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Produced β-hydroxybutyrate after β-hydroxy-β-methylbutyrate (HMB) administration may contribute HMB function in mice

Kazuki Ikeda [a,1], Masatomo Takahashi [b,1], Shunsuke Aburaya [b], Daiki Harada [a], Maki Ikeda [a], Yume Kitagawa [a], Yuki Soma [b], Yoshihiro Izumi [b], Takeshi Bamba [b], Mitsuhiro Furuse [a,*]

[a] Laboratory of Regulation in Metabolism and Behavior, Faculty of Agriculture, Kyushu University, Fukuoka, 819-0395, Japan
[b] Division of Metabolomics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, 812-8582, Japan

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ABSTRACT

β-Hydroxy-β-methylbutyrate (HMB) is an intermediate in the metabolism of the branched-chain amino acid leucine. HMB has several demonstrated effects on skeletal muscle function, some of which are contradictory. In addition, the effect of exogenous HMB intake on the levels of intermediate metabolites is not known. Therefore, we investigated changes in HMB metabolites after oral HMB administration in mice. First, ICR mice were treated with either distilled water or HMB (0.215 g/10 mL/kg). Sampling was performed at 0, 1, 6, 12, and 24 h after administration. Next, ICR mice were given distilled water or HMB (0.215 g/10 mL/kg/d) for 10 d. Mice given HMB shown a significant increase in liver β-methylcrotonyl-CoA and increased β-hydroxybutyrate in plasma and the gastrocnemius muscle 1 h after HMB administration. Mice administered HMB for 10 d showed significantly decreased food intake and body weight; however, the relative weight of the gastrocnemius muscle was significantly increased. These results may be attributed to an increase in β-hydroxybutyrate resulting from exogenous HMB, since β-hydroxybutyrate inhibits food intake and suppresses skeletal muscle catabolism. In conclusion, β-hydroxybutyrate, a metabolite of HMB, was found to play an important role in the function of HMB.

1. Introduction

β-Hydroxy-β-methylbutyrate (HMB) is a branched short-chain fatty acid (SCFA) produced from the metabolism of the branched chain amino acid (BCAA) leucine. It was hypothesized that HMB contributes to the skeletal muscle anti-catabolic effect of leucine and enhances the anabolic effect of skeletal muscle during resistance exercise [1]. Since those reports, substantial attention has been placed on the effects of HMB in increasing or decreasing muscle mass, but there remains a general lack of understanding of these processes [2]. However, anabolic effects of leucine and HMB via the mechanistic target of rapamycin complex1(mTORC1) are well established [3,4].

BCAAs, including leucine, are mainly metabolized in skeletal muscle. The primary metabolite of leucine is α-ketoisocaproate (KIC), and the transamination of α-ketoglutarate with nitrogen to form glutamate is metabolized by the metabolic enzyme BCAA transferase (BCAT) [5]. In the major BCAA pathway, KIC is metabolized in the mitochondria to form isovaleryl-CoA by branched-chain α-keto acid dehydrogenase [6, 7]. This is the committed step in leucine catabolism. Isovaleryl-CoA is then metabolized in succession to β-methylcrotonyl-CoA (MC CoA), β-methylglutaconyl-CoA (MG CoA), β-hydroxy-β-methylglutaryl-CoA (HMG CoA), and finally to acetoacetate and acetyl-CoA by HMG CoA lyase. Endogenous HMB is metabolized from KIC by KIC dioxygenase in the cytosol of the liver [8]. This is a minor pathway since it is estimated to utilize about 5–10% of available KIC [8]. HMB is metabolized to MC CoA through HMB CoA and feeds into the major pathway through isovaleryl-CoA. In other words, previous reports on the effect of HMB can be considered as a result of the administration of a large amount of HMB that is not normally produced during leucine metabolism. Therefore, we must understand how a large amount of HMB is metabolized.

