Mechanisms involved in the Enhancement of Mammalian Target of Rapamycin Signalling and Hypertrophy in Skeletal Muscle of Myostatin-deficient Mice

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Abstract

Myostatin deficiency leads to both an increased rate of protein synthesis and skeletal muscle hypertrophy. However, the mechanisms involved in mediating these effects are not yet fully understood. Here, we demonstrate that genetic loss of myostatin leads to enhanced muscle expression of both PKB and mTOR/S6K signalling components, consistent with their elevated activity. This is associated with a reduction in the expression of PGC1α and COXIV, proteins which play important roles in maintaining mitochondrial function. Furthermore, we show that these changes in signalling and protein expression are largely independent of alterations in intramuscular amino acid content. Our findings, therefore, reveal potential new mechanisms and further contribute to our understanding of myostatin-regulated skeletal muscle growth and function.

Keywords

growth differentiation factor-8 (GDF-8); mTOR; p70S6K1; PKB/Akt

1. Introduction

Muscle atrophy, or a loss in muscle mass, can occur in response to a variety of different conditions or metabolic disorders such as aging, impaired respiratory function, cancer and diabetes [1-4]. Among the unfavourable effects that may arise from a reduction in skeletal muscle mass include abated physical strength and mobility, as well as the development of insulin resistance due to its role as a metabolically active tissue.

Myostatin, or growth differentiation factor-8 (GDF-8), is a member of the transforming growth factor-β (TGF-β) family of related proteins which acts to negatively regulate skeletal muscle growth. This inhibitory effect has been demonstrated mainly through its genetic loss or

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mutation in a number of different species including humans [5-8]. More specifically, myostatin regulates the number of muscle fibres by inhibiting myogenic proliferation and differentiation, effects which result from its binding and signalling through cell-bound activin type II receptors expressed in this tissue [9-14].

Exposure of differentiated myotubes to growth factors such as insulin-like growth factor-I (IGF-I) leads to a hypertrophic response, characterised by an increase in myotube diameter and elevated rate of protein synthesis [15,16]. Importantly, the induction of protein synthesis by activation of PKB accompanied by mammalian target of rapamycin (mTOR)-mediated signalling is sufficient to promote hypertrophy in skeletal muscle both *in vitro* and *in vivo* [16,17].

mTOR, a key integrator of nutrient and growth factor signals, regulates protein synthesis by phosphorylating both p70 S6 kinase (S6K) and translational initiation factor 4E-binding protein-1 (4E-BP1), respectively leading to their activation and inhibition. A number of studies have demonstrated that myostatin acts as a negative regulator of mTOR-directed signalling [11,13,18-21], consistent with its inhibitory effect on protein synthesis, although the mechanisms by which it does so are not fully understood. Here, we provide further insight into the myostatin-mediated regulation of skeletal muscle mass by showing that genetic loss of myostatin leads to the upregulation of PKB expression and that of mTOR/S6K signalling components, namely S6K and its downstream target ribosomal S6 protein (rpS6), concomitant with an observed increase in their phosphorylation. Furthermore, we demonstrate this response occurs largely in the absence of any significant change in intramuscular free amino acid content.

In addition, both PKB and mTOR have been implicated in the regulation of mitochondrial function. However, whereas active PKB promotes the downregulation in expression of peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α), a transcription coactivator involved in regulating mitochondrial biogenesis [22], pharmacological inhibition of mTOR by rapamycin has been shown to suppress transcription of genes involved in mitochondrial oxidative function [23]. Therefore, we determined the expression of two key proteins important for mitochondrial oxidative function, namely PGC-1α and COX IV. We demonstrate here markedly reduced expression of both proteins under circumstances when PKB and mTOR/S6K signalling are simultaneously elevated in response to myostatin deficiency. Our observations are fully consistent with a switch towards an increased proportion of fast-twitch glycolytic type muscle fibres and also provide a possible explanation for the associated changes in protein synthesis, muscle mass and nutrient metabolism in skeletal muscle of myostatin deficient animals.

