

## Beta hydroxy beta methylbutyrate supplementation impairs peripheral insulin sensitivity in healthy sedentary Wistar rats

C. Y. Yonamine,<sup>1</sup> S. S. Teixeira,<sup>1</sup> R. S. Campello,<sup>1</sup> F. Gerlinger-Romero,<sup>1</sup> C. F. Rodrigues Jr,<sup>1</sup> L. Guimarães-Ferreira,<sup>2</sup> U. F. Machado<sup>1</sup> and M. T. Nunes<sup>1</sup>

<sup>1</sup> Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil

<sup>2</sup> Department of Sports, Center of Physical Education and Sports, Federal University of Espírito Santo, Vitória, Brazil

Received 16 December 2013,

revision requested 28 January 2014,

revision received 18 June 2014,

accepted 19 June 2014

Correspondence: C. Y. Yonamine, Department of Physiology and Biophysics, Room 129, Institute of Biomedical Sciences, University of São Paulo. Av. Prof. Lineu Prestes, 1524 – ICB-I – Cidade Universitária – Butantã – 05508-000 São Paulo/SP Brazil.

E-mail: caioyogi@icb.usp.br

### Abstract

**Aim:** Investigate, in healthy sedentary rats, the potential mechanisms involved on the effects of beta hydroxy beta methylbutyrate (HMB) supplementation upon the glycaemic homeostasis, by evaluating the insulin sensitivity in liver, skeletal muscle, and white adipose tissue.

**Methods:** Rats were supplemented with either beta hydroxy beta methylbutyrate (320 mg kg<sup>-1</sup> BW) or saline by gavage for 4 weeks. After the experimental period, the animals were subjected to the glucose tolerance test (GTT) and plasma non-esterified fatty acids (NEFA) concentration measurements. The soleus skeletal muscle, liver and white adipose tissue were removed for molecular (western blotting and RT-PCR) and histological analysis.

**Results:** The beta hydroxy beta methylbutyrate supplemented rats presented: (i) higher ratio between the area under the curve (AUC) of insulinaemia and glycaemia during glucose tolerance test; (ii) impairment of insulin sensitivity on liver and soleus skeletal muscle after insulin overload; (iii) reduction of glucose transporter 4 (GLUT 4) total and plasma membrane content on soleus; (iv) increased hormone-sensitive lipase (HSL) mRNA and protein expression on white adipose tissue and plasma NEFA levels and (v) reduction of fibre cross-sectional area of soleus muscle.

**Conclusion:** The data altogether indicate that beta hydroxy beta methylbutyrate supplementation impairs insulin sensitivity in healthy sedentary rats, which, in the long-term, could lead to an increased risk of developing type 2 diabetes.

**Keywords** beta hydroxy beta methylbutyrate, glucose transporter 4, glycaemic homeostasis, growth hormone, insulin resistance, non-esterified fatty acids.

Nutritional supplements have been highly commercialized and employed by the population in the last two decades (Bernstein *et al.* 2003). More than 3 million people in the USA use, or have used, ergogenic supplements. Moreover, supplement use is widespread among high school and college athletes as well as non-athletes, particularly adolescents (Moliner &

Márquez 2009), even though the possible side effects caused by its chronic administration on intermediary metabolism have not been investigated thoroughly (Loud *et al.* 2003, Baxter *et al.* 2005).

In this context, beta hydroxy beta methylbutyrate (HMB), a leucine metabolite, is one of the most used products by athletes (Portal *et al.* 2011) and people

engaged in physical activity or not, aiming to gain lean mass and/or to lose fat mass (Payne *et al.* 2006). Initial studies, proposed by (Nissen *et al.* 1996) demonstrated that HMB supplementation (1, 5 or 3 g day<sup>-1</sup>) in association with resistance training protocol (90 min, 3 days per week, during 3 weeks) was able to improve muscle strength and reduce exercise-induced muscle proteolysis in sedentary young men. However, the same results are not observed in resistance trained athletes, possibly due to suppression of protein breakdown induced by training adaptations, which may blunt the HMB effects (Slater & Jenkins 2000). This metabolite has also been employed for medical purposes, mainly in patients with muscle-wasting diseases, to reduce protein degradation and/or increase protein synthesis (Smith *et al.* 2005, Kuhls *et al.* 2007, Wilson *et al.* 2008).

Although HMB has been extensively used, effects of its chronic administration on glucose homeostasis are still unknown and should be explored. Several studies have hypothesized that chronic excess intake of amino acids aiming to enhance protein synthesis, could lead to inhibition of the first steps of insulin signalling, which in turn could result in insulin resistance status, a feature of type 2 diabetes (Promintzer & Krebs 2006, Um *et al.* 2006, Tremblay *et al.* 2007). In this context, a recent study carried out in our laboratory demonstrated that healthy sedentary rats supplemented with HMB, which is an amino acid derivative, presented hyperinsulinaemia and normoglycaemia, suggesting the development of insulin resistance status. Moreover, in the same study, the HMB-supplemented rats presented increased somatotrophic axis activity, demonstrated by increased growth hormone (GH) mRNA and protein expression, liver insulin-like growth factor I (IGF-I) mRNA expression and circulating levels (Gerlinger-Romero *et al.* 2011).

Based on these facts, this study attempted to investigate the effects of chronic supplementation of HMB (320 mg kg<sup>-1</sup> of BW day<sup>-1</sup>) upon glycaemic homeostasis and peripheral insulin sensitivity in healthy sedentary rats, as well as the potential involvement of GH.

## Material and methods

### Animals and procedures

Approximately 60 male Wistar rats of the same age (3 months) and weighing approx. 250 g were obtained from our own breeding colony. They were maintained on a standard rat chow containing 22% of protein (Nuvilab CR1; Nuvital Nutrientes S/A, Colombo, Paraná, Brazil) and tap water *ad libitum*

(Table S1). They were housed in a room kept at a constant temperature (23 ± 1 °C) and in a 12/12-h light/dark cycle (lights on at 07:00 h).

Hydroxy beta methylbutyrate (calcium salt: Dymatize Enterprises, Dallas, TX, USA) was administered daily in a dose of 320 mg kg<sup>-1</sup> of body weight (BW) by gavage, diluted in 1 mL of saline (0.9%), for 4 weeks as described by Gerlinger-Romero *et al.* (2011). The control animals (C group) received the same volume of vehicle (saline), by the same way. The rats were weighted throughout the experimental period, and the food intake was measured twice a week.

At the day of the sacrifice, they were subjected to a 4-h food restriction and subsequently killed. Part of the animals were subjected to the glucose tolerance test (GTT), and the remainder were subjected to tissue collection and after that killed by decapitation. The liver, soleus (SOL) skeletal muscle and white adipose tissue (WAT) were removed to evaluate the molecular parameters pointed below by western blotting and RT-PCR. Blood samples were also collected, and plasma was stored for later analysis of non-esterified fatty acids (NEFA) concentrations, as described by Chromy *et al.* (1977). Additionally, the morphological analysis of SOL muscle was performed, using eosin-haematoxylin staining protocol, as described by Harcourt *et al.* (2005). The experimental protocol is in accordance with the ethical principles in animal research adopted and approved by the Institute of Biomedical Sciences/University of São Paulo – Ethical Committee for Animal Research (001/42, book 2, 2007) and followed the National guidelines for ethical use of animals in research.

### Glucose tolerance test

The animals were anesthetized by i.p. injection of thiopental (Thiopentax, Cristália, Itapira, SP, Brazil) at the dose of 60 mg kg<sup>-1</sup> of BW and subjected to GTT. In this procedure, a cannula was inserted and properly immobilized into the jugular vein for glucose injection (0.75 g kg<sup>-1</sup> of BW) and blood collection before (0 min) and 4, 8, 16 and 30 min after the glucose overload, as described by Campello *et al.* (2012). The plasma was stored at -20 °C for further determination of glucose (glicose enzimática-CELM, São Paulo, Brazil) and insulin (Coat-a-Count<sup>®</sup> Insulin, DPC, Los Angeles, CA, USA) concentrations. The area under the curve of insulin and glucose plasma concentrations during the test was calculated, and the ratio between these areas [area under the curve (AUC) plasma insulin levels/AUC plasma glucose levels] indicated the insulin sensitivity. The area under the curve corresponds to the incremental area value, calculated over the basal value (time zero).