Recent studies have reported that MG CoA and HMG CoA also post-translational modify enzymes to non-enzymatically regulate energy metabolism [9]. Similarly, acetyl-CoA enzymatically activity of BCAT and other metabolic enzymes by acetylation [10]. The ketone bodies acetoacetate and β-hydroxybutyrate are also ligands for the SCFA receptors G protein-coupled receptor (GPR) 41 and GPR43, and function

* Corresponding author.
E-mail address: furuse@brs.kyushu-u.ac.jp (M. Furuse).
[1] These two authors contributed equally to this work.

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as signaling molecules that involved in metabolic homeostasis via the sympathetic nervous system [11]. Thus, HMB metabolism includes important metabolites that are involved in the regulation of energy metabolism as substrates and signaling molecules. However, it remains unclear how exogenous HMB is metabolized. To understand the mechanism of action of HMB, the present study was conducted to evaluate acute metabolic changes in HMB and investigate the effect of HMB on mouse health.

2. Materials and methods

2.1. Animals

Seven-week-old male ICR mice weighing 32–34 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Female mice have a shorter sex cycle, and female sex hormones are involved in the control of appetite, leading to body weight changes [12]. Therefore, considering the possibility that female mice have a larger inter-individual error in body weight compared to males, we used male mice. Mice were housed individually under a light/dark cycle (lights on at 08:00, off at 20:00) at a temperature of 23 °C ± 1 °C. Water and diet (MF, Oriental Yeast, Tokyo, Japan) for laboratory rodents were available *ad libitum* (Supplementary Table 1). The size of the cage used in this study was 175 × 245 × 125 mm and the type of cage was polymethylpentene. All animal experiments reported here were conducted in accordance with the Guidelines for Animal Experiments in the Faculty of Agriculture at Kyushu University, as well as by law and a notification by the Japanese Government.

2.2. Experimental procedure

In the first experiment, after one week of acclimation, mice were allocated to two groups (n = 3 per group). The control group was orally administered distilled water (10 mL/kg), and the HMB group was orally administered HMB (0.215 g/10 mL/kg). All mice were anesthetized with isoflurane before euthanasia. Blood, gastrocnemius, and liver were

