Chronic supplementation of beta-hydroxy-beta methylbutyrate (HMβ) increases the activity of the GH/IGF-I axis and induces hyperinsulinemia in rats

F. Gerlinger-Romero, L. Guimarães-Ferreira, G. Giannocco, M.T. Nunes *

Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo. Av. Prof. Lineu Prestes, 1524, ICB-I, Cidade Universitária, Butantã, São Paulo/SP, Brazil

ARTICLE INFO

Article history:
Received 20 September 2010
Received in revised form 7 December 2010
Accepted 20 December 2010
Available online xxxx

Keywords:
Beta-hydroxy-beta-methylbutyrate
Growth hormone
Insulin-like growth factor I
Myostatin
Insulin

ABSTRACT

Objective: Beta-hydroxy-beta-methylbutyrate (HMβ) is a metabolite of leucine widely used for improving sports performance. Although HMβ is recognized to promote anabolic or anti-catabolic effects on protein metabolism, the impact of its long-term use on skeletal muscle and/or genes that control the skeletal protein balance is not fully known. This study aimed to investigate whether chronic HMβ treatment affects the activity of GH/IGF-I axis and skeletal muscle IGF-I and myostatin mRNA expression.

Design: Rats were treated with HMβ (320 mg/kg BW) or vehicle, by gavage, for 4 weeks, and killed by decapitation. Blood was collected for evaluation of serum insulin, glucose and IGF-I concentrations. Samples of pituitary, liver, extensor digitorum longus (EDL) and soleus muscles were collected for total RNA or protein extraction to evaluate the expression of pituitary growth hormone (GH) gene (mRNA and protein), hepatic insulin-like growth factor I (IGF-I) mRNA, skeletal muscle IGF-I and myostatin mRNA by Northern blotting/real time-PCR, or Western blotting.

Results: Chronic HMβ treatment increased the content of pituitary GH mRNA and GH, hepatic IGF-I mRNA and serum IGF-I concentration. No changes were detected on skeletal muscle IGF-I and myostatin mRNA expression. However, the HMβ-treated rats although normoglycemic, exhibited hyperinsulinemia.

Conclusions: The data presented herein extend the body of evidence on the potential role of HMβ-treatment in stimulating GH/IGF-I axis activity. In spite of this effect, HMβ supplementation also induces an apparent insulin resistance state which might limit the beneficial aspects of the former results, at least in rats under normal nutritional status and health conditions.

© 2010 Growth Hormone Research Society. Published by Elsevier Ltd. All rights reserved.

1. Introduction

The branched-chain amino acids (BCAAs) leucine, isoleucine and valine comprise approximately one third of the total muscle protein content [1]. More recently, these amino acids and some of their metabolites (mainly leucine and α-ketoisocaproate – KIC), which are known for over 40 years as anti-catabolic compounds, are returning to the focus of several studies, based on an increasing body of evidence showing that they promote inhibition of muscle proteolysis in rats, leading to a decrease in nitrogen and protein loss [2]. Studies performed by Nissen et al. [3] have suggested that β-hydroxy-β-methylbutyrate (HMβ), which is another metabolite of the amino acid leucine, might be one of these anti-catabolic compounds.

Direct effects of HMβ on muscle metabolism were evidenced in sections of skeletal muscles of rats and chickens, incubated with various concentrations of HMβ, in which an inhibition of proteolysis concomitantly with an increase in protein synthesis were observed [4]. Studies have also shown that HMβ supplementation during immobilization of hind limbs in mice resulted in attenuation of skeletal muscle loss [5]. Increased bone mineral density was also detected in HMβ-treated turkeys, even though it was observed a concomitant increase in plasma amino acid concentration [6]. In addition, improvement of somatotropic axis function and acceleration of bone metabolism was detected in adult sheep subjected to HMβ-treatment during the neonatal period, and in offspring of HMβ-treated pregnant sows [7,8].