2. Materials and Methods

2.1 Materials

Antibodies against PKB, phospho-PKB-Ser473, phospho-p70S6K-Thr389, phospho-p44-42 MAPK-Thr202/Tyr204, p44-42 MAPK, phospho-S6-Ser240/244 and S6 were all from New England Biolabs (Beverley, MA). GAPDH antibody was from Sigma (Poole, UK). Antibody against COX IV was from Invitrogen (Madison, WI). PGC-1α antibody was purchased from Calbiochem (La Jolla, CA). HRP (horseradish peroxidase)-conjugated anti-(rabbit IgG) and anti-(mouse IgG) were obtained from New England Biolabs (Beverley, MA). All other chemicals were from Sigma-Aldrich unless otherwise stated.

2.2 Generation of Myostatin-deficient Mice

Animal studies were performed in accordance with the guidelines of the NIH Animal Care and Use Committee. Mice carrying a targeted mutation in the myostatin gene (MSTN-KO) [6] were

produced from matings between heterozygotes that had been backcrossed 6 times into the C57BL/6 genetic background and genotyped as described [24]. Only male mice aged between 30 and 32 weeks were used to obtain all data presented.

2.3 Western Blot Analysis

Gastrocnemius muscle was isolated from mice, snap frozen in liquid nitrogen and homogenized in lysis buffer [50 mM Tris/HCl (pH 7.4), 0.27 M sucrose, 1 mM sodium orthovanodate, 1 mM EDTA, 1 mM EGTA, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1% (v/v) Triton X-100, 0.1% 2-mercaptoethanol and protease inhibitors]. Cell lysates (40 μg) were subjected to SDS/PAGE on a 10% resolving gel and immunoblotted as previously described [25]. Immobilon-P membranes (Millipore, Bedford, MA) were probed with primary antibodies as indicated in the figure legends. Primary antibody detection was performed with the appropriate HRP (horseradish peroxidase)-conjugated anti-rabbit or anti-mouse IgG and resulting signals visualized using enhanced chemiluminescence by exposure to Konica Minolta X-ray autoradiographic film.

2.4 Amino Acid Analysis by HPLC

Muscle extracts were prepared for and analysed using HPLC as previously described [26]. Briefly, 20 mg of muscle tissue was homogenized in 12% PCA followed by derivatization using a mixture of ethanol, dH_2O, TEA and phenylisothiocyanate (PITC) in a 7:1:1:1 ratio. The resulting phenylthiocarbamyl peptides were separated by a Hewlett Packard 1050 HPLC system (Minnesota, USA) using standard protocols. Comparison of retention times using amino acid standards was used to identify individual amino acids together with relative changes in peak size to measure their abundance.

2.5 Statistical Analysis

Statistical significance was determined by one way analysis of variance (ANOVA) using GraphPad Prism software. Data was considered statistically significant at P-values < 0.05.

3. Results and Discussion

3.1 Elevated levels of PKB and mTOR/S6K signalling components present in skeletal muscle of myostatin-deficient mice

Activation of both the PI3K/PKB and mTOR/S6K pathways have been implicated as important signalling events involved in mediating increases in skeletal muscle mass, primarily by activating important downstream targets responsible for protein translation initiation and protein synthesis [15,27]. Previous studies have established that inhibiting myostatin function may lead to increased myofibrillar protein synthesis and skeletal muscle hypertrophy [5,6, 28-30]. To investigate the mechanism(s) by which myostatin acts to regulate these processes, we assessed the expression and phosphorylation status of PKB and components of the mTOR/ S6K pathway in myostatin-deficient mice (MSTN-KO). In comparison to wild type littermate control (WT) mice, PKB expression is significantly elevated by 1.8-fold along with a significant increase in PKB activity in the gastrocnemius muscle of MSTN-KO mice (Fig. 1). This is consistent with a previous study by Morissette et al. demonstrating a similar increase in PKB activity in accordance with elevated mRNA and protein levels of PKB in this same animal model [31]. However, in contrast to that study, we also observe a marked increase (both around 3-fold) in ribosomal protein S6 (rpS6) expression and that of its upstream kinase, S6K, thereby providing a likely explanation for the elevated levels of phosphorylated rpS6 at Ser240/244 in the MSTN-KOs (Fig. 1). It is unclear what may account for the observed differences in these studies, but it is plausible that differences in animal age as well as use of different muscle types may account for these contrasting findings. Our study used

gastrocnemius muscle extracts from mice of 30-32 weeks of age, whereas Morissette et al. used quadriceps muscle from much younger animals (8-20 weeks of age). Therefore, further work will be required to establish whether distinct mechanisms underlie the development of hypertrophy with age in different muscle types.

