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ORIGINAL PAPER

Effects of beta-hydroxy-beta-methylbutyrate (HMB) on the expression of ubiquitin ligases, protein synthesis pathways and contractile function in extensor digitorum longus (EDL) of fed and fasting rats

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Abstract Beta-hydroxy-beta-methylbutyrate (HMB), a leucine metabolite, enhances the gain of skeletal muscle mass by increasing protein synthesis or attenuating protein degradation or both. The aims of this study were to investigate the effect of HMB on molecular factors controlling skeletal muscle protein synthesis and degradation, as well as muscle contractile function, in fed and fasted conditions. Wistar rats were supplied daily with HMB (320 mg/kg body weight diluted in NaCl-0.9%) or vehicle only (control) by gavage for 28 days. After this period, some of the animals were subjected to a 24-h fasting, while others remained in the fed condition. The EDL muscle was then removed, weighed and used to evaluate the genes and proteins involved in protein synthesis (AKT/4E-BP1/S6) and degradation (*Fbxo32* and *Trim63*). A sub-set of rats were used to measure in vivo muscle contractile function. HMB supplementation increased AKT phosphorylation during fasting (three-fold). In the fed condition, no differences were detected in atrogenes expression between control and HMB supplemented group; however, HMB supplementation did attenuate the fasting-induced increase in their expression levels. Fasting animals receiving HMB showed improved sustained tetanic contraction times (one-fold) and an increased muscle to tibia length ratio (1.3-fold), without any cross-sectional area changes. These results suggest that HMB supplementation under fasting conditions increases AKT phosphorylation and attenuates the increased of atrogenes expression, followed by a functional improvement and gain of skeletal muscle weight, suggesting that HMB protects skeletal muscle against the deleterious effects of fasting.

Keywords HMB · Protein synthesis · Protein degradation · EDL · Muscle contraction

Abbreviations

AIDS	Acquired immune deficiency syndrome
ANOVA	Analysis of variance
AU	Arbitrary units
AUC	Area under the curve
CSA	Cross-sectional area
DEXA	Dexametasone
ECL	Enhanced chemiluminescence
EDL	Extensor digitorum longus
FOXO	Forkhead box O
HMB	Beta-hydroxy-beta-methylbutyrate
HRT	Half-relaxation time
LRT	Late-relaxation time
mTOR	Mammalian target of rapamycin
PMSF	Phenylmethanesulfonylfluoride
TTP	Time to peak tension
TSI	Time of sustained isometric contraction

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Table 1 Time parameters and acute fatigue index

Parameter (ms)	Fed:control	Fed:HMB	Fasting:control	Fasting:HMB
TTP	43.106 ± 6026	36.117 ± 3.816	48.435 ± 7.142	37.973 ± 2.200
HRT	24.122 ± 3.370	22.811 ± 2.759	30.415 ± 2.645	24.622 ± 1.071
LRT	17.141 ± 2.765	13.306 ± 1.579	20.608 ± 4.991	13.352 ± 1.896
TSI	5.103 ± 0.613	5.231 ± 0.835	6.032 ± 0.709[a]	9.373 ± 1.334[a,b]

Results are expressed as mean ± SEM of 6 observations

TTP time to peak of contraction, *HRT* half-relaxation time, *LRT* late-relaxation time, *TSI* time of sustained isometric contraction

[a] Effects of fasting ($p < 0.05$)

[b] $p < 0.05$ vs. Fed:Control and Fed: HMB

Introduction

Among nutritional strategies aiming to preserve skeletal muscle mass and contractile function, hydroxy-beta-methylbutyrate (HMB), a leucine derivative metabolite, is recognized as a compound that prevents skeletal muscle atrophy [1–3] and improves muscle function [4]. HMB supplementation in different models of skeletal muscle atrophy prevents proteolysis, mainly through inhibition of catabolic pathways [5, 6]. It also improves sports performance [7, 8].

The skeletal muscle mass is maintained by the balance between protein synthesis and degradation, which is controlled by the interaction of several intracellular pathways [9]. Many pathological and physiological conditions affect the homeostasis of skeletal muscle mass leading to atrophy; i.e. cancer, AIDS, diabetes, aging, fasting, and others [9–11]. However, there is a lack of investigation relating the skeletal muscle atrophy and contractile function. Understanding the mechanisms maintaining skeletal muscle mass and contractile function is important in developing effective strategies to combat and prevent skeletal muscle wasting. These strategies will ameliorate the well being of many patients with chronic diseases as well as improve rehabilitation and training strategies for athletes [3, 5, 8, 12–14].