#### *Evaluation of the insulin signalling pathway proteins (IR and AKT) by western blotting*

The SOL muscle, WAT and liver were removed before and after insulin overload (10 U) into penian vein as described by de Castro Barbosa *et al.* (2009). The tissues were homogenized in a hot buffer containing Trizma base 100 mM pH 7.5; EDTA 10 mM, 1% sodium dodecyl sulphate, sodium fluoride 100 mM, sodium pyrophosphate 10 mM, sodium orthovanadate 10 mM, also described by de Castro Barbosa *et al.* (2009) and centrifuged at 13.400 g for 40 min at 4 °C. Equal amounts of protein (60 µg) were subjected to electrophoresis and immunoblotted using antibodies for anti-phosphotyrosine (1 : 200; Cell Signaling Technology, Beverly, MA, USA), anti-IR total (1 : 500; Cell Signaling Technology), and antiphospho-Akt (Ser473) (1 : 500; Cell Signaling Technology) in 8% BSA/PBS solution. We used appropriated secondary peroxidase-conjugated antibody for band detection (1 : 5.000; Santa Cruz Biotechnology, Dallas, TX, USA) and Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Buckinghamshire, UK). SCION IMAGE software was used to analyse the intensity of blots (Scion Corp., Frederick, MD, USA). The Ponceau-stained nitrocellulose membrane was used for normalization, and results were expressed as arbitrary units (AUs).

#### *Evaluation of glucose transporter 4 protein expression and percentage of translocation to plasma membrane by western blotting*

Soleus skeletal muscle muscle and WAT were homogenized in 10 mM Trizma, 1 mM EDTA and 250 mM sucrose (pH 7.4) buffer. Plasma membrane (PM) and microsome (M) fractions of glucose transporter 4 (GLUT4) were separated by ultracentrifugation (de Castro Barbosa *et al.* 2009). The WAT samples were submitted to a subcellular fractionation according to Fabres-Machado & Saito (1995). Immunoblotting of the tissues protein extracts was performed using anti-GLUT4 antiserum (1 : 3.000/ Polyclonal Rabbit Raised Anti-GLUT4, AB1346; CHEMICON International, Temecula, CA, USA) in 8% BSA/PBS solution followed by the incubation with appropriated secondary peroxidase-conjugated antibody (1 : 5.000; Santa Cruz Biotechnology). The band detection was performed as previously described. The sum of the total protein yield of PM and M was considered the total GLUT4 content (PM and M). The percentage of total cellular GLUT4 present in the PM was then calculated as indicated:  $(100 \times \text{PM GLUT4}) / (\text{PM GLUT4} + \text{M GLUT4})$ . The Ponceau-stained nitrocellulose membrane was used for a loading control.

#### *Evaluation of SOCS3 and PEPCK protein expression on liver by western blotting*

A sample of the liver was removed and homogenized in a buffer containing Trizma 100 mM pH 7.5; EDTA 10 mM, 1% sodium dodecyl sulphate, sodium fluoride 100 mM, sodium pyrophosphate 10 mM, sodium orthovanadate 10 mM as described by de Castro Barbosa *et al.* (2009) and centrifuged at 13.400 g for 40 min at 4 °C. Equal amounts of protein (30 µg) were subjected to electrophoresis, and nitrocellulose membranes were immunoblotted with anti-SOCS3 (1 : 1.000 ab16030; Abcam, Cambridge, UK) and anti-PEPCK (1 : 1.000; Santa Cruz Biotechnology) in 8% BSA/PBS solution. We used appropriated secondary peroxidase-conjugated antibody for band detection (1 : 5.000; Santa Cruz Biotechnology) and ECL kit (Amersham Biosciences, Buckinghamshire, UK). SCION IMAGE software was used to analyse the intensity of blots (Scion Corp.). The Ponceau-stained nitrocellulose membrane was used for normalization, and results were expressed as AUs.

#### *Evaluation of pY STAT5 protein expression on liver and WAT by western blotting*

We have evaluated the expression of this protein on liver and WAT, using the same procedure described above. However, the membrane immunoblotting was performed using anti-pSTAT5 A/B (1 : 1.000; Cell Signaling Technology).

#### *Evaluation of hormone-sensitive lipase protein expression by western blotting*

White adipose tissue samples (0.05–0.3 g) were homogenized in 1.0 mL extraction buffer containing 100 mM Trizma, pH 7.5, 10 mM EDTA, 1% sodium dodecyl sulphate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride and 0.25 mg aprotinin. The lysates were incubated with 10% Triton X-100 for 30 min and centrifuged at 13.400 g for 30 min at 4 °C (Fain *et al.* 2008). The supernatant was collected, and the protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Immunoblotting was carried out using anti-hormone-sensitive lipase (HSL) antibody (1 : 1.000; Santa Cruz Biotechnology) or GAPDH (1 : 3.000; Santa Cruz Biotechnology) followed by the incubation with appropriated secondary peroxidase-conjugated antibody. The band detection was performed using enhanced chemiluminescent detection (ECL Western Blotting Detection; Amersham Pharmacia Biotech, Amersham, UK) and

analysed by the SCION IMAGE software (ScionCorp.), and the results were expressed as AUs.

#### Evaluation of HSL mRNA by real-time PCR

Hormone-sensitive lipase mRNA expression was analysed by real-time PCR as previously described by Fain *et al.* (2008). Total RNA was extracted from white adipose tissue (0.4 g). Two micrograms of total RNA was used to synthesize the first strand complementary DNA (cDNA) using 10 mM of each oligo-dT primers and the M-MLV reverse transcriptase kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Reverse transcription reaction was performed at 70 °C for 10 min, followed by 42 °C for 60 min, and 10 min at 95 °C. Real-time quantitative PCR amplification was performed using the SYBR<sup>®</sup> Green PCR master mix kit (Applied Biosystems, Foster City, CA, USA) and the primers for HSL (Forward: 5'TTTCCAC CCACGGCGCTCAA 3', Reverse: 5' TC CAGAGCGCAGCCACAAG 3'), or RPL19 (Forward: 5'CCAAT GAAACCAACGAAATCG3', Reverse: 5'TCAGGCCATCTTTGATCAGCT3'), 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. SYBR Green-based real-time PCR analysis was carried out with the ABI Prism 7300 sequence detector (Applied Biosystems), according to the manufacturer's instructions. The relative abundance of HSL and RPL19 mRNAs was calculated, using the  $2^{-\Delta\Delta C_t}$  method, and the results were expressed in arbitrary units (AU).

#### Evaluation of Non-esterified fatty acids concentration on plasma

The NEFA plasma concentration levels of control and HMB-supplemented rats were evaluated using the same protocol described by Chromy *et al.* (1977).

#### Evaluation of fibre cross-sectional area

Transverse muscle sections (12  $\mu$ m thick) were cut from the mid-belly region of each muscle using a cryostat (Jung Frigocut 2800N – Leica). After that, the samples were stored during 1 h at 37 °C and then at –80 °C until analysis. Sections were stained with Haematoxylin and Eosin for examination of fibre cross-sectional area (CSA) (Harcourt *et al.* 2005). Sections were imaged using an upright microscope at 5 $\times$  magnification (Leitz Aristoplan, Wetzlar, Germany) equipped with a camera (Spot model 1.3.0; Diagnostic Instruments, Sterling Heights, MI, USA), driven by Spot Diagnostic software (version 2.1; Diagnostic

Instruments). Digitized images were analysed using software IMAGE-PRO PLUS in a double-blinded manner. The mean CSA was determined by measuring the circumference of no less than 800 adjacent fibres per animal, and five animals were analysed in each group.

#### Statistical analysis

All data were expressed as Mean  $\pm$  SEM, and the significance level was set at 5%. The statistical analysis was performed by unpaired Student's *t*-test or two-way ANOVA with Bonferroni post-test (PRISM GRAPHPAD, Version: 5.0; GraphPad Software, San Diego, CA, USA).

