

Leucine minimizes denervation-induced skeletal muscle atrophy of rats through akt/mtor signaling pathways

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The aim of the present study was to evaluate the effect of leucine treatment (0.30 mM) on muscle weight and signaling of myoproteins related to synthesis and degradation pathways of soleus muscle following seven days of complete sciatic nerve lesion. Wistar rats ($n = 24$) of 3–4 months of age (192 ± 23 g) were used. The animals were randomly distributed into four experimental groups ($n = 6$ /group): control, treated with leucine (L), denervated (D) and denervated treated with leucine (DL). Dependent measures were proteins levels of AKT, AMPK, mTOR, and ACC performed by Western blot. Leucine induced a reduction in the phosphorylation of AMPK ($p < 0.05$) by 16% in the L and by 68% in the DL groups as compared with control group. Denervation increased AMPK by 24% in the D group as compared with the control group ($p < 0.05$). AKT was also modulated by denervation and leucine treatment, highlighted by the elevation of AKT phosphorylation in the D (65%), L (98%) and DL (146%) groups as compared with the control group ($p < 0.05$). AKT phosphorylation was 49% higher in the D group as compared with the DL group. Furthermore, denervation decreased mTOR phosphorylation by 29% in the D group as compared with the control group. However, leucine treatment induced an increase of 49% in the phosphorylation of mTOR in the L group as compared with the control group, and an increase of 154% in the DL as compared with the D group ($p < 0.05$). ACC phosphorylation was 20% greater in the D group than the control group. Furthermore, ACC in the soleus was 22% lower in the L group and 50% lower in the DL group than the respective control group ($p < 0.05$). In conclusion, leucine treatment minimized the deleterious effects of denervation on rat soleus muscle by increasing anabolic (AKT and mTOR) and decreasing catabolic (AMPK) pathways. These results may be interesting for muscle recovery following acute denervation, which may contribute to musculoskeletal rehabilitation after denervation.

Keywords: denervation, leucine, muscle atrophy, synthesis pathways, degradation pathways, rehabilitation

and then heated for 5–10 min. Equal quantities of proteins (75 µg) were submitted to eletroforesis in poliacrilamide SDS-PAGE gels at the BIO-RAD miniature slab gel apparatus (Mini-Protean, Bio-Rad Laboratories, Richmond, CA). The electrotransfer of gel proteins to the membrane lasted 120 min at 120 V in a miniaturized transference device (BIO-RAD). The binding of the antibodies to non-specific proteins was reduced by the pre-incubation of the membrane with blocking buffer under room temperature (5% BSA dissolved in basal solution) to 120 min. The nitrocellulose membrane was incubated, overnight, with specific antibodies: anti-pSer473AKT (#4058), anti-pAMPK (#2537), anti-pmTOR (#2971), and anti-pACC (#3661) obtained from Cell Signaling Technology (Beverly, MA), diluted in antibody solution (3% BSA); and then washed for 15 min with basal solution (150 mM NaCl, 10 mM Tris base and 0.02% of Tween 20). Detection of the antigen-antibody complex attached to the nitrocellulose membrane was determined via chemiluminescence by using a commercial kit from Amersham and following recommendations from the manufacturer. β -Actin was used as the internal control. Following the revelation of the autoradiographs, the identified bands were quantified by optical densitometry.

Statistical Analysis

All variables showed normal distribution (Shapiro–Wilk test) and homoscedasticity (Levene test). Thus, Two-Way ANOVA (denervation x treatment) followed by the Tukey multiple paired analysis was used for comparisons among treatments. Differences were considered significant when $P < 0.05$. All data were analyzed using the Statistica 7.0 software package (StatSoft Inc., Tulsa, OK, USA).

Results

There were group \times time interactions showing that denervation and leucine treatment interact with muscle weight ($p < 0.05$). Denervation decreased soleus muscle weight by 35.1% as compared with the control group ($p < 0.05$). Leucine treatment minimized muscle weight loss by 28% in the DL group as compared with the D group ($p < 0.05$). Moreover, soleus muscle weight was

TABLE 1 | Soleus muscle mass/body mass ratio (MM/BM) in the control group (C), treated with leucine (L), denervated (D), and denervated treated with leucine (DL).

Groups	MM/BM
C	0.55 \pm 0.01
L	0.65 \pm 0.01*
D	0.36 \pm 0.04*
DL	0.46 \pm 0.008*#~

Values are mean \pm standard error of the mean (SEM), $n = 6$. * $p < 0.05$ compared to C, # $p < 0.05$ compared to D; ~ $p < 0.05$ compared to L.