Moreover, in individuals subjected to resistance training, the HMβ supplementation led to a 20% decrease of 3-methylhistidine (3-MeHis) in urine and a 20 to 60% decrease in the levels and activities of enzymes, such as creatine kinase and lactate dehydrogenase (LDH), which are plasma indicators of skeletal muscle proteolysis/damage [49]. These effects were consistent with in vitro studies that suggested that leucine and its metabolites act directly on muscle to reduce protein loss [10]. Indeed, recent studies have shown that HMβ supplementation ameliorates cancer-induced cachexia, through activation of multiple intracellular mechanisms that lead to the reduction of protein degradation and increase in protein synthesis [11]. Similar results were obtained in rats, in which the effect of HMβ in skeletal muscle was related to inhibition of proteolysis in proteasome [12]. These data altogether strongly indicate that HMβ, acting directly or by indirect...
mechanisms, might be helpful for preventing muscle damage induced by intense exercise, severe stress conditions, starvation or burning [12,13].

The endogenous production of HMβ is small. An individual of 70 kg produces about 0.2–0.4 g of HMβ/day, depending on the content of leucine in the diet [4], and although it can be found in nature, the quantities required to promote inhibition of proteolysis and muscle mass gain cannot be achieved by usual diet. That is the reason why supplementation with HMβ has been used as an alternative strategy by resistance-exercise/bodybuilding practitioners [10,14], individuals under extreme muscle stress [13], in the elderly, as well as in patients carrying diseases that are associated with muscle loss, such as cancer [15].

Although the anti-catabolic and/or the anabolic effect of HMβ is well documented in the literature, the mechanisms involved in its triggering and the consequences of its long-term use have been poorly investigated [15].

Taking into account that the skeletal muscle mass is maintained by a balance between the rate of protein synthesis and catabolism [16,17], and that growth hormone (GH) as well as the insulin-like growth factor I (IGF-I) play a key role in these processes, it is possible that at least part of the effects of HMβ might occur by changes in the activity of GH/IGF-I axis, which has been suggested but not fully explored [7].

This study was undertaken in order to investigate this possibility, by evaluating the effects of chronic HMβ supplementation on the expression of pituitary GH gene (mRNA and protein), hepatic IGF-I mRNA, skeletal muscle IGF-I and myostatin mRNA, as well as the expression of pituitary GH gene (mRNA and protein), hepatic IGF-I mRNA, skeletal muscle IGF-I and myostatin mRNA, as well as in patients carrying diseases that are associated with muscle loss, such as cancer [15].

2. Materials and methods

2.1. Animals and treatments

Male Wistar rats, of the same age, weighting 200–250 g were obtained from our own breeding colony and maintained on rat chow and tap water ad libitum. They were housed in a room kept at constant temperature (23 ± 1 °C) and on a 12-h light, 12-h dark (lights on at 0700 h) schedule.

The experimental protocol (001/42, book 2, 2007) conforms with ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Institute of Biomedical Sciences/University of São Paulo-Ethical Committee for Animal Research (CEEA).

HMβ treatment (calcium salt: Optimum Nutrition, Sunrise, FL, USA) was carried out by gavage, at the dose of 320 mg/kg body weight/day, in a volume of 1 ml, for 4 weeks (HMβ group), Control group (C) received the same volume of vehicle (saline) by the same route [18]. The dose of HMβ used in the experiments was 10 times lower than that used in the study of Papet et al. [19] and 7 times higher than that used in the study of Tatara et al [8]. We have also taken into account that only approximately 2/3 of the ingested HMβ remain in the plasma [20].

The animals were weighted during the whole experimental period, and at the end of it, they were subjected to a 4-h-period of food restriction. Afterwards, they were anesthetized with thiopental sodium (6 mg/kg body) and killed by decapitation between 9:30–10:30 am. Blood was collected from the trunk for the evaluation of serum IGF-I, insulin and glucose concentration. Pituitary, liver and skeletal muscles were rapidly removed for analysis of GH at both the mRNA and protein levels in pituitary, IGF-I mRNA in liver and skeletal muscle, and myostatin mRNA in muscle. Epididymal adipose tissue was removed and weighted; heart was removed and the ventricle was excised and weighted for determining the ventricular/body weight ratio.

2.2. Evaluation of GH mRNA expression by Northern blotting analysis

Total RNA was extracted by the guanidinium thiocyanate–phenol–chloroform method, electrophoresed, transferred to a nylon membrane and subjected to Northern blotting analysis, as described [21,22]. The results were expressed as GH mRNA/18S rRNA ratio.