The upregulation of PKB/mTOR/S6K signalling in MSTN-KO mice is consistent with the reciprocal down-regulation of these proteins by myostatin and would, at least in part, explain the increase in protein synthesis and muscle hypertrophy observed in these animals. However, this increase in protein expression is not all-inclusive as, for instance, the levels of GAPDH remain unaltered whilst those of ERK1/2 actually significantly decrease (1.7-fold) in the MSTN-KOs (Fig. 1). The reduction in total ERK1/2 expression is also reflected by a lower level of its phosphorylation although the difference did not reach statistical significance in this case. This is a surprising observation given the role of ERK1/2 both as a key transcriptional regulator and in promoting cellular growth, whereby it has been shown to positively regulate protein synthesis [32,33]. Our data, therefore, suggests that inhibiting myostatin function does not promote growth through the elevation of ERK1/2 activity. This observation is, however, consistent with a previous study demonstrating that myostatin can activate rather than inhibit the Ras/Raf/ERK1/2-cascade in C2C12 myoblasts and, in doing so, implicates a role for this pathway in myostatin-mediated inhibition of muscle cell growth and differentiation [34]. Indeed, this may also be part of a counter-regulatory response to accelerated muscle growth. In addition to these changes in PKB/mTOR/S6K signalling components, we cannot exclude the possibility that autocrine production of upstream regulators such as muscle-derived growth factors may also be co-ordinately regulated. However, we observe no significant difference in the expression of mechano growth factor (MGF or IGF-1Eb in rodents) in muscle obtained from WT and MSTN-KO mice at the level of mRNA (data not shown), consistent with previous studies showing little change or even a slight decrease in IGF-1 expression with myostatin-deficiency [35-37]. On the other hand, these same studies demonstrate significant increases in IGF-2, a pro-myogenic growth factor, in response to loss of myostatin function [35-37]. Therefore, additional work will be required to determine the individual contribution of such upstream regulators upon cellular signalling and hypertrophy resulting from myostatin-deficiency.

What purpose may regulation of PKB/mTOR/S6K signalling by myostatin serve? Firstly, although induction of PKB/mTOR/S6K signalling promotes muscle hypertrophy, it has been shown that activation of PKB by either insulin or growth factors alone is not sufficient to induce a hypertrophic response even in the presence of amino acids [38]. In addition, inhibition of mTOR activity does not completely block hypertrophy in response to loss of myostatin function [30,31]. This therefore suggests that PKB and mTOR/S6K may, at least to some extent, act synergistically to promote a full hypertrophic response and that the induction of both PKB and S6K/rpS6 expression and activity may be required to convey the MSTN-KO phenotype. Secondly, as well as being important for the modulation of protein synthesis through effects on protein translation, it has also recently been shown that S6K1 can directly phosphorylate Rictor, a component of mTOR complex-2 (mTORC2) [39]. Although the phosphorylation of Rictor by S6K1 does not lead to major changes in mTORC2-kinase activity, it can alter interactions of this complex with other proteins including 14-3-3 proteins. Therefore, upregulation of S6K expression and activity can itself act as an additional signalling input into mTORC2 by potentially altering its activity towards other target proteins that may be involved in the hypertrophic response.

With regards to PKB, it is now well established that it can induce protein synthesis by phosphorylating and inhibiting the tuberous sclerosis (TSC1/TCS2) complex, a negative regulator of mTOR [40]. In addition, PKB also phosphorylates PRAS40 (proline-rich Akt substrate-40) [41]. Although the exact function(s) of PRAS40 is still unknown, it has been

shown to bind mTOR, via Raptor, leading to the suppression of mTOR activity [42,43]. Therefore, in both these cases, elevated PKB activity would further contribute towards induced mTOR/S6K signalling.