17.5% greater in the L group than the control group ($p < 0.05$; **Table 1**).

There were group \times time interactions showing that denervation and leucine treatment interact with to the AMPK, AKT, mTOR, and ACC proteins level. Leucine induced a reduction in the phosphorylation of AMPK ($p < 0.05$) by 16% in the L and by 68% in the DL groups as compared with control group. Denervation increased AMPK by 24% in the D group as compared with the control group ($p < 0.05$; **Figure 1**). AKT was also modulated by denervation and leucine treatment, highlighted by the elevation of AKT phosphorylation in the D (65%), L (98%), and DL (146%) groups as compared with the control group ($p < 0.05$). AKT phosphorylation was 49% higher in the D group as compared with the DL group (**Figure 2**; $p < 0.05$).

Furthermore, denervation decreased mTOR phosphorylation by 29% in the D group as compared with the control group. However, leucine treatment induced an increase of 49% in the phosphorylation of mTOR in the L group as compared with the control group, and an increase of 154% in the DL as compared with the D group (**Figure 3**; $p < 0.05$). ACC phosphorylation was 20% greater in the D group than the control group. Furthermore, ACC in the soleus was 22% lower in the in the L group and 50% lower in the DL group than the respective control group (**Figure 4**; $p < 0.05$).

Discussion

To the best of our knowledge, this is the first study to investigate the effects of leucine treatment following 7 days of denervation on the molecular pathways of muscle protein synthesis and degradation of rats. The initial hypothesis was confirmed, since leucine minimized the early deleterious effects of denervation on the soleus muscle by increasing important molecular pathways