2.3. Evaluation of GH protein expression by Western blotting analysis

The pituitaries were removed, homogenized in 0.25 M sucrose; 2 mM MgCl2; 20 mM Tris–HCl buffer and centrifuged at 100 × g for 10 min at 4 °C; the supernatant was centrifuged at 800 × g for 10 min, at 4 °C. Immunoblotting of pituitary protein extracts was performed using anti-GH antibody (1:5000) (National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Torrance, CA), followed by the incubation with appropriated secondary peroxidase-conjugated antibody. The bands detection was performed using Enhanced Chemiluminescence (ECL) Kit (Amer sham Biosciences, Buckinghamshire, UK), and bands were analysed with Scion Image software (Scion Corp, Frederick, MD, USA) as described [16]. Results were expressed as arbitrary units (AU).

2.4. IGF-I mRNA expression by real time-PCR

Total RNA was extracted from liver (~50 mg) as described [17]. Two micrograms of total RNA were used to synthesize the First strand complementary DNA (cDNA) using oligo-dT primers and the M–MLV reverse transcriptase kit (Invitrogen Corp., Carlsbad, CA), according to the manufacturer’s recommendations. Reverse transcription reaction was performed at 70 °C for 10 min, followed by 37 °C for 60 min, and at 95 °C. Real-time quantitative PCR amplification was performed using the SYBR® Green PCR master mix kit (Applied Biosystems, UK) and the primers for IGF-I (Forward: 5′AAGCTTA-CAAAGTACAGTGCG3′, Reverse: 5′GGTTGTGTTTCCTGCACTT3′) and Cyclophilin (Forward: 5′GATTCTAGTGCCAGGGTGC3′, Reverse: 5′CACATGCCTGCACTCAGCG3′), as internal control. 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 [17]. SYBR Green-based real-time PCR analysis was carried out with the ABI Prism 7300 sequence detector (Applied Biosystems, UK), according to the manufacturer’s instructions. RNA aliquots are routinely sham reverse transcribed (i.e. reverse transcriptase omitted) to ensure the absence of products other than those from the reverse transcribed mRNAs. The relative abundance of IGF-I and cyclophilin mRNAs was calculated, using the 2 −ΔΔCt method [23], and the results were expressed in arbitrary units (AU).

2.5. Myostatin and IGF-I mRNA expression by real time-PCR

Total RNA was extracted from Soleus and EDL muscles, reverse transcribed and amplified by real time PCR, using specific primers for Myostatin (Forward: 5′AGTGAGCGCTCTTGGGAAGATG3′, Reverse: 5′AGTCAGACTCGTGTTAGCCG3′), IGF-I (described above) and beta actin (Forward: 5′AGATTGCGACACACATTTCTACA3′, Reverse: 5′CCGTGACAGCACACCG3′), in the same conditions described above. The relative abundance of myostatin, IGF-I and beta actin mRNAs was calculated, using the 2 −ΔΔCt method, and the results were expressed in arbitrary units (AU), as described earlier [23].
2.6. Serum determinations

The IGF-I concentration was measured by chemiluminescence immunoassay using the Luminex xMAP technology (LINCOplex kit; Luminex Corp., Austin, TX, USA) as described [24]. The glucose levels were determined by the enzyme-colorimetric method, according to the manufacturer’s instructions (CELM — Cia. Equipadora de Laboratórios Modernos, Barueri — SP), and the insulin concentration was determined by RIA, as described [21].

2.7. Statistical analysis

All data were expressed as mean ± SEM, and the significance level was set at 5% (P < 0.05) with 6 to 16 animals per group (n = 6–16). The statistical analysis was performed using an unpaired nonparametric Student’s t test (Prism GraphPad, Version: 5.0; GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Effect of HMβ treatment on morphometric tissue parameters and body weight gain

Except for the epididymal adipose tissue/body weight ratio, that was reduced in HMβ-treated rats, the other parameters studied as: body weight gain (final body weight — initial body weight), the dry/wet weight ratio of Soleus and EDL muscles and the ventricular/body weight ratio did not differ between the experimental groups, as shown in the Table 1.

3.2. Effect of the HMβ treatment on the GH/IGF-I axis activity

The Fig. 1 shows the effect of the HMβ supplementation on pituitary GH mRNA and protein expression. It was observed that the HMβ treatment induced an increase in GH mRNA by 65% (P < 0.001) and in GH content by 20% (P < 0.05) compared to control group. The IGF-I mRNA expression in liver, as well as the serum IGF-I concentration was also significantly increased in the HMβ-treated animals, as shown in the Fig. 2A and B, respectively.