Furthermore, PKB also has an important role in the regulation of protein degradation. One such target of PKB in this process are the forkhead box O (FoxO) transcription factors. PKB phosphorylates FoxO proteins preventing their translocation into the nucleus, thereby suppressing their transcriptional activity towards genes encoding the E3 ubiquitin ligases MuRF1 and MAFbx, both of which mediate ubiquitin-mediated proteasomal degradation and are thought to be involved in the development of muscle atrophy [44]. In the case of MSTN-KO mice, the higher levels of PKB activity would be expected to suppress transcription of these atrogenes, although surprisingly, a previous study has shown that their levels either remain unaltered or are actually increased in myostatin-deficient mice, possibly representing a counter-regulatory response to an increase in muscle mass [31]. Consistent with this, although overexpressing myostatin causes skeletal muscle atrophy it does not appear to alter activity of the ubiquitin-proteasome pathway [19]. Likewise, exposing cultured myotubes to myostatin results in reduced protein synthesis but has no significant effect on the rate of protein degradation [10]. This therefore raises the issue as to whether manipulating myostatin function is itself sufficient to modulate proteasomal-mediated degradation, or whether there may be additional factors involved that it may act in conjunction with to mediate its effects. Also, we cannot exclude the possibility that myostatin acts to regulate other proteolytic mechanisms (e.g. lysosmal protein degradation) *in vivo*.

3.2 Myostatin modulation of amino acid composition in skeletal muscle

Due to the marked effects of myostatin deficiency upon muscle mass and signalling involved in regulating protein synthesis, we wanted to further explore the possibility that loss of myostatin function may also cause enhanced muscle growth by modulating amino acid composition within this tissue. To do this, gastrocnemius muscle was isolated from five wild type and five MSTN-KO non-fasted mice, and then subjected to HPLC analysis in order to compare the relative abundance of the 12 amino acids indicated in Fig. 2. By this analysis, despite the increase in mean gastrocnemius muscle mass (70% from 156 mg to 265 mg) and corresponding overall intramuscular amino acid pool size in MSTN-KOs, there was little difference in total intramuscular amino acid content between MSTN-KO and WT mice when expressed as an amount per mg of muscle tissue (Fig. 2A). This therefore suggests that enhanced muscle growth and changes in myocellular signalling observed in MSTN-KO mice occur independently of total intramuscular amino acid concentration.

However, three out of the 12 amino acids analysed were found to have their concentrations significantly altered in response to myostatin deficiency, with levels of isoleucine increased by 56% and those of aspartate and glycine reduced and increased by around 40% respectively (Fig. 2B). Isoleucine, a branched amino acid, has previously been shown to activate S6K and regulators of mRNA translation in skeletal muscle [45,46]. Therefore, its elevated levels in MSTN-KO mice may contribute towards enhanced protein synthesis and the hypertrophic phenotype of these animals. In addition, administration of isoleucine has also been demonstrated to convey a hypoglycaemic effect involving increased glucose uptake into skeletal muscle – one of the phenotypic attributes of MSTN-KO mice [47,48]. The fact that glycine is selectively upregulated in MSTN-KO mice is surprising but a previous study has shown that administering glycine can counteract myofibrillar proteolysis by downregulating atrophic and proteolytic-related genes [49]. Therefore, it is possible that this increase in muscle glycine content may further augment the hypertrophic response by suppressing proteolysis.

3.3 Myostatin deficiency reduces oxidative capacity in skeletal muscle

The multifarious functions of skeletal muscle arise from differences in fibre type distribution which is primarily responsible for the metabolic diversity found in this tissue. This variation in metabolic makeup can be further altered under different pathophysiological conditions. For instance, factors such as physical inactivity and insulin resistance all coincide with an overall reduced oxidative capacity [50,51]. In contrast, increased physical activity, associated with improved insulin sensitivity, elevates expression of oxidative enzymes whilst reducing those with glycolytic activity [50-52]. Therefore, we wanted to determine whether myostatin deficiency alters the expression of peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC-1α), a key regulator of mitochondrial biogenesis, and of cytochrome c oxidase IV (COX IV), the terminal enzyme in the electron transport chain in mitochondria.