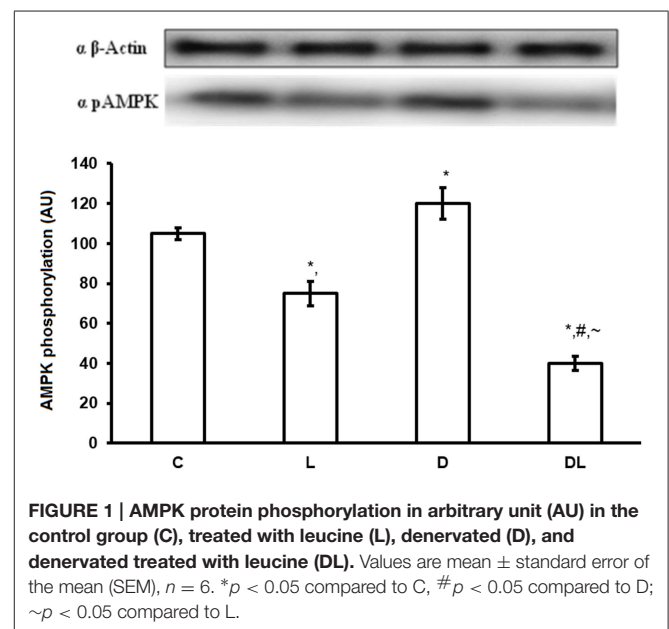
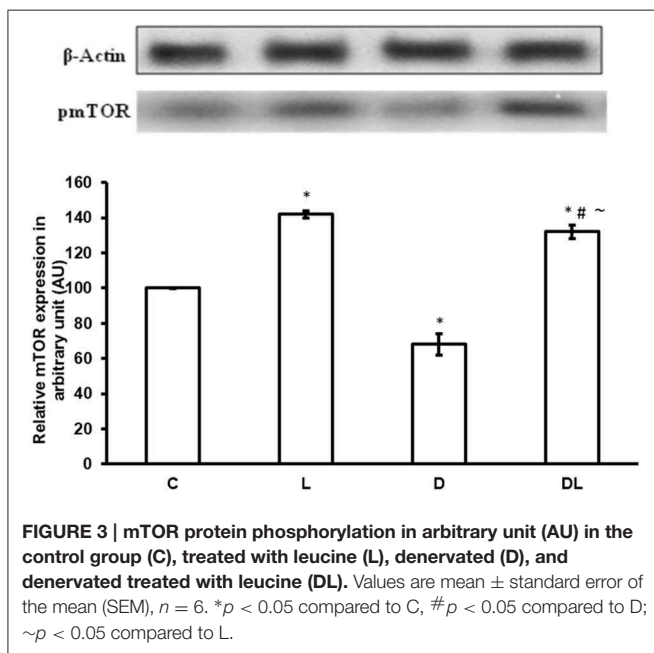
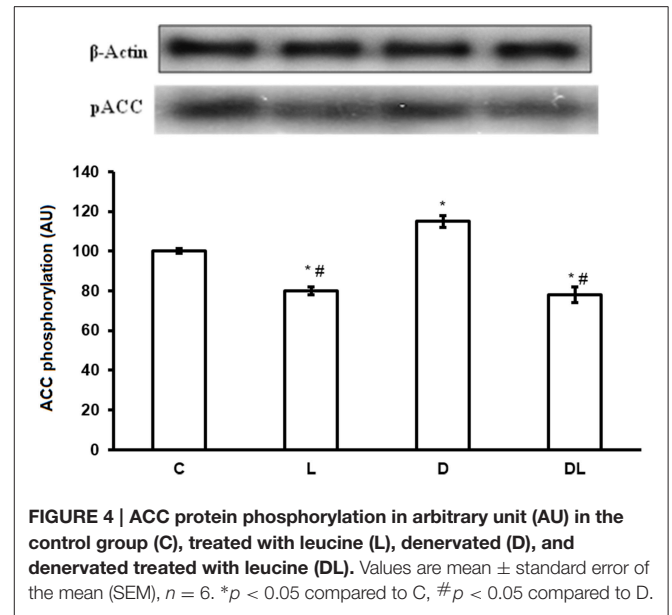
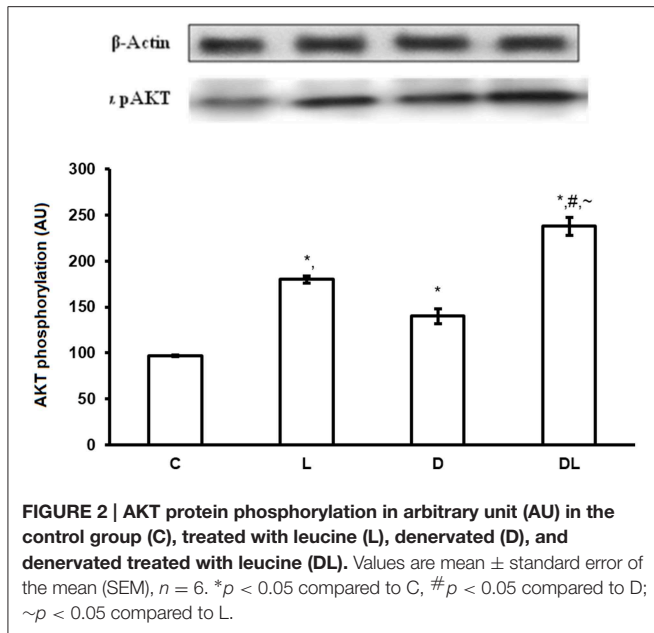


FIGURE 1 | AMPK protein phosphorylation in arbitrary unit (AU) in the control group (C), treated with leucine (L), denervated (D), and denervated treated with leucine (DL). Values are mean \pm standard error of the mean (SEM), $n = 6$. * $p < 0.05$ compared to C, # $p < 0.05$ compared to D; ~ $p < 0.05$ compared to L.



involved with muscle protein synthesis. Although this study was performed in animals, it has clinical relevance and indicates the importance of leucine treatment in the acute phase of muscle atrophy with the attempt to reduce the deleterious effects related to denervated muscles.

Leucine is known to regulate protein turnover of muscle cells, inhibiting protein degradation and promoting protein synthesis (Norton and Layman, 2006; Tom and Nair, 2006; Maki et al., 2012; Kelleher et al., 2013). Among the possible mechanisms involved, leucine can activate protein synthesis in the muscle, heart, liver and kidney by mTOR-dependent pathways (mTOR,

S6K1, and 4E-BP1) and mTOR-independent pathways (Norton and Layman, 2006). Moreover, it has been shown that the ability of leucine to phosphorylate p70^{S6K} (serine/threonine kinase that acts downstream PI3-K pathway) is blocked by rapamycin, an inhibitor of mTOR and phosphatidylinositol 3-kinase (PI3-K) (Tom and Nair, 2006).