## Results

#### Effect of the HMB supplementation on body weight gain and food intake

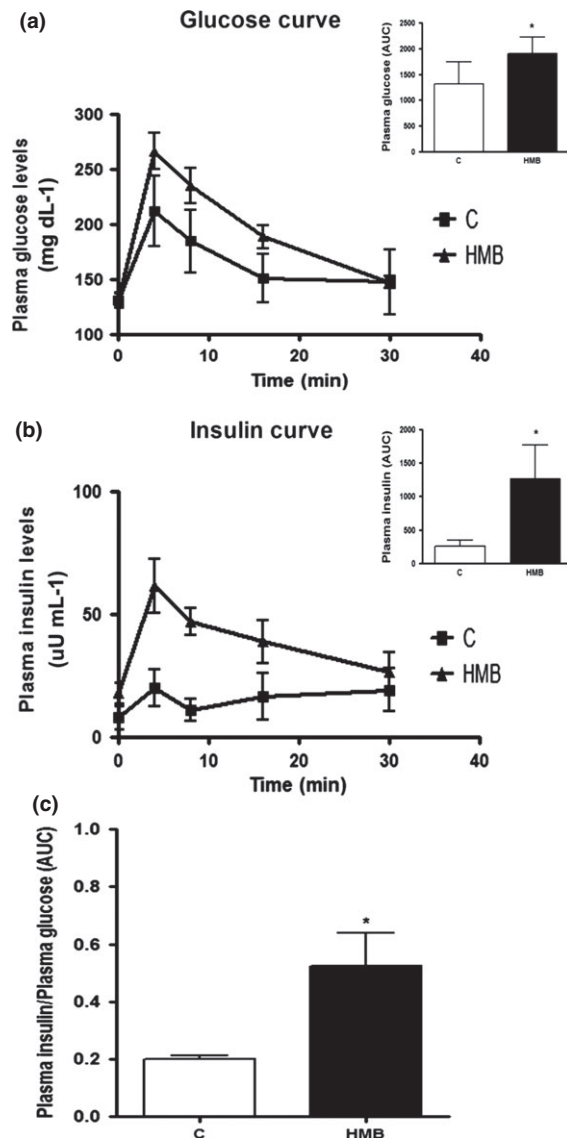
The body weight gain (final – initial body weight) (HMB: 80.5  $\pm$  6.4 vs. C: 71.3  $\pm$  5.5 g) and food intake (HMB: 25.79  $\pm$  1.11 vs. C: 26.82  $\pm$  2.99 g day<sup>-1</sup>) were the same between groups.

#### Effect of the HMB supplementation on the whole-body insulin sensitivity

Results of the GTT are shown in Figure 1. The AUC of variation of plasma glucose (1899.0  $\pm$  327.5 vs. 1317.0  $\pm$  419.8,  $P < 0.05$ ) and insulin levels (1271.0  $\pm$  499.0 vs. 259.6  $\pm$  85.9,  $P < 0.05$ ) was greater in the HMB-supplemented rats when compared to the control rats (Fig. 1a,b). Finally, the AUC plasma insulin levels/AUC plasma glucose levels ratio was significantly higher in the HMB-supplemented rats (0.523  $\pm$  0.117 vs. 0.198  $\pm$  0.013,  $P = 0.021$ ) (Fig. 1c). The post-prandial plasma glucose levels were the same between HMB-supplemented and control rats (131.1  $\pm$  5.6 vs. 131.0  $\pm$  6.9 mg dL<sup>-1</sup>,  $P = 0.98$ ).

#### Effect of the HMB supplementation on the phosphorylation of insulin receptor (pY IR) and AKT (pS AKT) in liver, SOL skeletal muscle and WAT

The results indicate that the liver of HMB-supplemented rats presented a reduction on pY IR (approx. 23%) and pS AKT (approx. 32%) in response to insulin overload vs. control group (Fig. 2a). However, no differences were detected on pY IR in the SOL, even though a reduction was detected on pS AKT (approx. 45%) in response to insulin overload in HMB-supplemented group (Fig. 2b). With regard to WAT, no differences were observed on pY IR and pS AKT activity between the HMB vs. control group after insulin overload (Fig. 2c).



**Figure 1** Glucose Tolerance Test (GTT). A glucose load ( $0.75 \text{ g kg}^{-1} \text{ BW}$ ) was injected into the jugular vein, and the plasma levels of glucose and insulin were evaluated in hydroxy beta methylbutyrate (HMB)-supplemented and control rats from 0 to 30 min. (a). shows the variation of plasma glucose levels, and the insert shows the area under the curve (AUC); (b). shows the variation of plasma insulin levels, and the insert shows the AUC; (c). shows the ratio between the AUC of insulin and glucose plasma levels (AUC ins/AUC glu). Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$  vs. control ( $n = 6$  animals per group).

#### Effect of the HMB supplementation on GLUT4 translocation to plasma membrane, total and plasma membrane GLUT4 content in SOL and WAT

No differences were detected on GLUT4 translocation to plasma membrane in SOL between HMB-supplemented and control rats. However, both SOL total

and PM GLUT4 protein content were reduced by HMB (approx. 45% and approx. 57% respectively) (Fig. 3a). These parameters were not altered in WAT after HMB supplementation (Fig. 3b).

#### Effect of HMB supplementation on SOCS3 and PEPCK content in liver

No significant differences were detected on SOCS3 and PEPCK content in the liver of HMB-supplemented vs. control rats (Fig. 4a and b respectively).

#### Effect of HMB supplementation on tyrosine phosphorylation of STAT5 (pY STAT5) of liver and WAT

Even though we have not detected a significant alteration on pY STAT5 content on liver and WAT (Fig. 5a,b), it should be stressed that there is a statistical tendency of increase on pY STAT5 content in WAT, considering that the  $P$  value was 0.1.

#### Effect of the HMB supplementation on HSL mRNA and protein content on WAT and NEFA plasma levels

As seen in Figure 6, the HSL gene (a) and protein expression (b) in WAT and plasma NEFA levels (c) were increased in rats subjected to chronic HMB supplementation (approx. 52, 88 and 293% respectively).

#### Effect of the HMB supplementation on CSA

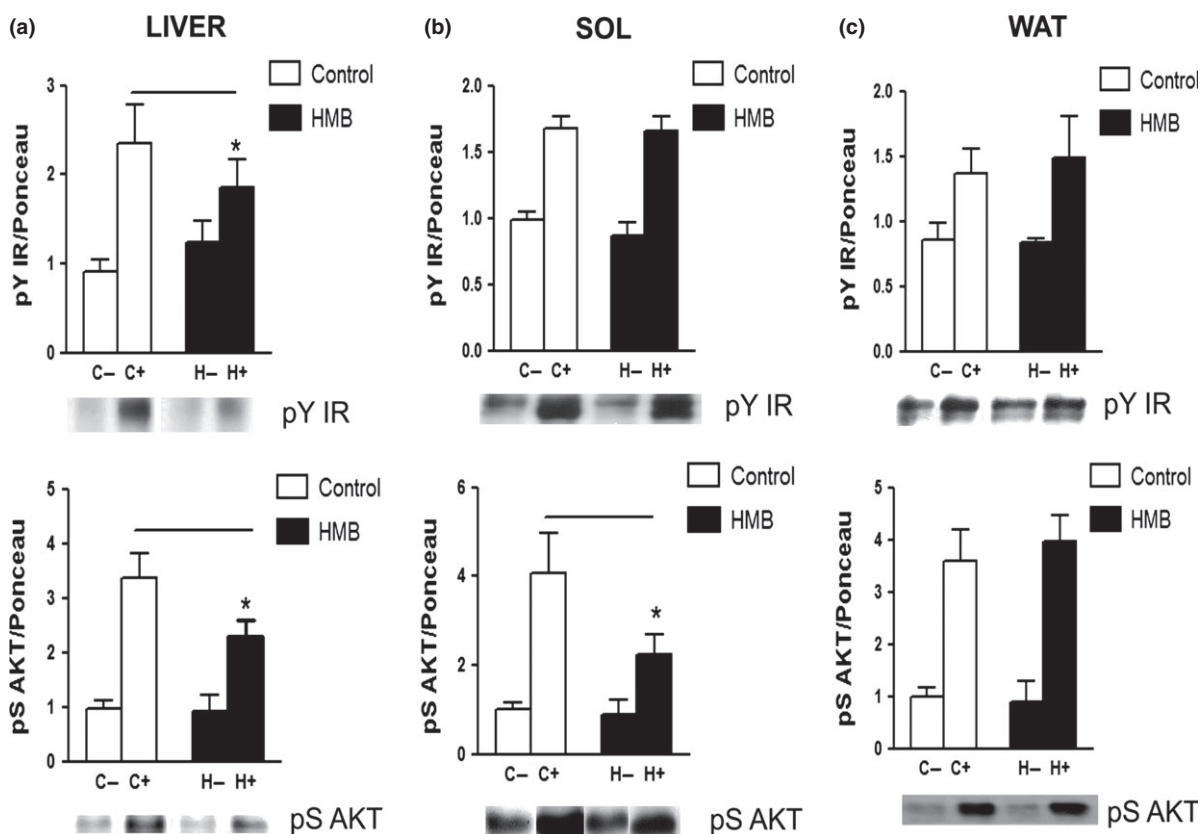
The histological analysis of SOL skeletal muscle revealed that chronic supplementation with HMB reduces the fibres cross-sectional area (approx. 18%) (Fig. 7).