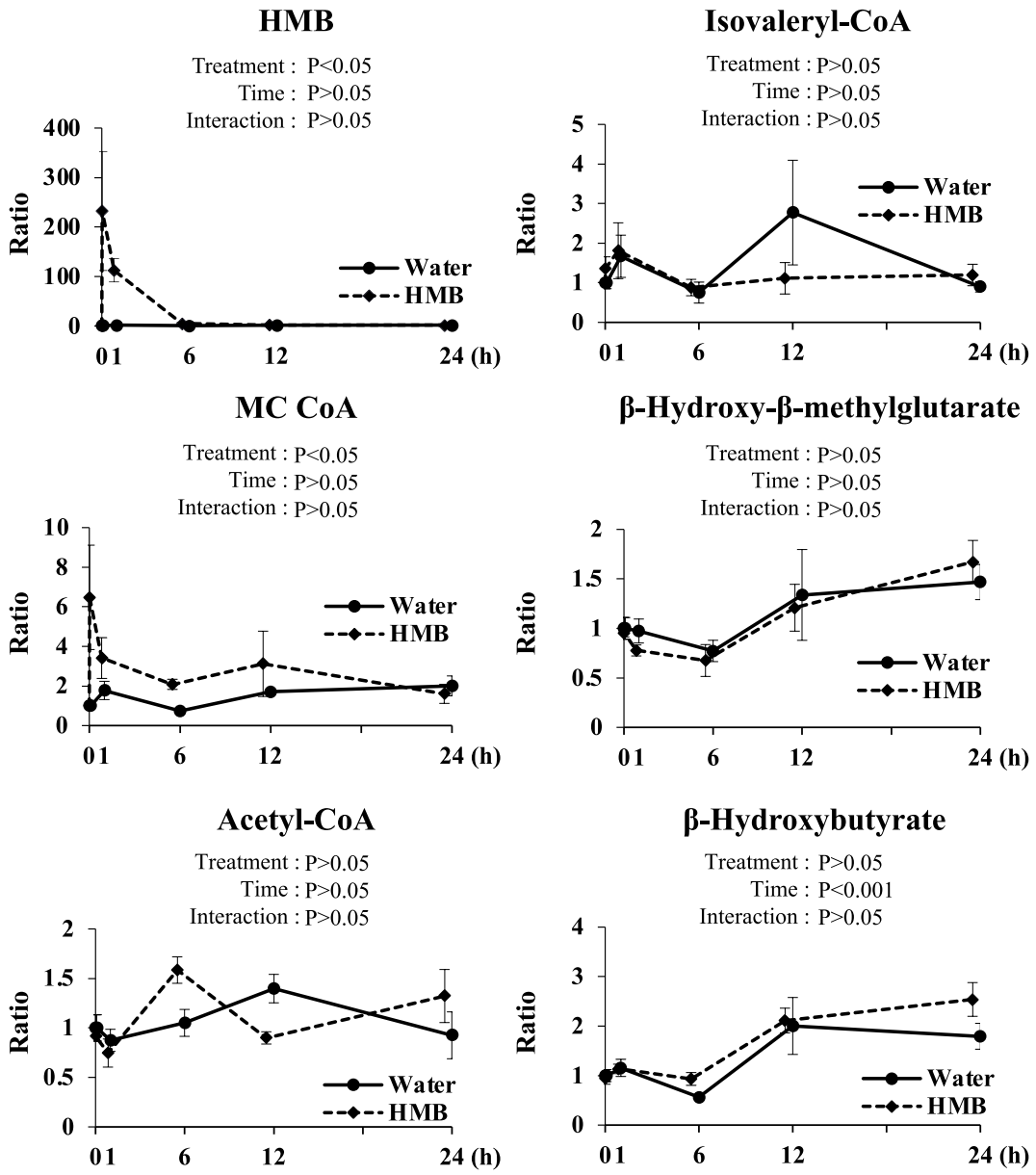

Fig. 1. Acute effect of oral HMB administration on the ratios of leucine metabolites in the liver. The ratio indicates the fold change for each group, with 0 h in the control group as 1. Values are expressed as means ± SEM; n = 3 in the water group; n = 3 in the HMB group. HMB: β-Hydroxy-β-methylbutyrate.