Fasting is a condition characterized by a simultaneous increase in protein degradation and decrease in protein synthesis, resulting in a loss of skeletal muscle mass. As there is a known positive relationship between skeletal muscle mass and force production, fasting may also impair muscle contractile properties [15, 16]. Since the effect of HMB supplementation on force production and acute fatigue index in fasting is unknown, it seems attractive to investigate this possibility.

The skeletal muscle atrophy during fasting has been ascribed to the increased expression of FBXO32 by Forkhead box O (FOXO) and decreased activity of the PI3K/AKT pathway [9]. However, there is evidence that suggests the involvement of nutrient-sensing pathways, including reduced activation of the mammalian target of rapamycin (mTOR) and, consequently, some of their downstream proteins such as 4EBP1 and S6 [17]. Increased activation of the ubiquitin proteasome system is seen in many skeletal muscle atrophy models. However, there are studies pointing in the other direction [18]. Recently, Bodine et al. [19] showed that FBXO32 and TRIM63 are two ubiquin E3 ligases that actively participate in protein degradation; however, their role in the regulation of skeletal muscle functional properties remains unclear [20].

The present study aimed to investigate the effect of a 29-day HMB supplementation on the molecular mechanisms involved in muscle protein synthesis and degradation and associated contractile function in fed and fasted rats.

Materials and methods

The experimental protocol (#93/02) was approved and follows the ethical principles in animal research adopted by the National Council for the Control of Animal Experimentation of the Institute of Biomedical Sciences/University of São Paulo.

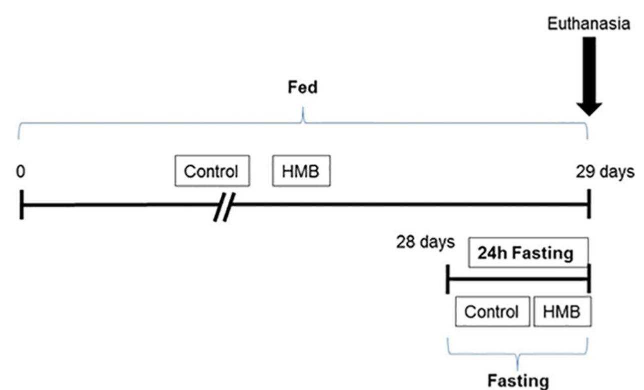

Fig. 1 Experimental design

Animals

Male Wistar rats, of the same age (3 months), weighing 200–250 g were obtained from our own breeding colony provided by the Central Animal Breeding House of the Institute of Biomedical Sciences of the University of São Paulo. The animals were maintained on rat chow, tap water ad libitum and housed in a room kept at a constant temperature (23 ± 1 °C) and on a 12-h light, 12-h dark cycle plan (lights on at 0700 hours).

Experimental design

Animals of the same age (3 months), weighing 200–250 g ($n = 5$–14 animals/group) were supplemented for 29 days with HMB at 320 mg/kg body weight/day (Fed:HMB group) (calcium salt: Laboratory Metabolic Technologies, IA, USA) or vehicle (NaCl 0.9%) (Fed:Control group) by oral gavage, as described [4, 21]. One day before the rats were culled (at day 28), a group of animals from each supplementation group and were fasted for 24 h (Fasting:HMB and Fasting:Control groups) as a stimulus for muscle proteolysis [22]. The remaining rats were kept on rat chow and tap water ad libitum. During the fasting period, the gavage was performed for all groups (Fig. 1).

Tissue samples

The rats were anesthetized with thiopental (6 mg/kg body) and killed by decapitation. The extensor digitorum longus (EDL) muscles were rapidly removed and transversely cut in half. The segment was immersed in cold isopentane for 30 s, cooled in liquid nitrogen and stored at −80 °C for histochemistry, and subsequently morphological analysis was performed, using the eosin–haematoxylin staining protocol, as described [23]. The other segment was snap-frozen in liquid nitrogen and stored at −80 °C for RNA and protein expression analysis. After excision of the muscles, the left tibia was removed and freed from connective tissue. Maximal tibia length was measured to verify the ratio of muscle weight and tibia length [24].

Total mRNA extraction and real-time PCR

Total RNA was extracted from the EDL as described previously [21]. Two micrograms of total RNA were used to synthesize the first strand complementary DNA (cDNA) using oligo-dT primers and the MMLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. The reverse transcription reaction was performed at 70 °C for 10 min, followed by 37 °C for 60 min, and 10 min at 95 °C. Real-time quantitative PCR was performed using the SYBR® Green PCR master mix kit (Applied Biosystems, UK) and the primers for *Fbxo32* (Forward: 5́TACTAAGGAGCGCCATGGATACT3́, Reverse: 5́GTTGAATCTTCTGGAATCCAGGAT3́); *Trim63* (Forward:5́TGACCAAGGAAAACAGCCACCAG3́, Reverse: 5́TCACTCCTTCTTCTCGTCCAGGATGG3́) and *Gapdh* (Forward: 5′GATGGGTGTGAACCACGAGAAA3′, Reverse: 5′ACGGATACATTGGGGGTAGGA3′) as a reference gene. The reaction conditions consisted of two steps at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of three steps: 20 s denaturation at 95 °C, 60 s annealing at 58 °C and 20 s at 72 °C, as described [21]. The relative abundance of *Fbxo32*, *Trim63* and *Gapdh* mRNA was calculated, using the $2^{\Delta\Delta Ct}$ method [25], and the results were expressed in arbitrary units (AU).