## Discussion

Previous studies of our laboratory have suggested an association of HMB supplementation with insulin resistance state in healthy sedentary rats. It was detected in this condition an increased activity of the GH/IGF-I axis (Gerlinger-Romero *et al.* 2011), which is known to impair the insulin sensitivity (Smith *et al.* 1997, Cho *et al.* 2006).

In the present study, we have confirmed that HMB-supplemented rats presented an insulin resistance state. This was revealed by the GTT that showed a threefold higher AUC glucose/AUC insulin ratio vs. control rats, indicating that the relative intolerance to glucose observed was due to peripheral insulin resistance and not by lack of insulin secretion (Fig. 1).

Considering that insulin binds to its receptor and promotes its phosphorylation and recruitment of insulin receptor substrates (IRS), which are also positively



**Figure 2** Western blotting analysis of total content of pY IR and pS AKT phosphorylation activity of liver (a); soleus skeletal muscle (SOL) (b); and white adipose tissue (WAT) (c) from HMB-supplemented [hydroxy beta methylbutyrate (HMB)] and control (C) rats, before (–) and after (+) insulin overload into penian vein. The data obtained in all experiments are represented in the graphics and expressed as mean  $\pm$  SEM. At the bottom, typical autoradiograms of one experiment in a total of two are shown. \* $P < 0.05$  vs. control. ( $n = 9$  animals per group). The Ponceau-stained nitrocellulose membrane was used as housekeeping.

regulated when phosphorylated and trans-phosphorylated in tyrosine sites (Taniguchi *et al.* 2006), we firstly evaluated the pY IR content in liver, SOL and WAT.

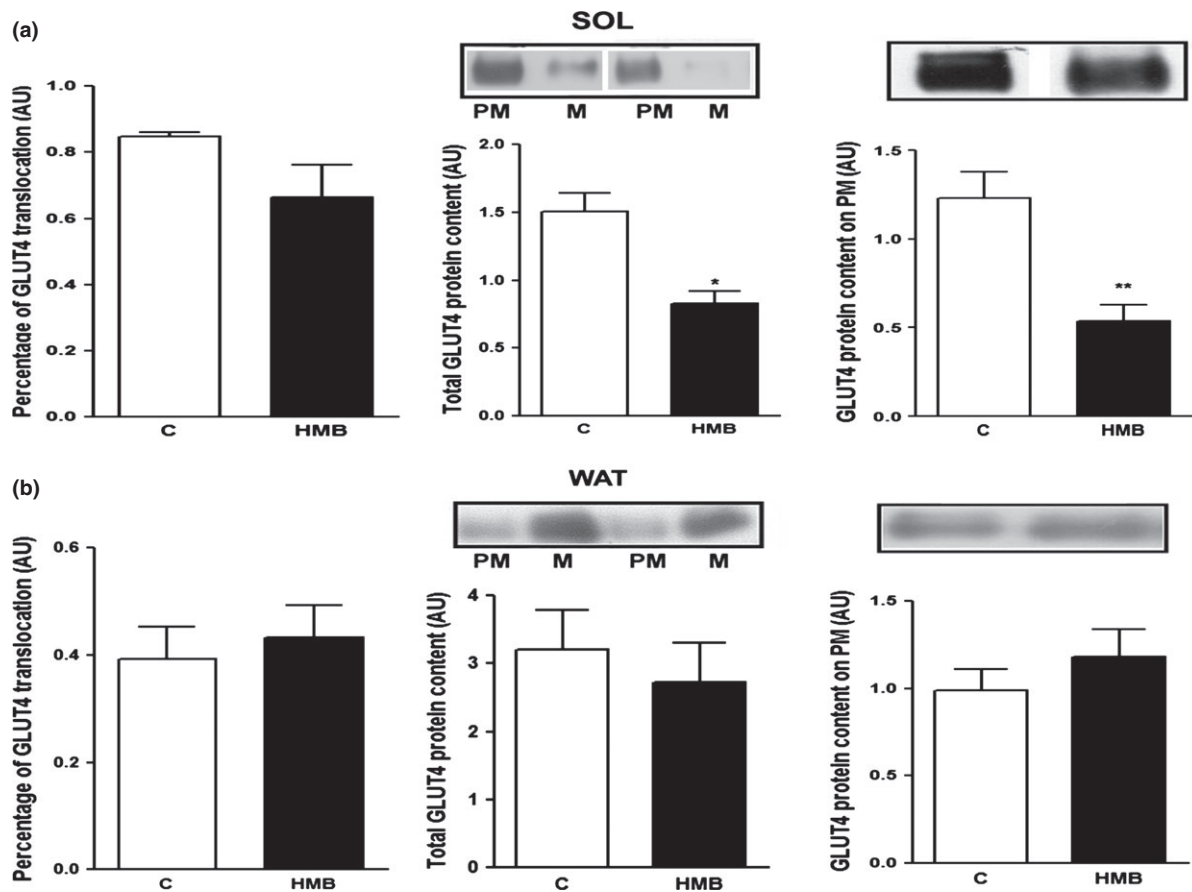
In liver, it was observed a decreased pY IR content, which indicates that insulin sensitivity is affected by HMB supplementation at the receptor level. The evaluation of pS AKT content also showed that insulin signalling is affected at post-receptor level, as a reduction of its content was observed in HMB-supplemented rats (Fig. 2a).

Taking into account that AKT/PKB is known to suppress hepatic glucose production, by reducing the expression of phosphoenolpyruvate carboxykinase (PEPCK), a crucial enzyme of gluconeogenesis pathway (Xing & Quinn 1993, Oakes *et al.* 1997), we evaluated PEPCK protein content in liver. However, even though pS AKT was shown to be reduced in this tissue by HMB supplementation, we did not detect any alterations in PEPCK content (Fig. 4b). This result indicates that although liver insulin sensitivity is

impaired, as shown by the decreased pY IR and pS AKT content, the expected repercussions on PEPCK content were not established yet (Michael *et al.* 2000).

The evaluation of pS AKT content in SOL muscle in response to insulin overload showed that insulin sensitivity is also impaired in this tissue after HMB supplementation period, but only at post-receptor level, as no alterations were detected on pY IR content (Fig. 2b). The reduction of pS AKT in skeletal muscle is observed in insulin resistance status and is accompanied by downstream defects, because AKT appears to be essential for regulation of AKT substrate of 160 kDa (AS160) which is involved in GLUT4 translocation to the plasma membrane (van Dam *et al.* 2005, Tonks *et al.* 2013).

In this context, we evaluated the GLUT4 translocation index from microsome (M) to PM, as well as, GLUT4 total protein content, in SOL of rats subjected to HMB supplementation. No significant alterations in GLUT4 translocation index were observed, but a reduction of SOL total GLUT4 content was detected



**Figure 3** Percentage of GLUT4 translocation to plasma membrane, total GLUT4 content [sum of plasma membrane (PM) and microsome (M) fractions] and GLUT4 protein content in PM from HMB-supplemented [hydroxy beta methylbutyrate (HMB)] and control (C) rats in SOL and white adipose tissue (WAT) are shown in (a) and (b) respectively. The data obtained in all experiments are represented in the graphics and expressed as mean  $\pm$  SEM. The top shows the autoradiogram of one in a total of three experiments. \* $P < 0.05$  vs. control and \*\* $P < 0.01$  vs. control ( $n = 9$  animals per group). The Ponceau-stained nitrocellulose membrane was used as housekeeping.