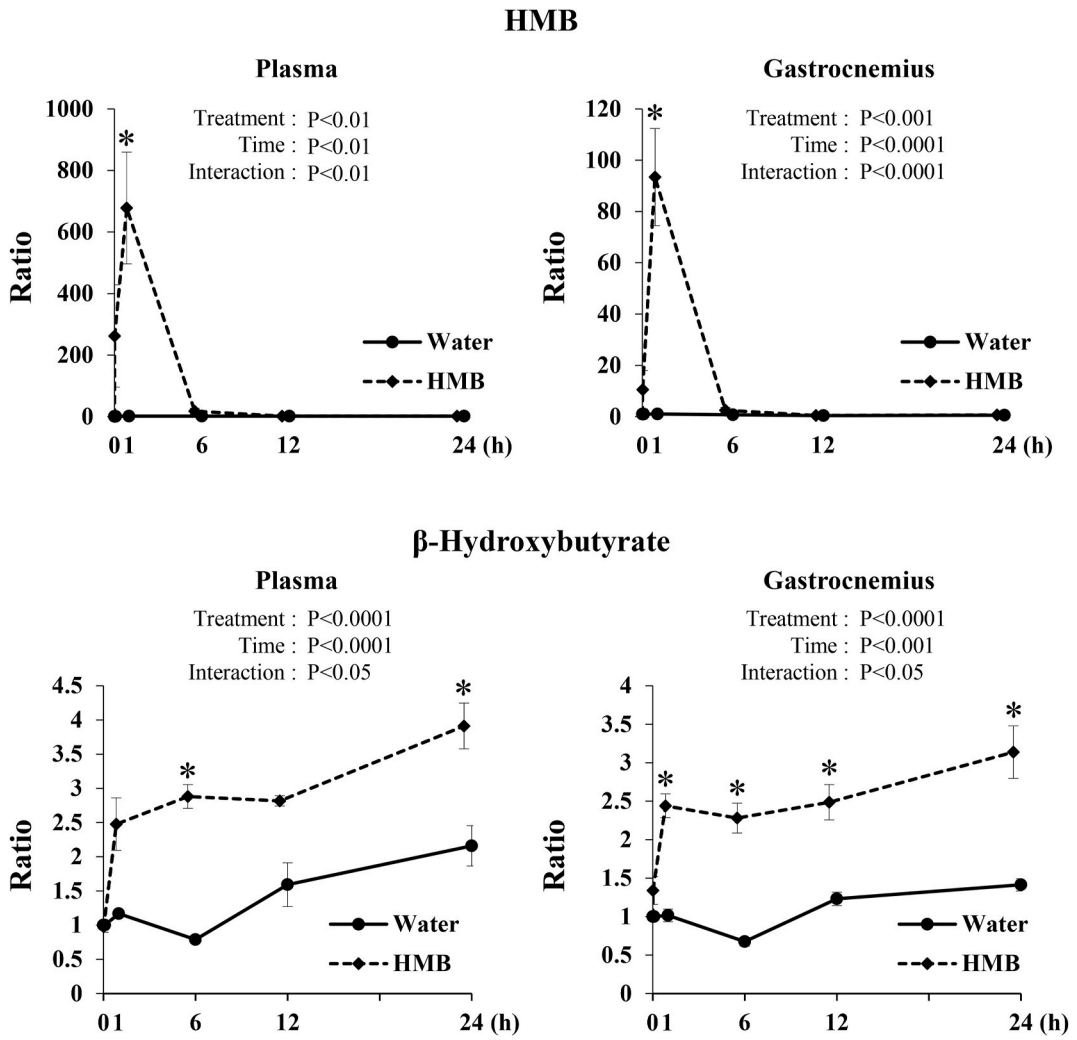

Fig. 2. Acute effect of oral HMB administration on the ratios of HMB and β-hydroxybutyrate in the plasma and gastrocnemius. Values are expressed as means ± SEM; n = 3 in the water group; n = 3 in the HMB group. HMB: β-Hydroxy-β-methylbutyrate. *P < 0.05 compared with values for the water group at the same time.

collected at 0, 1, 6, 12, and 24 h after administration. The purity of HMB (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was 96%. Since errors due to the use of a small number of animals were a concern, we used early stage of post-sexual maturation when inter-individual errors in body weight are relatively low, and fasted 3 h before oral administration to reduce the effects of feeding. In the analysis of metabolites, the internal standard method was used to correct for variations in the sensitivity of the analytical equipment and the injected sample volume. Thus, errors due to the use of a small number of animals were minimized.

In the second experiment, after one week of acclimation, mice were allocated to two groups (n = 7 per group). The control group was orally administered distilled water (10 mL/kg/d) and the HMB group orally administered HMB (0.215 g/10 mL/kg/d). Doses and days of HMB administration were determined according to Smith et al. [13]. Food consumption and body weight of each mouse were measured daily. After 10 d, all mice were anesthetized with isoflurane before euthanasia.

2.3. Tissue dissection

In the first experiment, the gastrocnemius and liver were collected immediately after euthanasia, placed into 2 mL safe-lock tubes (Eppendorf, Hamburg, Germany), flash frozen in liquid nitrogen, and stored at −80 °C. In the second experiment, the gastrocnemius muscle was dissected immediately after euthanasia.