Protein extraction and western blotting

EDL was homogenized in a buffer containing NaCl 137 mM, KCl 2.7 mM, $MgCl_2$ 1 mM, Tris pH 7.8 20 mM, EDTA 1 mM, sodium pyrophosphate 5 mM, NaF 10 mM, Triton X-100 1%,glycerol 10%, PMSF (phenylmethanesulfonylfluoride) 0.2 mM; Na_3VO_4 (sodium orthovanadate) 0.5 mM and PIC 1:100 (Protease Inhibitor Cocktail), and centrifuged at 13,400*g* for 40 min at 4 °C [26]. Equal amounts of protein (35 µg) were subjected to electrophoresis and immunoblotted using antibodies for anti-phospho (Ser473) and total-AKT (1:1000; Cell Signaling Technology, MA, USA), anti-phospho (Thr37/46) and total 4EBP1 (1:1000; Cell Signaling Technology) and anti-phospho (Ser240/244) and total S6 (1:1000; Cell Signaling Technology) in 5% BSA/basal solution (100 mM Trizma, pH 7.5; 150 mM NaCl; e 0.05% Tween 20). We used appropriated secondary peroxidase conjugated antibodies for band detection (1:5000; Santa Cruz Biotechnology, Dallas, TX, USA) and the Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Buckinghamshire, UK). Scion Image software was used to analyze the intensity of blots (Scion, Frederick, MD, USA). The Ponceau-stained nitrocellulose membrane was used for normalization, and the results were expressed as arbitrary units (AU).

Analysis of fiber cross-sectional area (CSA)

The morphological analysis of the EDL muscle was performed, using the eosin–haematoxylin staining protocol as described [23, 27].

Analysis of skeletal muscle strength and contractile properties

For this evaluation, both hindlimbs were removed and fixed on an acrylic platform. The stimulated limb was placed on