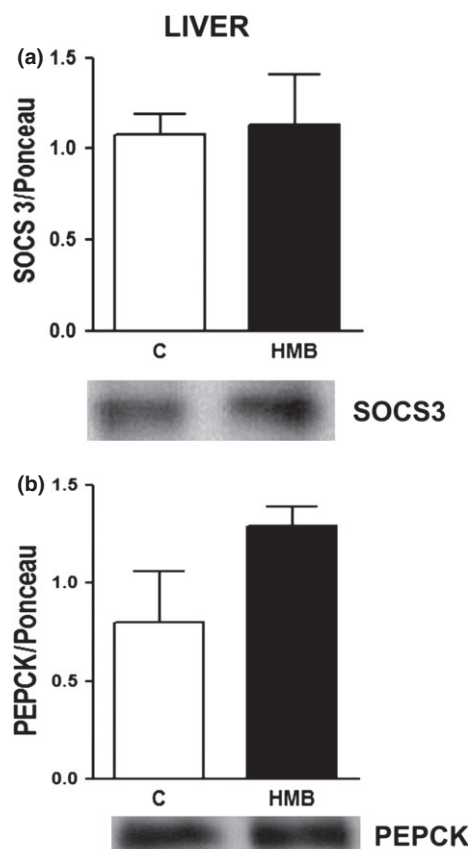
in HMB-supplemented rats (Fig. 3a). These data indicate that, even though the translocation index of GLUT4 to PM was unaltered, less GLUT4 was available for glucose uptake, as shown by the reduced GLUT4 protein content on PM (Fig. 3a). This might explain the reduction of glucose plasma clearance and higher insulin secretion observed in the HMB-supplemented rats, during GTT. In fact, the GLUT4 is the isoform of glucose transporters most expressed in skeletal muscle and WAT (Stöckli *et al.* 2011), and its overexpression was shown to enhance the glucose uptake, ameliorating glucose homeostasis *in vivo* (Wallberg-Henriksson & Zierath 2001). Likewise, muscle GLUT4 KO mice develop insulin resistance (Kim *et al.* 2001).

However, in WAT of HMB-supplemented rats, we did not observe any alterations in insulin signalling, GLUT4 total protein expression and GLUT4 translocation index (Fig. 3b). On the other hand, it was

detected an increase of mRNA and protein expression of HSL, which is known to enhance triacylglycerol hydrolysis, leading to glycerol and free fatty acids release to bloodstream (Lafontan & Langin 2009). These findings led us to measure the plasma NEFA, which was shown to be increased, indicating that HMB supplementation enhanced the WAT lipolytic activity (Fig. 6).

Previous studies of our group have pointed that HMB supplementation to rats leads to an increased activity of GH-IGF-I axis (Gerlinger-Romero *et al.* 2011). It is known that GH exerts a recognized lipolytic effect, by stimulating the activity of HSL, and enhancing NEFA plasma levels and fat oxidation (Bergan *et al.* 2013). Therefore, the data presented may be resultant of a direct effect of GH on WAT (Carrel & Allen 2000, Fain *et al.* 2008).

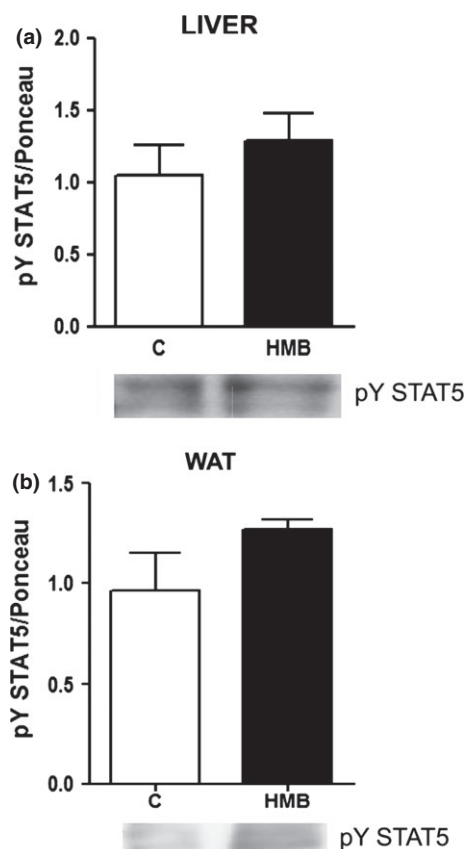
Considering that STAT5 is involved in the GH canonical signalling pathway, and it is also a crucial



**Figure 4** Total content of SOCS3 (a) and phosphoenolpyruvate carboxykinase (PEPCK) (b) of liver from HMB-supplemented [hydroxy beta methylbutyrate (HMB)] and control (C) rats. The data obtained in all experiments are represented in the graphics and expressed as mean  $\pm$  SEM. The bottom shows the autoradiogram of one in a total of two experiments. \* $P < 0.05$  vs. control ( $n = 9$  animals per group). The Ponceau-stained nitrocellulose membrane was used as housekeeping.

protein involved in lipolysis (Fain *et al.* 2008), we have evaluated its activity by measuring the pY STAT5 content in WAT. We detected an increase of pY STAT5 content in WAT after HMB supplementation period; however, it failed to reach statistical significance ( $P = 0.1$ ) (Fig. 5b). We cannot discard the possibility that other proteins could mediate the GH lipolytic effects. In fact, Bergan *et al.* (2013) have shown that GH stimulates HSL expression, by activating phospholipase C (PLC)/protein kinase C (PKC) and the MEK/ERK pathways, instead of the JAK/STAT pathway. Moreover, we cannot discard a direct action of HMB increasing the lipolytic activity on WAT (Panton *et al.* 2000). The current findings may explain previous reports of HMB-induced reduction of WAT weight (Vukovich *et al.* 2001).

Taking into account that high circulating levels of NEFA are known to impair insulin signalling, and

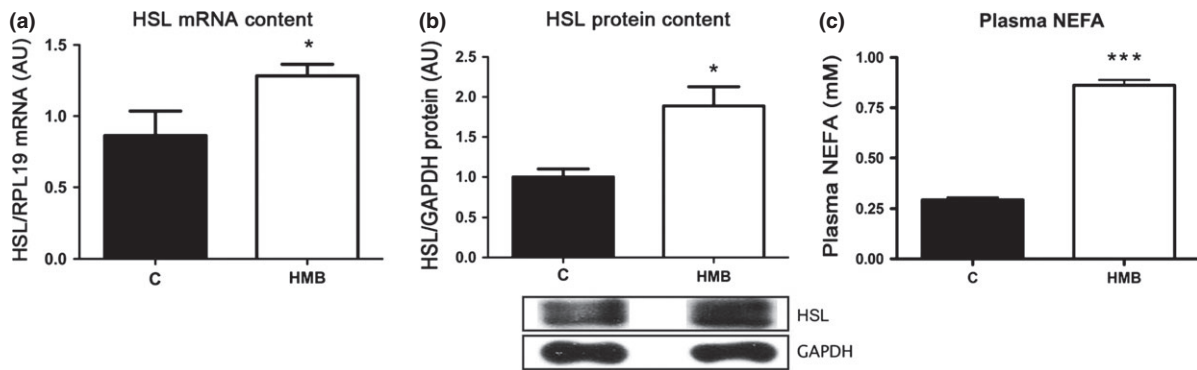


**Figure 5** Western blotting analysis of total content of pY STAT5 phosphorylation activity in liver (a) and white adipose tissue (WAT) (b) from hydroxy beta methylbutyrate (HMB)-supplemented [hydroxy beta methylbutyrate (HMB)] and control (C) rats. The data obtained in all experiments are represented in the graphics and expressed as mean  $\pm$  SEM. The bottom shows the autoradiogram of one in a total of two experiments. \* $P < 0.05$  vs. control ( $n = 9$  animals per group). The Ponceau-stained nitrocellulose membrane was used as housekeeping.