As shown in Fig. 3, the expression of both PGC-1α and COX IV is significantly reduced by 3 and 2.2-fold respectively in the gastrocnemius muscle of MSTN-KO compared to wild type mice, indicative of a reduction in oxidative capacity. Therefore, interestingly, our data suggests that loss of myostatin function leads to a fibre type distribution more characteristic of an insulin resistant state, despite myostatin-deficient mice displaying increased insulin sensitivity and resistance to diet-induced obesity [48,51,53]. However, our observations are consistent with previous work demonstrating that genetic loss of myostatin promotes a shift towards a fast glycolytic phenotype [36,54-56]. In addition, the downregulation of PGC-1α and COX IV expression is in accordance with reduced lipid oxidation in skeletal muscle of MSTN-KO mice [48,57,58]. Further work will be required to determine whether the loss in expression of these mitochondrial markers is simply due to the lower proportion of oxidative Type I fibres present or if more acute inhibition of myostatin signalling, perhaps in cultured myotubes, can mimic this response. Again, the question remains as to whether the loss of myostatin signalling is directly causing the fibre type switch or if this is part of a feedback response to accelerated muscle growth.

In conclusion, maintenance of skeletal muscle mass and function is crucial for proper regulation of blood glucose levels and lipid metabolism. Importantly, myostatin plasma levels and expression in muscle have been shown to be elevated in obesity and with progressive aging [59-61]. In accordance with this, recent studies have shown that inhibition of myostatin signalling either by the development of pharmacological inhibitors or otherwise may prove to be beneficial in the treatment of metabolic disorders such as Type II diabetes or for increasing skeletal muscle mass in patients with muscle-wasting diseases [48,62,63]. The findings presented in this study demonstrate some degree of selectivity by which such a course of treatment may have upon cellular signalling, although further work will be required to fully elucidate the consequences of modulating myostatin function upon other signalling systems. Only then can a more comprehensive understanding of its downstream effects and therefore its viability as a potential therapeutic target be acquired.

Acknowledgments

Abbreviations

PKB	protein kinase B
mTOR	mammalian target of rapamycin
GDF-8	growth differentiation factor-8

TGF-β	transforming growth factor -β
4E-BP1	eukaryotic translation initiation factor 4E binding protein 1
rpS6	ribosomal protein S6
COX IV	cytochrome c oxidase subunit IV
S6K	p70 ribosomal protein S6 kinase
TSC	tuberous sclerosis complex
PRAS40	proline-rich Akt substrate
OXPHOS	oxidative phosphorylation
MuRF1	muscle RING finger protein-1
MAFbx	muscle atrophy F-box

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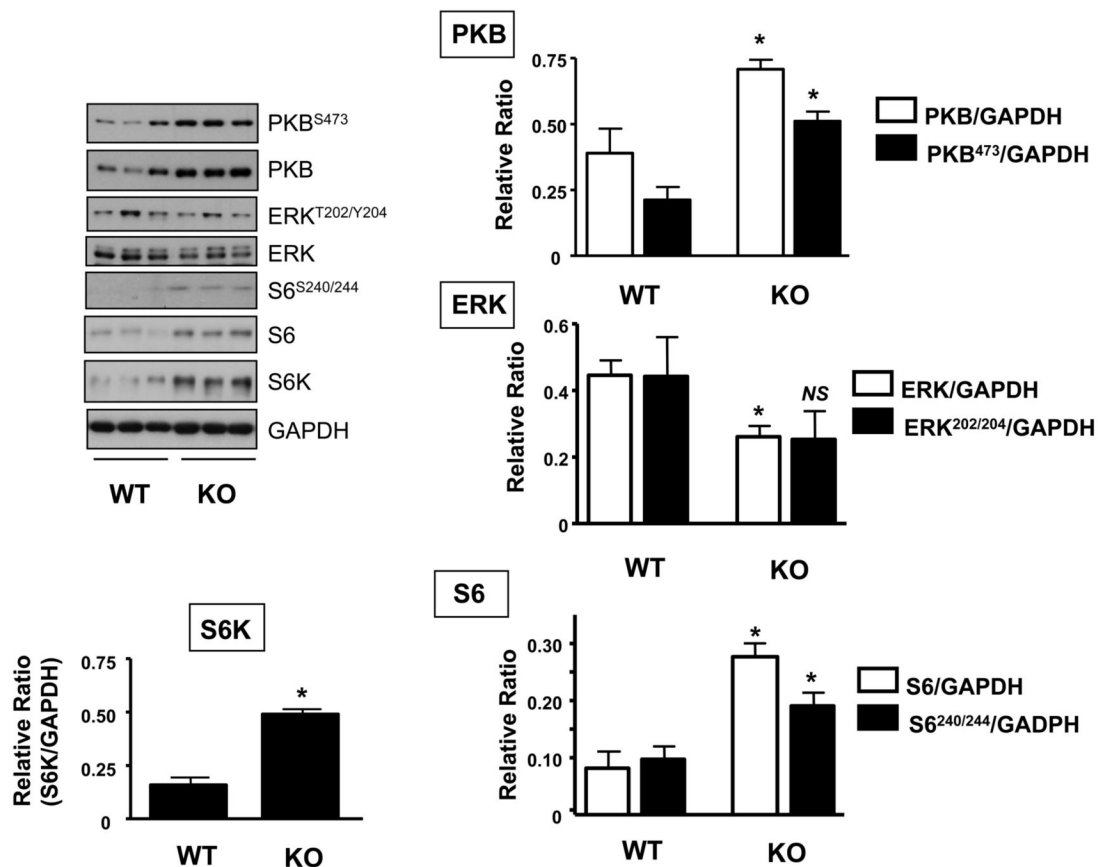
Figure 1. Effect of myostatin deficiency on PKB, S6K and ERK1/2 protein expression and activity in skeletal muscle