Escobar et al. (2006), found that leucine increases the phosphorylation of S6K1 and rpS6 in glycolytic muscles, such as the type II myosin containing latissimus dorsi. Furthermore, the elevation of plasma leucine induced an increase in the phosphorylation of 4E-BP1 at the Thr⁷⁰, with a concomitant decrease of 4E-BP1 and eIF4E complex; and an increase of the eIF4G complex and IF4E in skeletal muscle (Escobar et al., 2006). Similarly, the present study revealed that leucine treatment increased the phosphorylation of AKT, mTOR and reduced ACC in the soleus muscle following 7 days of denervation, indicating the anti-catabolic effect of leucine during denervation-induced atrophy.

Leucine also know to affect the autophagy-lysosome system. Mordier et al. (2000) showed that leucine restriction induces the formation of autophagy and activation of lysosomal-dependent proteolysis in C2C12 myotube. Kadowaki and Kanazawa (2003) suggest that leucine has direct regulatory potential in liver autophagic proteolysis. In addition, a reduction in muscle mass was suppressed by leucine-supplementation (1,5% leucine) in rats fed protein-free diet for 7 days. The results showed that the expression of a very specific marker of autophagen, microtubule-associated protein light chain 3 active form (LC3-II) was significantly decreased, wherein atrogin-1 and MuRF1 were not suppressed. It is suggested that inhibition of autophagy-lysosome is likely the system involved in the suppression of myofibrillar protein degradation by leucine (Sugawara et al., 2009).

Interestingly, our results revealed that denervation decreased mTOR and increased AMPK and ACC, which are activated during energy catabolism, such as decreased ATP levels. It

has been proposed that p70^{S6k} controls AMPK signaling and affects intracellular energetic metabolism, represented by the AMPK/ATP ratio. Although leucine may modulate the AMPK/ATP ratio and mTOR/p70^{S6k} pathways, the increase of AMPK phosphorylation may occur without a relevant modification of muscle energy status (Deshmukh et al., 2009; Iwanaka et al., 2010). This fact may explain the reduction of mTOR signaling and consequently a reduction of muscle atrophy observed in denervated and treated animals.

Furthermore, Filiputti et al. (2010) investigated the islet cells of rats treated with different diets, and found that leucine treatment increased mTOR activation by 2.5 times in a group with diet restriction (composed of 6% of protein) and in the normal diet group. Leucine treatment also minimized the decrease of S6K-1 ribosomal protein induced by protein restriction. These results indicate that the activation of PI-3K and mTOR induced by leucine may be involved in insulin secretion during protein restriction.

In the present study, denervation increased AMP-activated protein kinase expression, indicating that the autophagy protein breakdown system may be involved in muscle atrophy induced by denervation. Along this line, Sugawara et al. (2009) showed that L-leucine reversed a reduction in gastrocnemius muscle weight and reversed increased LC3II expression (an autophagy marker) induced by a protein-free diet for 7 days in rats. The difference between their study and ours is likely due to the differences in the experimental methods. They selected a protein-free diet for an induction of muscle atrophy, which markedly increases autophagy, while we demonstrated, for the first time, the role of AMP-activated protein kinase expression after denervation-induced atrophy. Our results

suggest that leucine treatment may protect muscle mass via the autophagy pathway, and increasing AKT/mTOR signaling pathways.

It is important to consider some limitations of the present study. Movement restriction inherent to the denervation surgery process could exert some influence on the results of the denervation groups, possibly potentiating the effects of disuse atrophy, especially during the first few days of denervation. Future studies should consider a follow up of animals to assess pain and movement restriction during the first days after the denervation process and correlate these findings with muscle disuse adaptation. Moreover, we have a limitation of AKT normalization in the western blotting assay. This may explain the surprising increase of AKT in the phosphorylated states of denervated muscles. Future studies should also include non-phosphorylated forms to normalize the phospho isoforms of AKT and also include different AKT isoforms. Moreover, antiphosphatases inhibitors should be used to prevent degradation of the phosphoisoforms of AKT and mTOR. Muscle cross sectional area analysis should also be included. The results revealed atrophy in the entire soleus muscle, and selective atrophy on different types of muscle fibers could be missed.

Conclusion

In conclusion, leucine treatment minimized the deleterious effects of denervation on rat soleus muscle by increasing anabolic (AKT and mTOR) and decreasing catabolic (AMPK) pathways. These results may be interesting for muscle recovery following acute denervation. Future studies investigating additional molecular pathways, muscle function and morphology should be conducted.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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