3.3. Effect of the HMβ treatment on IGF-I and myostatin mRNA expression in S and EDL muscles

The HMβ treatment did not induce any alteration in the IGF-I and myostatin mRNA expression in both Soleus and EDL muscles, as shown in Figs. 3 (A and B) and 4 (A and B), respectively.

3.4. Effect of the HMβ treatment on serum glucose and insulin concentrations

These data are presented in the Fig. 5, which shows that the HMβ supplementation did not change the serum glucose concentration (A), but induced a significant increase (P < 0.05) in serum insulin levels (B).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HMβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW gain (g)</td>
<td>62.18 ± 3.91</td>
<td>63.07 ± 3.76</td>
</tr>
<tr>
<td>Dry/wet weight ratio Soleus</td>
<td>0.248 ± 0.002</td>
<td>0.255 ± 0.001</td>
</tr>
<tr>
<td>Dry/wet weight ratio EDL</td>
<td>0.252 ± 0.001</td>
<td>0.254 ± 0.001</td>
</tr>
<tr>
<td>Heart/body weight ratio (%)</td>
<td>0.333 ± 0.008</td>
<td>0.333 ± 0.006</td>
</tr>
<tr>
<td>Epididymal adipose tissue/body weight ratio (%)</td>
<td>2.093 ± 0.064</td>
<td>2.786 ± 0.047</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM, *P < 0.05, HMβ vs. control (n = 10–15 per group).
4. Discussion

This study shows that, in parallel to its recognized anti-catabolic effects, the chronic administration of HMβ increases the activity of the GH/IGF-I axis in adult rats, which might contribute to its anabolic effects. These data extends the findings of Tatara et al.[7,8], who pointed to the involvement of somatotropic axis on prenatal and neonatal programming of skeletal development in pigs and sheep, respectively, triggered by HMβ chronic supplementation at these critical periods of development. Although we are concerned with the differences between these studies and our experimental approach, all point towards a stimulatory effect of this metabolite on the GH–IGF-I axis.

The increased GH mRNA and protein expression in pituitaries of HMβ-treated rats strongly indicate that GH synthesis was stimulated under this condition. Moreover, the increase of hepatic IGF-I mRNA expression and serum IGF-I concentration clearly show that GH secretion was stimulated by HMβ supplementation, and acted on this target organ, inducing IGF-I synthesis and release, as pointed by earlier reports that demonstrate that hepatic IGF-I mRNA levels, and in a lesser degree the IGF-I plasma levels, are directly related to GH concentration in serum[22,25–27]. Even though there is no evidence that indicates that HMβ presents GH releasing activity, it is worth mentioning that some amino acids, as arginine and leucine, induce GH release in experimental and human models[21,28,29]. Considering that HMβ is a metabolite derived from leucine, it is possible that it could exert this effect, which remains to be elucidated. Thus, the increased GH synthesis and release observed in rats underwent HMβ chronic treatment strength the possibility that HMβ supplementation could activate the somatotropic axis, as does arginine and leucine supplementation.

Despite the increased GH synthesis and serum IGF-I concentration induced by HMβ treatment, no alterations in the body, skeletal muscle and heart weight were detected (Table 1). This finding appears to be paradoxical, considering the GH and IGF-I effects on the mass gain; however it was already expected since changes in body and tissues

Fig. 3. Real-time PCR analysis of IGF-I mRNA expression in skeletal muscles of control (C) and HMβ-treated (HMβ) rats. The quantitative representation of the IGF-I mRNA abundance in EDL and Soleus muscle normalized to β actin is shown in A and B, respectively. Data are expressed as mean ± SEM, in arbitrary units (AU). P<0.05 HMβ vs. control (n=6–9 animals per group).

Fig. 4. Real-time PCR analysis of skeletal muscles myostatin mRNA expression of control (C) and HMβ-treated (HMβ) rats. The quantitative representation of the myostatin mRNA abundance in EDL and Soleus muscle normalized to β actin is shown in A and B, respectively. Data are expressed as mean ± SEM, in arbitrary units (AU). P<0.05 HMβ vs. control (n=9–16 animals per group).