Gastrocnemius muscle was isolated from wild type control (WT) or homozygous myostatin knockout (MSTN-KO) mice and resulting tissue extracts immunoblotted using the indicated antibodies. Representative immunoblots and corresponding quantifications are shown. Each bar represents the mean ± S.E.M. for four individual animals. Asterisks indicate statistically significant differences versus wild type (WT) animals. *, P < 0.05; NS, not significant.
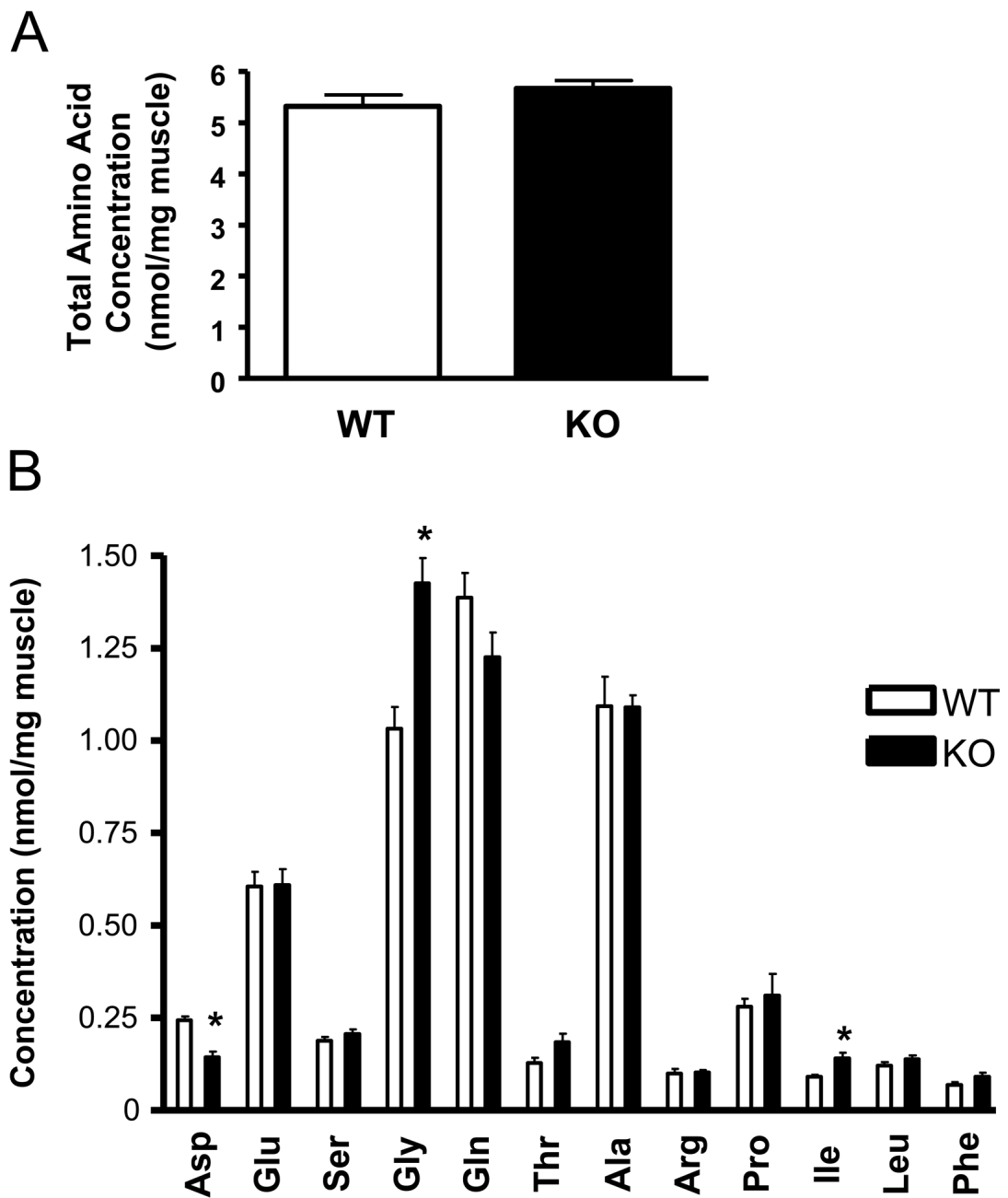
A