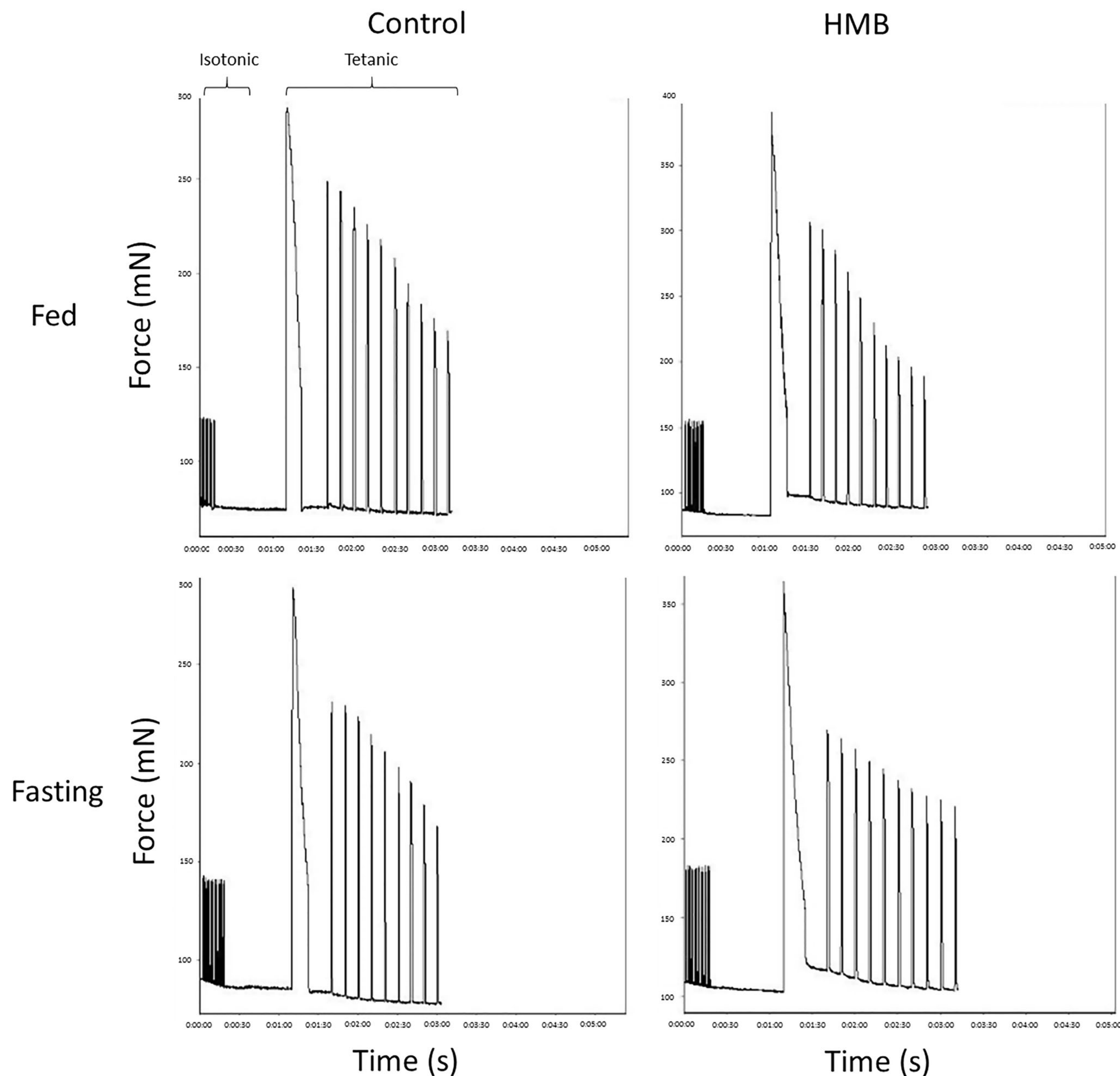

Fig. 2 Myogram representative images. Isotonic contractions were analyzed and are shown in Fig. 6 and time parameters (TTP, HRT, LRT) in Table 1. Tetanic contractions were analyzed and shown in Fig. 7 and times of sustained isometric contraction (TSI) in Table 1

the platform with the hip and knee joint at a 60° angle. A hook was placed under the Achilles tendon and connected to an isometric force transducer (Grass Technologies, West Warwick, RI, USA). The skin was excised and a platinum electrode was placed at the sciatic nerve. The maximum isotonic contractions (muscle twitches) and tetanic force were induced by electrical stimulation at a low (1 Hz) and high (100 Hz) stimulation frequency, respectively [4, 28] (Fig. 2). The muscle strength and contractile properties were analyzed using the AqAnalysis® software (v.4.16; Lynx Tecnologia Eletrônica, São Paulo, Brazil). The twitches time parameters such as time to peak tension (TTP), half-relaxation time (HRT) and late-relaxation time (LRT) were evaluated at 1 Hz as we have described previously [4].

Analysis of acute skeletal muscle fatigue

The acute muscle fatigue was evaluated using the curve decline of the initial tetanic force (100%) during ten successive tetanic contractions, and fatigue index was determined as the area under the muscle tension, as described by

Pinheiro et al. [4]. The time of sustained isometric contraction (TSI) was considered as the maximum time (millisecond) to develop and sustain 100% of the tetanic force.

Statistical analysis

The results are presented as mean ± SEM and analyzed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Two-way ANOVA was used for comparison between the groups, followed by Tukey's post-test. Differences between values were considered statistically significant for $p < 0.05$.

Results

Effect of fasting on morphometric parameters

No differences were detected in muscle to tibia length ratio in the fed condition (control vs. HMB) (Fig. 2). However, as expected, fasting condition reduced the muscle mass around 1.3-fold while HMB supplementation preserved it.

Effect of HMB supplementation on Fbxo32 and Trim63 gene expression

In the fed condition, no differences were detected in atrogenes expression between the control and the HMB-supplemented group. In the fasting condition, the Fbxo32 and Trim63 gene expression of the control groups were increased by approximately 4-fold and 3-fold, respectively. The effect of fasting was attenuated in the HMB-treated group, which presented a reduced expression of Fbxo32 (~50%) and Trim63 (~32%) versus the control group (Fig. 3a, b).

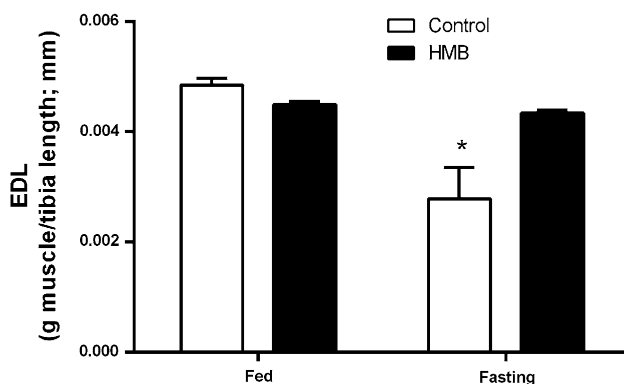

Fig. 3 Weight of EDL muscle; in *white* Fed:Control and Fasting:Control and in *black* Fed:HMB and Fasting:HMB; values are mean ± SEM ($n = 9$–14 animals/group); *asterisk* vs. Fed:HMB; Control ($p < 0.0001$) and Fasting:HMB ($p < 0.001$); effect of supplementation ($p < 0.05$); effect of fasting ($p < 0.0001$) and interaction: supplementation and fasting ($p < 0.001$)

