to HIV was directly linked to increased MAFbx, but not MuRF1, expression in the skeletal muscle of infected rats. Similarly, our data suggest that β GPA feeding lead to increased ubiquitin-dependent protein degradation that was due, at least in part, to increased MAFbx, but not MuRF1, expression in the gastrocnemius (Fig. 4). The increase in MAFbx protein expression was consistent with the significant increase in total protein ubiquitination in the gastrocnemius of β GPA-fed mice at 8 weeks (Fig. 4c). In addition, the significant decrease in total protein carbonylation in the gastrocnemius of β GPA-supplemented mice is consistent with increased ubiquitin-dependent protein degradation at 8 weeks. The ubiquitin–proteasome system is the dominant means of removing oxidatively damaged carbonylated proteins in mammalian skeletal muscle [43].

Our results therefore indicate that in a normal juvenile mouse treated with β GPA there is decreased protein synthesis and increased degradation as expected. However, many of the effects of β GPA do not coincide with the timing of AMPK activation and mTORC1 inhibition. We propose that the reduced whole-body and skeletal muscle mass at 2 weeks (when AMPK is active but P-S6K1, MAFbx, ubiquitin, and carbonylation are unaltered) is induced primarily by energy allocation being diverted from hypertrophic fiber growth to muscle remodeling, including mitochondrial biogenesis, angiogenesis, and shifts toward more aerobic fiber types. Thus, at the 2-week time point the mTORC1 pathway

“outcompetes” the β GPA-enhanced AMPK pathway, leading to high rates of net protein synthesis, but much of that synthetic effort is not directed to muscle hypertrophy. At 4 weeks, as growth rate slows somewhat, the situation reverses as signs of reduced protein synthesis (S6K1) and increased degradation (MAFbx) appear, although AMPK activation is no longer elevated. However, the fact that the AMPK target ACC remained elevated at 4 weeks may indicate that residual effects of AMPK activation remained for some time after AMPK phosphorylation returned to control levels. The apparent increase in protein turnover seen at 8 weeks likely results from the higher rates of protein turnover found in more aerobic muscle [44, 45], which begins to appear after extended treatment with β GPA [21, 22].

Even though it remains unclear if dietary β GPA supplementation can significantly affect whole-body and skeletal muscle growth in children and young adults, the current study presents evidence that long-term use of AMPK-activating supplements, such as β GPA, may increase protein catabolism and thus limit anabolic growth of skeletal muscle.

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Fig. 1.

The effect of dietary β GPA supplementation on whole-body and skeletal muscle growth in juvenile CD1 mice ($n = 6$). Dietary β GPA supplementation significantly reduced whole-body (a) and gastrocnemius mass (b) in a similar manner. β GPA feeding promoted reduced soleus mass (c) at 2 weeks and reduced EDL mass (d) at 8 weeks (* $P < 0.05$)

Fig. 2.

The effect of dietary β GPA supplementation on AMPK signaling in the gastrocnemius of juvenile CD1 mice. **a** Dietary β GPA supplementation promoted increased phosphorylation of AMPK (T172) at 2 weeks when normalized to total AMPK protein levels. **b** β GPA feeding increased ACC (S79) phosphorylation at all three time points when normalized to total ACC proteins levels, though the difference was statistically significant at 4 weeks (* $P < 0.05$)

Fig. 3.

The effect of dietary βGPA supplementation on mTORC1 signaling in the gastrocnemius of juvenile CD1 mice. **a** βGPA feeding promoted reduced S6K1 (T389) phosphorylation at 4 weeks **b** but had no significant effect on 4EBP1 (T37/46) phosphorylation at any time point (* $P < 0.05$)

Fig. 4.

The effect of dietary β GPA supplementation on markers of ubiquitin-dependent protein degradation in the gastrocnemius of CD1 mice. **a** β GPA feeding had no significant effect on MuRF1 protein levels at any time point. **b** Dietary β GPA supplementation increased MAFbx protein levels at 4 and 8 weeks, though the difference was only statistically significant at 8 weeks. **c** β GPA feeding significantly increased total protein ubiquitination levels at 8 weeks while **d** significantly reducing total protein carbonylation levels at 8 weeks (* $P < 0.05$)