B

Figure 2. Comparison of amino acid profiles of gastrocnemius muscle obtained from wild type and myostatin-deficient mice
Gastrocnemius muscle isolated from individual wild type (WT) and myostatin deficient (KO) mice was prepared for and subjected to HPLC analysis in order to determine; (A) the total content of amino acids or (B) the concentration of the individual amino acids indicated. Data are presented as mean ± S.E.M. (n = 5 animals group). *, P < 0.05.
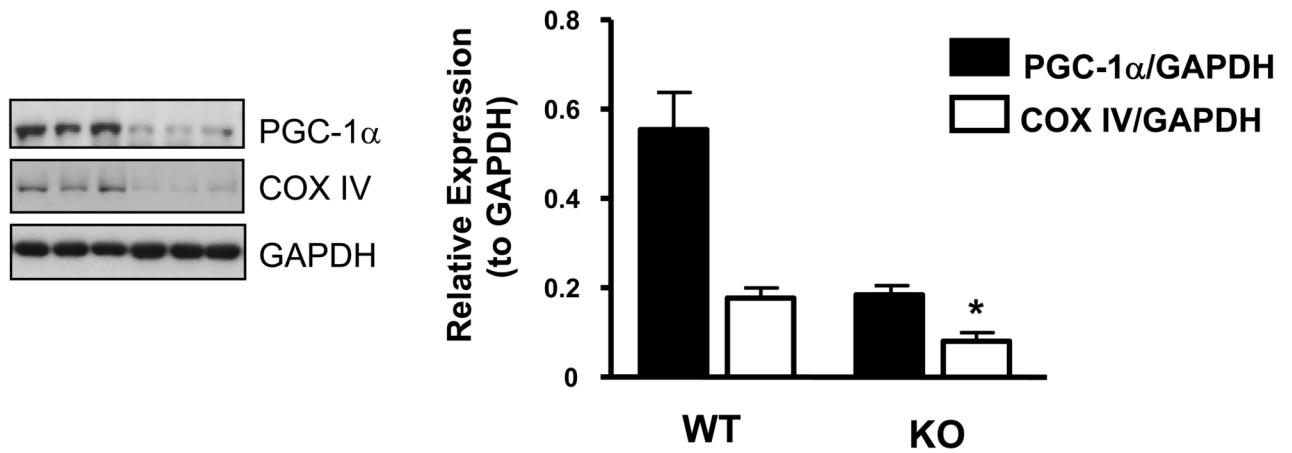
Figure 3. Effect of myostatin deficiency on PGC-1α and COX IV protein expression in skeletal muscle
Gastrocnemius muscle was isolated from wild type control (WT) or homozygous myostatin knockout (KO) mice and resulting tissue extracts immunoblotted for PGC-1α and COX IV. Representative immunoblots and corresponding quantifications are shown. Each bar represents the mean ± S.E.M. for three individual animals. Asterisks indicate statistically significant differences versus wild type (WT) animals. *, $P < 0.05$.