Fig. 5. Effect of the HMβ-treatment on serum glucose (A) and Insulin concentrations (B). Data are expressed as mean ± SEM. *P<0.05 (n=9–16 animals per group).
weight occur as late events. Furthermore, studies in mice, in which the total deletion of the igf1 gene in a liver-specific manner was performed, resulted in abrogation of liver IGF-I mRNA expression and a significant reduction in serum IGFI levels, even though growth and development of these animals did not differ from wild-type littersmates, suggesting that the role of circulating IGF-I during this stage of development needs to be elucidated [30].

However, the increased hepatic IGF-I mRNA content, and decreased periepiydidimal adipose tissue weight observed in HMβ3-treated rats strongly indicate that both tissues are being targeted by GH under this experimental condition, and it is worth mentioning that these effects are currently observed in a relatively short period of time in rats under GH treatment [25,26,31].

It is important to stress that benefits of GH administration have been reported for those who suffer from GH deficiency. Besides, there is currently very little evidence to support an anabolic role for supraphysiolog-ical levels of systemic GH or IGF-I in skeletal muscle of healthy individuals [32]. However, it is possible that such effect might be detected when HMβ3 treatment is associated with muscle atrophy [15].

Considering that GH has been pointed to increase IGFI [25,26] and decrease myostatin mRNA [33,34] expression in skeletal muscle, we evaluated whereas the HMβ3 treatment induced similar alterations on skeletal muscle IGF-I and myostatin mRNA expression. We did not find any alteration in the expression of both genes in soleus and EDL muscles. However it should be stressed that the increase in IGF-I mRNA expression in response to HMβ3 was observed only in in vitro models [35], and that the decreased myostatin gene expression was detected in patients with GH deficiency chronically treated with GH [35], or in hypophysectomised mice [34], and mouse C2C12 skeletal muscle cell line under acute GH treatment [33].

These experimental models completely differ from ours, in which the GH–IGF-I axis activity of the animals was not manipulated. Furthermore, the skeletal muscles of rats under HMβ3 treatment were supposed to be under continuous and not so strong GH stimulus, as pointed by the 20% increase in serum IGF-I levels, in contrast to the acute exposition of GH, in high doses, observed in the studies with the C2C12 skeletal muscle cell line. Moreover, it has been reported that an increase in IGF-I mRNA expression in muscles is more effectively observed with pulsatile than continuous GH treatment, being the later condition much more similar to that observed in our experiments [36].

Although the use of this metabolite has been shown to be safe [37], it was observed in the HMβ3–treated rats an increased serum insulin concentration, despite normoglycemia, which suggests that a certain degree of insulin resistance is taking place. A potential candidate for this effect could be the GH itself, whose increased levels were shown to decrease IRS-1 tyrosine phosphorylation and IRS-1/P3-kinase association in skeletal muscle, adipose tissue and liver, impairing the insulin signaling [22], a finding that was also observed in rats chronically treated with GH, transgenic mice overexpressing GH and acromegalic patients [38,39]. The hyperinsulinemia also might account for the apparent insulin resistance, since chronic exposure to insulin was shown to provoke serine phosphorylation of IRS1 and inhibit PI3-K activity, proteins that are essential in signaling mechanisms involved in insulin action [40,41]. It is interesting to stress that some amino acids, like leucine, are able to increase insulin secretion, as pointed beforehand [42], and it is possible that its metabolite HMβ3 could also exert this action, which remains to be addressed.

In summary, the data presented herein show that chronic administration of HMβ3 for 4 weeks enhances the GH/IGF-I axis activity, but, in parallel, induces an apparent insulin resistance state, in a stage in which the increased insulin secretion seemed to account for normoglycemia. Even though the insulin resistance is taking place, body, skeletal muscle and heart weights remain unaltered, which suggest a positive influence of HMβ3 in preventing protein wasting. It is possible that in conditions like progressive loss of skeletal muscle, as cancer, sepsis, diabetes, and exercise-induced proteolysis, HMβ3 treatment could promote its anabolic effects, in parallel to its effects in attenuating protein wasting. This study adds to a growing body of evidence indicating that dietary constituents can modulate events that control gene expression pattern and therefore, affecting specific aspects of cellular function.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

Funding for this project was from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), Brazil. F.G.-R. and LG-F. were recipients of fellowships from FAPESP and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). M.T.N. is recipient of fellowship from CNPq.

References