2.4. Extraction procedure for metabolome analysis

Metabolite extraction was performed according to the method described by Bligh and Dyer (1959) with some modifications [14]. Briefly, 50 μL of plasma was transferred into a 2 mL Eppendorf tube and mixed with 950 μL cold methanol (−30 °C) containing propionyl-CoA (1.5 nmol) and 10-camphorsulfonic acid (1.5 nmol) (Merck, Darmstadt, Germany) as internal standards (ISs) for mass spectrometry-based metabolome analysis. The gastrocnemius and liver were homogenized on ice to a paste using a Power Masher II (Nippi, Tokyo, Japan) for 5 s. After crushing, the homogenate was transferred into a 2 mL Eppendorf tube and mixed with 1000 μL cold methanol (−30 °C) containing ISs. The samples were vigorously mixed by vortexing for 1 min, followed by 5 min for sonication, and placed on ice for 5 min. The samples were centrifuged at 16,000×*g* at 4 °C for 5 min and the supernatant (400 μL) was collected in clean tubes. After mixing with 400 μL chloroform and 320 μL water, phase separation of the aqueous and organic layers was performed via centrifugation (16,000×*g*, 4 °C, 5 min). The aqueous (upper) layer (500 μL) was transferred to a clean tube. After the aqueous layer extracts were evaporated under vacuum (TAITEC, Aichi, Japan), the dried extracts were stored at −80 °C until analysis of hydrophilic metabolites. Prior to metabolome analysis, the dried aqueous layer was reconstituted in 50 μL water.

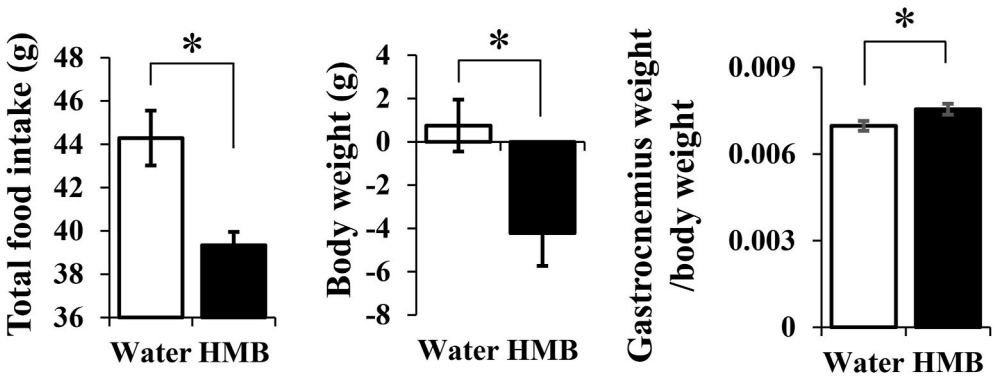

Fig. 3. Changes in total food intake, body weight, and relative gastrocnemius weight in mice after oral HMB administration. Results are expressed as means ± SEM; n = 7 in the water group; n = 7 in the HMB group. HMB: β-Hydroxy-β-methylbutyrate; *P < 0.05 compared with values for the water group.

2.5. Metabolome analysis

Three liquid chromatography mass spectrometry methods for metabolome analysis were employed as described previously [15,16]. Anionic polar metabolites (i.e., citrate, *cis*-aconitate, isocitrate, α-ketoglutarate, succinate, malate, fumarate, glucose) were analyzed using ion chromatography (Dionex ICS-5000$^+$ HPIC system, Thermo Fisher Scientific, Waltham, MA, USA) with a Dionex IonPac AS11-HC-4 μm column (2 mm i.d. × 250 mm, 4 μm particle size, Thermo Fisher Scientific) coupled with a Q Exactive, high-performance benchtop quadrupole Orbitrap high-resolution tandem mass spectrometer (Thermo Fisher Scientific) (IC/HRMS/MS). Cationic polar metabolites (i.e., leucine, KIC, HMB, β-hydroxy-β-methylglutarate, β-hydroxybutyrate, acetoacetate) were analyzed via liquid chromatography (Nexera X2 UHPLC system, Shimadzu, Kyoto, Japan) with a Discovery HS F5 column (2.1 mm i.d. × 150 mm, 3 μm particle size, Merck) coupled with a Q Exactive instrument (PFPP-LC/HRMS/MS). Acetyl-CoA, isovaleryl-CoA, and MC CoA were analyzed using LC (Shimadzu Co.) with a metal-free peek-coated InertSustain C18 column (2.1 mm i.d. × 150 mm, 3 μm particle size, GL Sciences Inc., Tokyo, Japan) coupled with a Q Exactive instrument (metal-free C18-LC/HRMS/MS). The three analytical platforms (i.e., IC/HRMS/MS, PFPP-LC/HRMS/MS, and metal-free C18-LC/HRMS/MS) for hydrophilic metabolite analysis were controlled using LabSolutions (version 5.80; Shimadzu Co.) and Xcalibur 4.2.47 (Thermo Fisher Scientific).