Effect of HMB supplementation on protein synthesis

In fed condition, no differences were observed in the pAKT/tAKT ratio between control and HMB supplemented group. Fasting condition did not alter the pAKT/tAKT ratio in control group. However, this ratio was increased by 3-fold in HMB fasted group, as shown in Fig. 4a. Regarding p4EBP-1/t4EBP-1 ratio, in the fed condition no alterations were detected between HMB and control group. However, in the fasting condition, a significant enhancement of p4EBP-1/t4EBP-1 ratio was detected and this effect was attenuated in HMB supplemented group, as shown in Fig. 4b. Considering pS6/tS6 ratio, no differences were observed among the experimental groups (Fig. 4c).

Effect of HMB supplementation on CSA

No alterations in CSA were observed between groups in fed and fasting conditions, and the HMB supplementation did not change this, as shown in Fig. 5a, b.

Effect of HMB supplementation on contractile muscle function

The absolute muscle twitch and tetanic forces (mN) did not change between control and HMB groups in the alimentary conditions studied (Fig. 6a, b). Likewise, the specific muscle twitch and tetanic forces (mN/mm^2) remained unaltered (Fig. 6c, d).An increase was detected in the parameters studied in the HMB-fed group; however, it failed to reach statistical significance. The resistance to the fatigue index, shown by muscle tension development along 10 successive muscle tetanic contractions (Fig. 7a) and the area under the curve (Fig. 7b)was not changed between groups and alimentary states, even though a tendency to increase was detected in the fasting:HMB-supplemented group ($p = 0.0878$). Additional parameters of contractile muscle function are illustrated in Table 1. No significant changes were detected regarding the the twitch parameters between fed and fasting conditions in all experimental groups (TTP, HRT, LRT). There was a significant increase in the time of sustained isometric contraction (TSI) in the fasting:control (~1.2-fold) and in fasting:HMB groups (~1.8-fold), in comparison to the fed:control condition (Table 1).

Discussion

Skeletal muscle hypertrophy occurs when the rate of protein synthesis exceeds the rate of protein degradation, whereas skeletal muscle atrophy occurs when protein

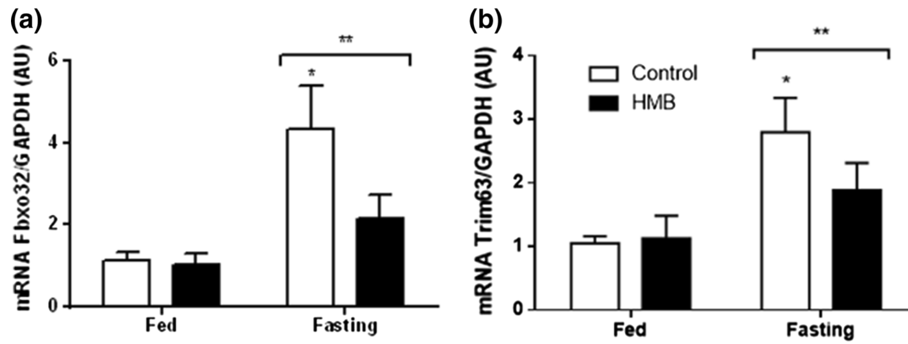

Fig. 4 Real-time PCR analysis of *Fbxo32* and *Trim63* mRNA expression, respectively, in EDL muscle normalized to *Gapdh*. Data are expressed as mean ± SEM ($n = 8$–10 animals/group), in arbitrary units (AU). In *white Fed*:*Control* and *Fasting*:*Control* and in *black Fed*:*HMB* and *Fasting*:*HMB*; *double asterisks* vs. Fed ($p < 0.001$); in (**a**) *asterisk* vs. Fed:Control and HMB ($p < 0.05$) and in (**b**) *asterisk* vs. Fed:Control ($p < 0.05$)