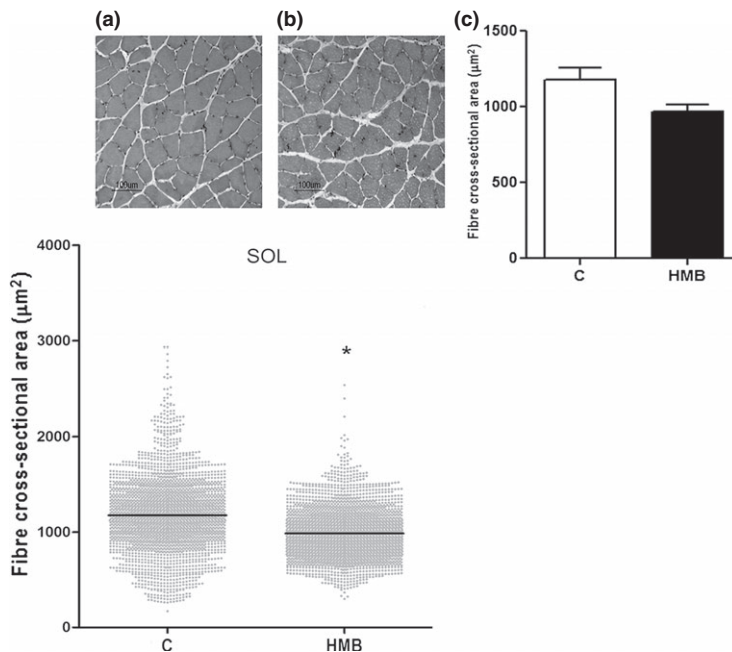
interfere with GLUT4 expression (Armoni *et al.* 2007, Martins *et al.* 2012), and considering that we have detected a reduction of total GLUT4 protein content in SOL muscle, as well as a reduction of peripheral insulin sensitivity, we could point out the increased plasma NEFA levels as an additional mechanism for the establishment of insulin resistance in HMB-supplemented rats, a point which is supported by several studies (Kelley *et al.* 2002, Raz *et al.* 2005, Deng *et al.* 2012). Indeed, type 2 diabetic patients present an increased plasma NEFA levels which is associated with accumulation of lipid inside the muscle cells, compromising their glucose uptake (Phielix & Mensink 2008).

Moreover, the diabetogenic effect of long-term GH exposition is well recognized (Vijayakumar *et al.* 2010). Impairment of insulin action has been observed





**Figure 6** Hormone-sensitive lipase (HSL) mRNA (a) and protein (b) expression in white adipose tissue (WAT), and plasma concentration of non-esterified fatty acids (NEFA) (c) from HMB-supplemented [hydroxy beta methylbutyrate (HMB)] and control (C) rats. Data are expressed as mean  $\pm$  SEM. \* $P < 0.05$ /\*\* $P < 0.001$  vs. control ( $n = 6$  animals per group). In (b), the bottom shows the autoradiogram of one in a total of two experiments. GAPDH and RPL-19 were used as internal control for protein and mRNA normalization respectively.



**Figure 7** Fibre Cross-sectional area (CSA) analysis of soleus skeletal muscle (SOL) from hydroxy beta methylbutyrate (HMB)-supplemented (HMB) and control (C) rats. The figure illustrates transverse muscle sections stained with hematoxylin and eosin from SOL of control (a) and HMB-supplemented (b) rats. Quantitative analysis of the CSA in  $\mu\text{m}^2$  is presented in (c). \* $P < 0.05$  vs. control ( $n = 5$  animals per group).

in rats chronically supplemented with GH (Thirone *et al.* 1997), in mice overexpressing human GH (Boparai *et al.* 2010), and in acromegalic patients (Hansen *et al.* 1986, Kinoshita *et al.* 2011). Additionally, increased GH levels by arginine supplementation in healthy sedentary rats were able to reduce insulin signalling in liver, skeletal muscle and WAT, and total GLUT4 content in SOL muscle (de Castro Barbosa *et al.* 2009). In fact, it is well described that there is a cross-talk between GH and insulin intracellular signalling pathways, which leads to an impairment of insulin action, mainly on liver, in conditions of high GH levels (Dominici *et al.* 2005, Taniguchi *et al.* 2006). This also might support the insulin resistance observed in HMB-supplemented rats in the present study.

Growth hormone interacts with members of cytokine receptors recruiting JAK2 which, in turn, phosphorylates tyrosine residues of GH receptor (GHR). This event permits the phosphorylation and dimerization of STATs which is translocated into the nucleus enhancing the expression of several genes, as those encoding SOCS protein which exerts a negative feedback loop in JAK/STAT signalling pathway (Rawlings *et al.* 2004). In cases of chronic GH excess, an overexpression of SOCS3 is observed, which blocks the tyrosine phosphorylation sites of IR, impairing the insulin signalling pathway (Dominici *et al.* 2005). Moreover, the overexpression of SOCS1 and SOCS3 enhances pS IR and IRS-1, increases proteasomal degradation of IRS-1 and reduces the interaction between IR and IRS

by blocking tyrosine residues which are classical mechanisms associated with insulin resistance (Krebs & Hilton 2003, Dominici *et al.* 2005).

Considering that, we also evaluated the JAK/STAT signalling pathway on the liver of HMB-supplemented animals. However, we did not observe any significant alteration in SOCS3 and pY STAT5 content between HMB-supplemented and control rats (Figs 4a and 5a respectively), excluding the participation of both proteins in the insulin resistant state detected.

It should be pointed that, besides the high NEFA levels, the HMB-supplemented rats present hyperinsulinaemia, which is also known to contribute to the development of insulin resistance (Thirone *et al.* 1997, Zhande *et al.* 2002, DeFronzo 2004, Gerlinger-Romero *et al.* 2011). Furthermore, decreased total GLUT4 protein content in muscle has also been associated to chronic hyperinsulinaemia, partially explaining our data (Machado *et al.* 1992, Papa *et al.* 1997, de Carvalho Papa *et al.* 2002, Ledwig *et al.* 2002).

We also observed a reduction in CSA fibre of SOL muscle. This event has also been described in insulin resistant mice and related to a reduction of PI3-kinase activity and pS AKT content, leading to increased activation of caspase-3 and ubiquitin-proteasome pathway, both contributing to imbalanced proteogenic/proteolytic activity (Wang *et al.* 2006, Chaudhary *et al.* 2012). These reports corroborate our results, which showed a reduction on pS AKT content on SOL of HMB-supplemented rats (Fig. 2b), but seem not to be in agreement with reports showing that HMB increases muscle mass (Wilson *et al.* 2008). However, most of these reports refer to conditions of muscle wasting, like cancer and sepsis (Smith *et al.* 2005, Kuhls *et al.* 2007), in which proteolysis is increased, and the energetic metabolism is seriously impaired. Interestingly, HMB supplementation was reported to activate S6K1 in Wistar rats carrying tumour (Aversa *et al.* 2011), and the S6K1 is known to phosphorylate the IRS1 in serine, thus inactivating this substrate and impairing insulin signalling pathway (Tremblay & Marette 2001). This could explain the presently described insulin resistance, which, in long-term, could impair the whole-body glucose homeostasis.

Some investigations of HMB effects have been performed in other animals than rats, aiming at commercial profit by improving meat production. These studies include swine, bovine, ovine and chicken, and it is important to consider that: (i) improvement in muscle development was clearly observed only in broiler chicken breasts (Qiao *et al.* 2013); (ii) hyperinsulinaemia in response to food ingestion, a signal of insulin resistance, was observed in lambs (Papet *et al.*

1997); and (iii) hyperthyroidism was clearly demonstrated in chicken (Qiao *et al.* 2013).

In summary, the HMB effectiveness in humans seems to be restricted to physical exercise or skeletal muscle proteolytic conditions, and the absence of adverse effects has been restricted to haematological parameters (Baxter *et al.* 2005, Molfino *et al.* 2013, Fuller *et al.* 2014). Furthermore, glycaemic homeostasis and/or insulin sensitivity impairments have not been investigated yet, and based on the present results, this analysis should be initiated immediately in human studies involving HMB supplementation.

## Conclusions

This study showed that rats chronically supplemented with HMB without engaged to physical exercise protocol or under conditions of severe muscle loss (cancer and AIDS) presents decreased peripheral insulin sensitivity detected mainly in liver and SOL skeletal muscle, which in long-term could lead to an increased risk of developing type 2 diabetes.

## Ethical standards

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

## Conflict of interest

The authors declare that they have no conflict of interest.

This research was supported by Fundação de Amparo Pesquisa do Estado de São Paulo (FAPESP), and Conselho Nacional de Pesquisa e Desenvolvimento (CNPq), Brazil. C. Y. Y. is recipient of fellowship from FAPESP. F. G-R., L. G-F and C. R. F. Jr. are recipients of fellowships from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). R. S. C. and S. S. T. are recipients of fellowships from FAPESP. M. T. N. is recipient of fellowship from CNPq. The authors would like to thank Dr. Aداوري Brezolin for English revision of the manuscript and Prof. Dr. José Donato Júnior from Institute of Biomedical Sciences of University of São Paulo for providing the primary antibodies (pY STAT5 and SOCS3) for Western Blot analysis.

## References

- Armoni, M., Harel, C. & Karnieli, E. 2007. Transcriptional regulation of the GLUT4 gene: from PPAR-gamma and FOXO1 to FFA and inflammation. *Trends Endocrinol Metab* 18, 100–107.