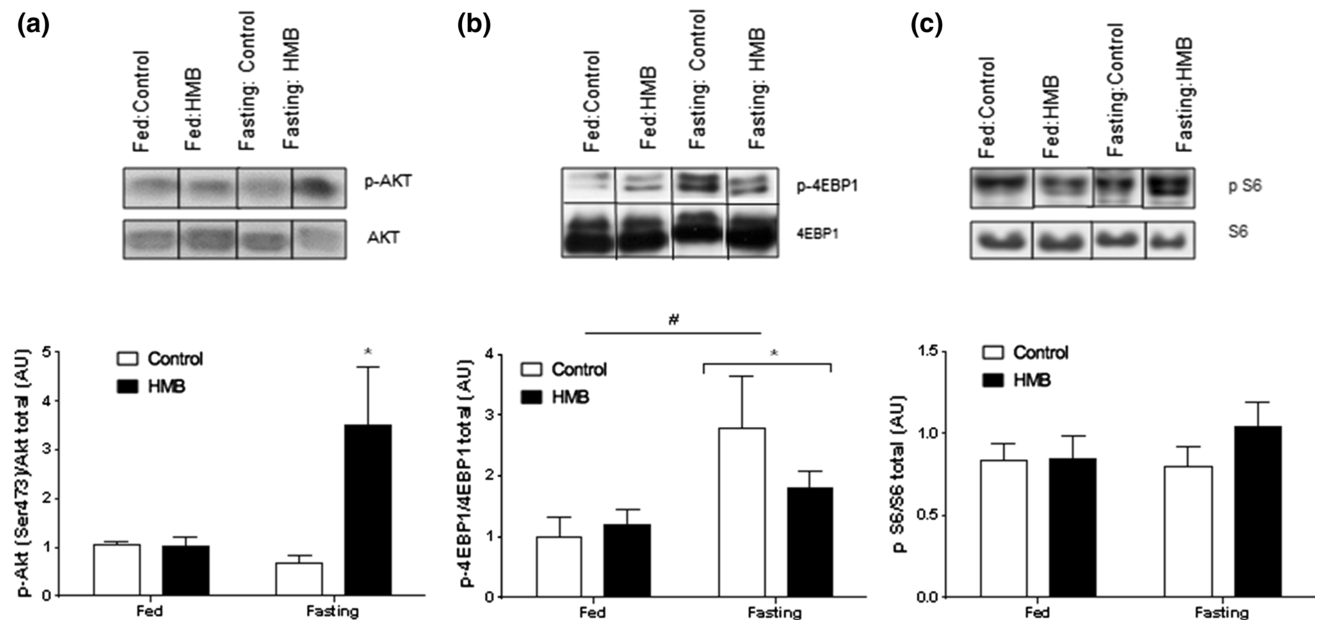

Fig. 5 Western blotting analysis of AKT, 4EBP1 and S6; content of phosphorylation/total (**a**, **b**, **c**) for each protein, respectively. Quantitative representation obtained by densitometric analysis is shown. Data are expressed as mean ± SEM ($n = 6$–9 animals/group), in arbitrary units (AU). in *white Fed:Control* and *Fasting:Control* and in *black Fed:HMB* and *Fasting:HMB*. *Asterisk* effect of supplementation ($p = 0.0316$) (**a**); *hash* interaction supplementation and fasting ($p = 0.0281$) and *asterisk* effect of fasting ($p = 0.0329$) (**b**)

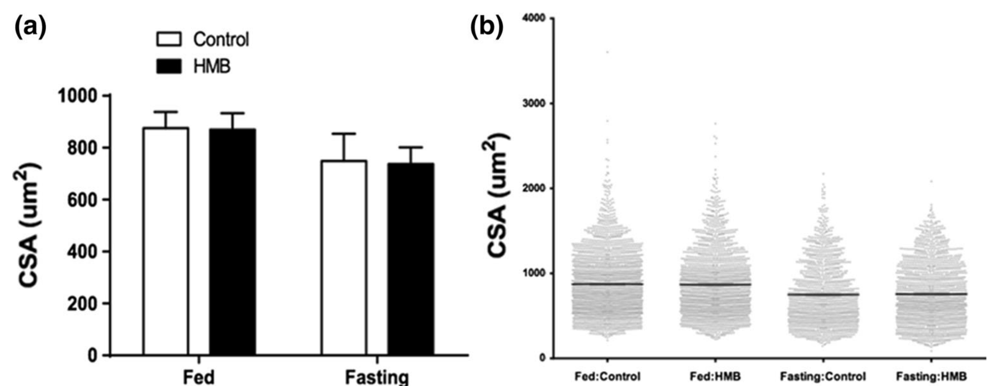

Fig. 6 Fiber cross-sectional area (CSA) analysis of EDL muscle. In *white Fed:Control* and *Fasting:Control* and in *black Fed:HMB* and *Fasting:HMB*. **a** Quantitative analysis of the CSA in μm^2, measuring the circumference of no less than 800 adjacent fibers per animal ($n = 5$ animals/group); **b** showing the distribution of CSAs

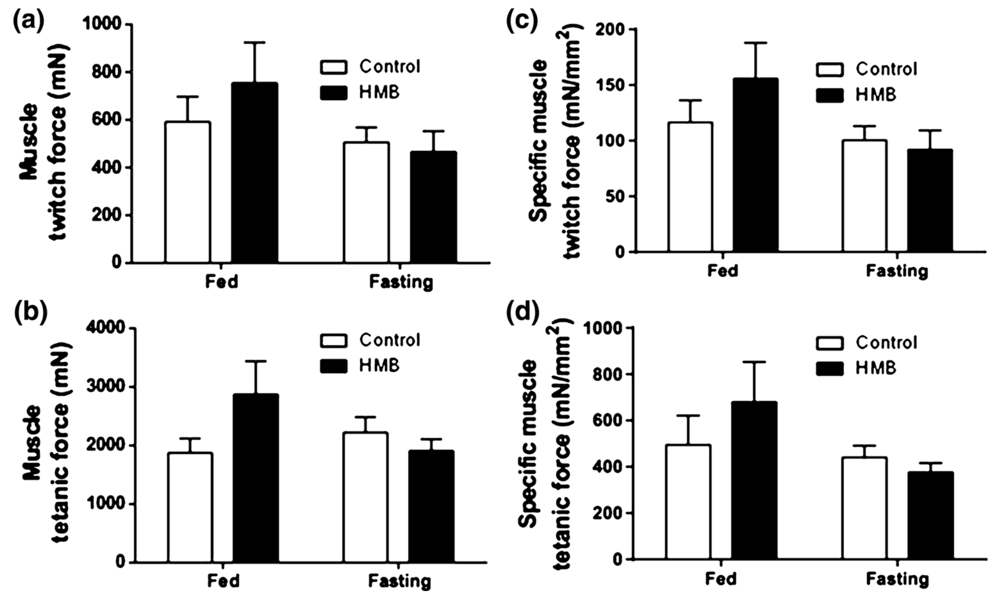

Fig. 7 Data of maximum skeletal muscle strength production in EDL. Muscle twitch force was determined at 1 Hz and tetanic force was at 100 Hz electrical stimulation frequency. **a** Muscle twitch force; **b** muscle tetanic force; **c** specific muscle twitch force normalized per CSA (fed vs. fasting, $p = 0.07$); **d** muscle tetanic force normalized per CSA. Results are expressed as mean ± SEM ($n = 6$ animals/group); in *white Fed:Control* and *Fasting:Control* and in *black Fed:HMB* and *Fasting:HMB*

degradation exceeds synthesis. This can be observed in numerous conditions, such as sarcopenia, cancer, AIDS, immobilization, absolute rest and increase production/use of glucocorticoids [9, 19]. Fasting condition simultaneously increases the rate of skeletal muscle protein degradation and decreases protein synthesis [10, 29]. However, this rate changes accordingly to muscle specificity and contractile properties [10, 30].

HMB is a leucine metabolite and considered an important muscle mass inducer [31, 32]. Here, we found out that chronic HMB supplementation in fasting increased AKT phosphorylation in EDL muscle and prevented the increase of atrogenes expression (*Fbxo32* and *Trim63*) usually observed in this condition, thus preventing muscle weight loss, as shown by Whitehouse et al. [33] and Lecker et al. [34]. Fasting is known to induce a decline in IGF-I content, which leads to a reduction in the PI3 K and AKT activity, while the opposite is observed in muscle hypertrophy [10, 35]. In this study, as pointed out, we detected a 3-fold increase in pAKT content in animals subjected to fasting plus HMB supplementation. In fact, previous data from our group and others demonstrated that HMB supplementation was able to activate the GH-IGF-I axis [21, 36], which could justify the increased pAKT levels.

In this study, we hypothesize that the AKT activation could exert two different actions: (1) an increase in protein synthesis and/or improvement of intramuscular metabolism, and (2) an increase in FoxO phosphorylation, which decreases *Fbxo32* and *Trim63* expression.

Considering the first possibility, HMB supplementation was shown to increase p70S6K1 and 4EBP1 phosphorylation which are related to an enhancement of protein synthesis [12, 37]. However, in our study, we did not find any alteration in these proteins. In contrast, fasting led to an increase of p4EBP1 content, which might be related to the increased atrogenes expression [38]. Muscle atrophy is characterized by a decrease in overall protein. On the other hand, if even protein synthesis is reduced, the atrophy program has to maintain or increase the expression of key proteins [34].