- Aversa, Z., Bonetto, A., Costelli, P., Minero, V.G., Penna, F., Baccino, F.M., Lucia, S., Rossi Fanelli, F. & Muscaritoli, M. 2011.  $\beta$ -hydroxy- $\beta$ -methylbutyrate (HMB) attenuates muscle and body weight loss in experimental cancer cachexia. *Int J Oncol* 38, 713–720.
- Baxter, J.H., Carlos, J.L., Thurmond, J., Rehani, R.N., Bultman, J. & Frost, D. 2005. Dietary toxicity of calcium beta-hydroxy-beta-methyl butyrate (CaHMB). *Food Chem Toxicol* 43, 1731–1741.
- Bergan, H.E., Kittilson, J.D. & Sheridan, M.A. 2013. PKC and ERK mediate GH-stimulated lipolysis. *J Mol Endocrinol* 51, 213–224.
- Bernstein, A., Safirstein, J. & Rosen, J.E. 2003. Athletic ergogenic aids. *Bull Hosp Jt Dis* 61, 164–171.
- Boparai, R.K., Arum, O., Khardori, R. & Bartke, A. 2010. Glucose homeostasis and insulin sensitivity in growth hormone-transgenic mice: a cross-sectional analysis. *Biol Chem* 391, 1149–1155.
- Campello, R.S., Alves-Wagner, A.B., Abdulkader, F., Mori, R.C. & Machado, U.F. 2012. Carbohydrate- and lipid-enriched meals acutely disrupt glycemic homeostasis by inducing transient insulin resistance in rats. *Can J Physiol Pharmacol* 90, 537–545.
- Carrel, A.L. & Allen, D.B. 2000. Effects of growth hormone on adipose tissue. *J Pediatr Endocrinol Metab* 13(Suppl 2), 1003–1009.
- de Carvalho Papa, P., Vargas, A.M., da Silva, J.L., Nunes, M.T. & Machado, U.F. 2002. GLUT4 protein is differently modulated during development of obesity in monosodium glutamate-treated mice. *Life Sci* 71, 1917–1928.
- de Castro Barbosa, T., de Carvalho, J.E., Poyares, L.L., Bordin, S., Machado, U.F. & Nunes, M.T. 2009. Potential role of growth hormone in impairment of insulin signaling in skeletal muscle, adipose tissue, and liver of rats chronically treated with arginine. *Endocrinology* 150, 2080–2086.
- Chaudhary, P., Suryakumar, G., Prasad, R., Singh, S.N., Ali, S. & Ilavazhagan, G. 2012. Chronic hypobaric hypoxia mediated skeletal muscle atrophy: role of ubiquitin-proteasome pathway and calpains. *Mol Cell Biochem* 364, 101–113.
- Cho, Y., Ariga, M., Uchijima, Y., Kimura, K., Rho, J.-Y., Furuhashi, Y., Hakuno, F., Yamanouchi, K., Nishihara, M. & Takahashi, S.-I. 2006. The novel roles of liver for compensation of insulin resistance in human growth hormone transgenic rats. *Endocrinology* 147, 5374–5384.
- Chromy, V., Gergel, J., Voznicek, J., Krombholzova, L. & Musil, J. 1977. Assay of serum free fatty acids by extraction-photometric procedure. *Clin Chim Acta* 80, 327–332.
- van Dam, E.M., Govers, R. & James, D.E. 2005. Akt activation is required at a late stage of insulin-induced GLUT4 translocation to the plasma membrane. *Mol Endocrinol* 19, 1067–1077.
- DeFronzo, R.A. 2004. Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am* 88, 787–835, ix.
- Deng, Y.-T., Chang, T.-W., Lee, M.-S. & Lin, J.-K. 2012. Suppression of free fatty acid-induced insulin resistance by phytopolyphenols in C2C12 mouse skeletal muscle cells. *J Agric Food Chem* 60, 1059–1066.
- Dominici, F.P., Argentino, D.P., Muñoz, M.C., Miquet, J.G., Sotelo, A.I. & Turyn, D. 2005. Influence of the crosstalk between growth hormone and insulin signalling on the modulation of insulin sensitivity. *Growth Horm IGF Res* 15, 324–336.
- Fabres-Machado, U. & Saito, M. 1995. The effect of adipose cell size on the measurement of GLUT 4 in white adipose tissue of obese mice. *Braz J Med Biol Res* 28, 369–376.
- Fain, J.N., Cheema, P., Tichansky, D.S. & Madan, A.K. 2008. Stimulation of human omental adipose tissue lipolysis by growth hormone plus dexamethasone. *Mol Cell Endocrinol* 295, 101–105.
- Fuller, J.C. Jr, Arp, L.H., Diehl, L.M., Landin, K.L., Baier, S.M. & Rathmacher, J.A. 2014. Subchronic toxicity study of beta-hydroxy-beta-methylbutyric free acid in Sprague-Dawley rats. *Food Chem Toxicol* 67, 145–153.
- Gerlinger-Romero, F., Guimarães-Ferreira, L., Giannocco, G. & Nunes, M.T. 2011. Chronic supplementation of beta-hydroxy-beta methylbutyrate (HMB) increases the activity of the GH/IGF-I axis and induces hyperinsulinemia in rats. *Growth Horm IGF Res* 21, 57–62.
- Hansen, I., Tsalikian, E., Beaufre, B., Gerich, J., Haymond, M. & Rizza, R. 1986. Insulin resistance in acromegaly: defects in both hepatic and extrahepatic insulin action. *Am J Physiol* 250, E269–E273.
- Harcourt, L.J., Holmes, A.G., Gregorevic, P., Schertzer, J.D., Stupka, N., Plant, D.R. & Lynch, G.S. 2005. Interleukin-15 administration improves diaphragm muscle pathology and function in dystrophic mdx mice. *Am J Pathol* 166, 1131–1141.
- Kelley, D.E., Goodpaster, B.H. & Storlien, L. 2002. Muscle triglyceride and insulin resistance. *Annu Rev Nutr* 22, 325–346.
- Kim, J.K., Zisman, A., Fillmore, J.J., Peroni, O.D., Kotani, K., Perret, P., Zong, H., Dong, J., Kahn, C.R., Kahn, B.B. & Shulman, G.I. 2001. Glucose toxicity and the development of diabetes in mice with muscle-specific inactivation of GLUT4. *J Clin Invest* 108, 153–160.
- Kinoshita, Y., Fujii, H., Takeshita, A., Taguchi, M., Miyakawa, M., Oyama, K., Yamada, S. & Takeuchi, Y. 2011. Impaired glucose metabolism in Japanese patients with acromegaly is restored after successful pituitary surgery if pancreatic  $\beta$ -cell function is preserved. *Eur J Endocrinol* 164, 467–473.
- Krebs, D.L. & Hilton, D.J. 2003. A new role for SOCS in insulin action. Suppressor of cytokine signaling. *Sci STKE* 2003, PE6.
- Kuhls, D.A., Rathmacher, J.A., Musngi, M.D., Frisch, D.A., Nielson, J., Barber, A., MacIntyre, A.D., Coates, J.E. & Fildes, J.J. 2007. Beta-hydroxy-beta-methylbutyrate supplementation in critically ill trauma patients. *J Trauma* 62, 125–131; discussion 131–132.
- Lafontan, M. & Langin, D. 2009. Lipolysis and lipid mobilization in human adipose tissue. *Prog Lipid Res* 48, 275–297.
- Ledwig, D., Muller, H., Bischoff, H. & Eckel, J. 2002. Early acarbose treatment ameliorates resistance of insulin-regulated GLUT4 trafficking in obese Zucker rats. *Eur J Pharmacol* 445, 141–148.

- Loud, K.J., Field, A.E. & Micheli, L.J. 2003. Primary care of the elite or elite-emulating adolescent male athlete. *Adolesc Med* 14, 647–661, vii.
- Machado, U.F., Nogueira, C.R. & Carpinelli, A.R. 1992. Obesity is the major cause of alterations in insulin secretion and calcium fluxes by isolated islets from aged rats. *Physiol Behav* 52, 717–721.
- Martins, A. R., Nachbar, R. T., Gorjao, R., Vinolo, M. A., Festuccia, W. T., Lambertucci, R. H., Cury-Boaventura, M. F., Silveira, L. R., Curi, R. & Hirabara, S. M. 2012. Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. *Lipids Health Dis* 11, 30.
- Michael, M.D., Kulkarni, R.N., Postic, C., Previs, S.F., Shulman, G.I., Magnuson, M.A. & Kahn, C.R. 2000. Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol Cell* 6, 87–97.
- Molfino, A., Gioia, G., Rossi Fanelli, F. & Muscaritoli, M. 2013. Beta-hydroxy-beta-methylbutyrate supplementation in health and disease: a systematic review of randomized trials. *Amino Acids* 45, 1273–1292.
- Molinero, O. & Márquez, S. 2009. Use of nutritional supplements in sports: risks, knowledge, and behavioural-related factors. *Nutr Hosp* 24, 128–134.
- Nissen, S., Sharp, R., Ray, M., Rathmacher, J.A., Rice, D., Fuller, J.C., Connelly, A.S. & Abumrad, N. 1996. Effect of leucine metabolite beta-hydroxy-beta-methylbutyrate on muscle metabolism during resistance-exercise training. *J Appl Physiol* (1985) 81, 2095–2104.
- Oakes, N.D., Cooney, G.J., Camilleri, S., Chisholm, D.J. & Kraegen, E.W. 1997. Mechanisms of liver and muscle insulin resistance induced by chronic high-fat feeding. *Diabetes* 46, 1768–1774.
- Panton, L.B., Rathmacher, J.A., Baier, S. & Nissen, S. 2000. Nutritional supplementation of the leucine metabolite beta-hydroxy-beta-methylbutyrate (hmb) during resistance training. *Nutrition* 16, 734–739.
- Papa, P.C., Seraphim, P.M. & Machado, U.F. 1997. Loss of weight restores GLUT 4 content in insulin-sensitive tissues of monosodium glutamate-treated obese mice. *Int J Obes Relat Metab Disord* 21, 1065–1070.
- Papet, I., Ostaszewski, P., Glomot, F., Obled, C., Faure, M., Bayle, G., Nissen, S., Arnal, M. & Grizard, J. 1997. The effect of a high dose of 3-hydroxy-3-methylbutyrate on protein metabolism in growing lambs. *Br J Nutr* 77, 885–896.
- Payne, E.T., Yasuda, N., Bourgeois, J.M., Devries, M.C., Rodriguez, M.C., Yousuf, J. & Tarnopolsky, M.A. 2006. Nutritional therapy improves function and complements corticosteroid intervention in mdx mice. *Muscle Nerve* 33, 66–77.
- Phielix, E. & Mensink, M. 2008. Type 2 diabetes mellitus and skeletal muscle metabolic function. *Physiol Behav* 94, 252–258.
- Portal, S., Zadik, Z., Rabinowitz, J., Pilz-Burstein, R., Adler-Portal, D., Meckel, Y., Cooper, D.M., Eliakim, A. & Nemet, D. 2011. The effect of HMB supplementation on body composition, fitness, hormonal and inflammatory mediators in elite adolescent volleyball players: a prospective randomized, double-blind, placebo-controlled study. *Eur J Appl Physiol* 111, 2261–2269.
- Promintzer, M. & Krebs, M. 2006. Effects of dietary protein on glucose homeostasis. *Curr Opin Clin Nutr Metab Care* 9, 463–468.
- Qiao, X., Zhang, H.J., Wu, S.G., Yue, H.Y., Zuo, J.J., Feng, D.Y. & Qi, G.H. 2013. Effect of beta-hydroxy-beta-methylbutyrate calcium on growth, blood parameters, and carcass qualities of broiler chickens. *Poult Sci* 92, 753–759.
- Rawlings, J.S., Rosler, K.M. & Harrison, D.A. 2004. The JAK/STAT signaling pathway. *J Cell Sci* 117, 1281–1283.
- Raz, I., Eldor, R., Cernea, S. & Shafir, E. 2005. Diabetes: insulin resistance and derangements in lipid metabolism. Cure through intervention in fat transport and storage. *Diabetes Metab Res Rev* 21, 3–14.
- Slater, G.J. & Jenkins, D. 2000. Beta-hydroxy-beta-methylbutyrate (HMB) supplementation and the promotion of muscle growth and strength. *Sports Med* 30, 105–116.
- Smith, T.R., Elmendorf, J.S., David, T.S. & Turinsky, J. 1997. Growth hormone-induced insulin resistance: role of the insulin receptor, IRS-1, GLUT-1, and GLUT-4. *Am J Physiol* 272, E1071–E1079.
- Smith, H.J., Mukerji, P. & Tisdale, M.J. 2005. Attenuation of proteasome-induced proteolysis in skeletal muscle by {beta}-hydroxy-{beta}-methylbutyrate in cancer-induced muscle loss. *Cancer Res* 65, 277–283.
- Stöckli, J., Fazakerley, D.J. & James, D.E. 2011. GLUT4 exocytosis. *J Cell Sci* 124, 4147–4159.
- Taniguchi, C.M., Emanuelli, B. & Kahn, C.R. 2006. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7, 85–96.
- Thirone, A.C., Carvalho, C.R., Brenelli, S.L., Velloso, L.A. & Saad, M.J. 1997. Effect of chronic growth hormone treatment on insulin signal transduction in rat tissues. *Mol Cell Endocrinol* 130, 33–42.
- Tonks, K. T., Ng, Y., Miller, S., Coster, A. C. F., Samochar-Bonet, D., Iseli, T. J., Xu, A., Patrick, E., Yang, J. Y. H., Junutula, J. R. et al. 2013. Impaired Akt phosphorylation in insulin-resistant human muscle is accompanied by selective and heterogeneous downstream defects. *Diabetologia* 56, 875–885.
- Tremblay, F. & Marette, A. 2001. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. *J Biol Chem* 276, 38052–38060.
- Tremblay, F., Lavigne, C., Jacques, H. & Marette, A. 2007. Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu Rev Nutr* 27, 293–310.
- Um, S.H., D'Alessio, D. & Thomas, G. 2006. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. *Cell Metab* 3, 393–402.
- Vijayakumar, A., Novosyadlyy, R., Wu, Y., Yakar, S. & LeRoith, D. 2010. Biological effects of growth hormone on carbohydrate and lipid metabolism. *Growth Horm IGF Res* 20, 1–7.
- Vukovich, M.D., Stubbs, N.B. & Bohlken, R.M. 2001. Body composition in 70-year-old adults responds to dietary beta-hydroxy-beta-methylbutyrate similarly to that of young adults. *J Nutr* 131, 2049–2052.

- Wallberg-Henriksson, H. & Zierath, J.R. 2001. GLUT4: a key player regulating glucose homeostasis? Insights from transgenic and knockout mice. *Mol Membr Biol* **18**, 205–211.
- Wang, X., Hu, Z., Hu, J., Du, J. & Mitch, W.E. 2006. Insulin resistance accelerates muscle protein degradation: activation of the ubiquitin-proteasome pathway by defects in muscle cell signaling. *Endocrinology* **147**, 4160–4168.
- Wilson, G.J., Wilson, J.M. & Manninen, A.H. 2008. Effects of beta-hydroxy-beta-methylbutyrate (HMB) on exercise performance and body composition across varying levels of age, sex, and training experience: a review. *Nutr Metab (Lond)* **5**, 1.
- Xing, L. & Quinn, P.G. 1993. Involvement of 3',5'-cyclic adenosine monophosphate regulatory element binding protein (CREB) in both basal and hormone-mediated expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene. *Mol Endocrinol* **7**, 1484–1494.
- Zhande, R., Mitchell, J.J., Wu, J. & Sun, X.J. 2002. Molecular mechanism of insulin-induced degradation of insulin receptor substrate 1. *Mol Cell Biol* **22**, 1016–1026.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Diet Composition of control and HMB-supplemented rats. The amount of each component *per* Kg of ration.

**Figure S1.** (a) Ponceau image of pY IR (Liver) and Ponceau image of pS AKT (Liver) (b) Ponceau image of pY IR (SOL) and Ponceau image of pS AKT (SOL), (c) Ponceau image of pY IR (WAT) and Ponceau image of pS AKT (WAT).

**Figure S2.** (a) Ponceau image of GLUT4 (SOL).

**Figure S3.** (a) Ponceau image of PEPCK (Liver), (b) Ponceau image of SOCS3 (Liver).

**Figure S4.** (a) Ponceau image of pY STAT5 (Liver), (b) Ponceau image of pY STAT5 (WAT).