Moreover, AKT activation could lead to the inactivation of glycogen synthase kinase-3 (GSK-3), thereby increasing glycogen synthase activity, which in turn increases the intramuscular glycogen content [4, 39]. Indeed, a previous study by our group showed that HMB supplementation increased muscle glycogen content [4]. This could contribute to explain the trend of an increasing acute fatigue index (Fig. 7; $p = 0.08$) and the increase in skeletal muscle time of development force (TDF) observed when fasted rats were supplemented with HMB (Table 1). In fact, HMB supplementation has been associated with reduced exercise-induced muscle damage and increased resistance to acute fatigue [7, 8]. Moreover, the fasting group supplemented with HMB presented an increased time of sustained isometric contraction (TSI), supporting our evidence of beneficial effects of HMB supplementation under muscle atrophy situations. The second possible effect of AKT activation involves the regulation of atrogenes expression by FoxO phosphorylation. The phosphorylation of transcription factors FoxO1 and FoxO3 prevents their migration to the nucleus, which in turn reduces atrogenes expression [40]. As mentioned before, HMB supplementation attenuated the increase on the *Fbxo32* and *Trim63* expression in fasting muscle (Fig. 3a, b), which could account for the maintenance of skeletal muscle mass. Aversa et al. [41] demonstrated similar HMB effects in

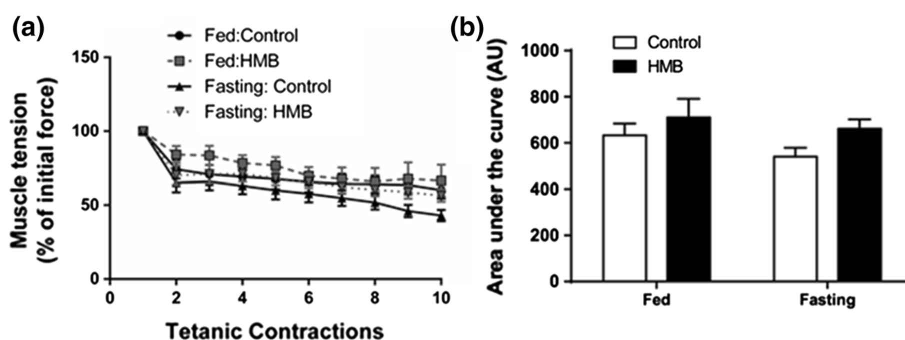

Fig. 8 Resistance to acute fatigue in contracting EDL muscle. Successive tetanic contractions were evocked at 100 Hz each 10 s of interval. **a** Muscle tension during successive tetanic contractions; **b** resistance to fadigue index was calculated as area under the curve to each animal. In *white Fed:Control* and *Fasting:Control* and in *black Fed:HMB* and *Fasting:HMB*. Data are expressed as mean ± SEM ($n = 6$ animals/group)

muscle cells incubated with dexametasone (DEXA), thus indicating that HMB supplementation was able to attenuate DEXA-induced increases in the atrogenes expression, preventing protein degradation. Eley et al. [6] also observed similar effects in a rodent model of sepsis. In a classic study, Li and Goldberg [42] suggested that fast and slow muscles exhibit different protein synthesis and degradation rates. In the former, the rate of protein synthesis and degradation is more affected than the latter under acute fasting conditions. Corroborating these data, in the present study, an increase in both atrogenes (*Fbxo32* and *Trim63*) was detected in EDL muscle, which suggests a rise of proteolysis in the fasting model. The effects were less expressive in the soleus muscle (data not shown). Additionally, our group has found that HMB supplementation in animal models under normal nutritional conditions without an imbalance between protein synthesis and degradation has been able to stimulate an insulin resistance state in soleus muscle [27]. These different effects in distinct muscles could make it difficult to draw overall conclusions about HMB effects in muscle (Fig. 8).

In the fasting condition, there was also a dramatic reduction in the muscle weight in the rats, which was prevented by HMB supplementation; however, this effect did not induce an improvement in the specific twitch force. Consequently, this effect might explain the weight muscle gain without any changes in CSA (Fig. 5). This was the second study that used HMB supplementation and in vivo electrical stimulation in rats. The first study found similar effects of HMB supplementation in muscle with mixed glycolytic and oxidative fibers (gastrocnemius) showing improved effects in parameters of isometric contractions [4]. The current study used EDL, a glycolytic muscle that exhibits a distinct balance between protein synthesis and degradation [10, 42] with chronic HMB supplementation and fasting.

In conclusion, these findings and observations indicate that HMB supplementation prevents the increase of atrogenes expression following fasting, while at the same time improves muscle contractions performance, suggesting an improvement of intramuscular metabolism. HMB supplementation results in metabolic and skeletal muscle function changes which may have a beneficial effect in patients with diseases, as well as improving rehabilitation or athlete performance. However, more investigations with HMB supplementation are required to identify more details about this strategy as well as possible different effects between the muscles.

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Compliance with ethical standards

Conflict of interest The authors declare they have no conflict of interest.

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