2.6. Statistical analysis

Data were analyzed using a two-way analysis of variance (ANOVA) in the first experiment and one-way ANOVA in the second. When significant ($P < 0.05$) effects were detected, the Dunnett test was used to evaluate differences. Analyses were performed using StatView version 5 (SAS Institute, Cary, NC, USA). Outlier data were eliminated using Thompson's test criterion for outlier observations ($P < 0.01$).

3. Results

3.1. Metabolism of HMB

The acute effects of oral administration of HMB on the ratios of leucine metabolites in the liver are shown in Fig. 1. HMB and MC CoA levels were significantly increased by HMB treatment. β-Hydroxybutyrate gradually increased over time, but this effect was not statistically significant. No other effects or interactions were observed. We also determined the ratios of TCA cycle intermediates, but no significant effects were observed (Supplementary Fig. 1).

The effect of transient high doses of HMB on the ratios of leucine and KIC upstream of HMB was also investigated. No significant effects of HMB treatment on leucine and KIC ratios were found in either the plasma or gastrocnemius (Supplementary Fig. 2).

3.2. HMB and β-hydroxybutyrate in the plasma and skeletal muscle

The acute effects of oral HMB administration on the ratios of HMB and β-hydroxybutyrate in the plasma and gastrocnemius of mice are shown in Fig. 2. In the plasma and gastrocnemius, a significant interaction between HMB treatment and time implied that HMB levels elevated quickly and disappeared within 6 h.

Significant main effects of HMB treatment and time, and a significant interaction between HMB treatment and time in plasma and gastrocnemius, suggest that β-hydroxybutyrate levels were quickly increased after HMB treatment and the differences between the treatment and control groups were maintained throughout the experimental time fram.

Acetoacetate in the plasma gradually but significantly increased during the experiment, but no significant effect of HMB treatment was observed. In contrast, acetoacetate was not detected in the gastrocnemius muscle (Supplementary Fig. 3).

3.3. Body weight, food intake, and relative weight of gastrocnemius muscle

The effects of oral administration of HMB on total food intake, body weight change, and relative weight of the gastrocnemius muscle are shown in Fig. 3. Total food intake was significantly decreased, and as a result, body weight decrased after treatment with HMB. However, the gastrocnemius muscle was significantly heavier than that in the control group.

4. Discussion

Previous studies have reported that HMB assists in increasing muscle strength in resistance exercise [1,17] and sports activities [18]. HMB also relieves the loss of skeletal muscle mass in old age [19], cancer cachexia [20], and immobility [21] based on gene and protein expression. However, depending on the subject and treatment, some reports suggest that HMB has beneficial effects, while others suggest that it does not [2]. To further elucidate the mechanism of action, it is necessary to consider and discuss changes in metabolism caused by HMB. Therefore, we investigated acute effects of HMB metabolism and longer term effects of oral administration of HMB on body weight, food intake, and skeletal muscle in mice.

First, the effect of HMB was investigated both upstream and downstream of HMB metabolism. Upstream, leucine and KIC ratios in the plasma and gastrocnemius were not significantly altered by large amounts of exogenous HMB (Supplementary Fig. 2). These results suggest that the functions of HMB are dependent on HMB itself and

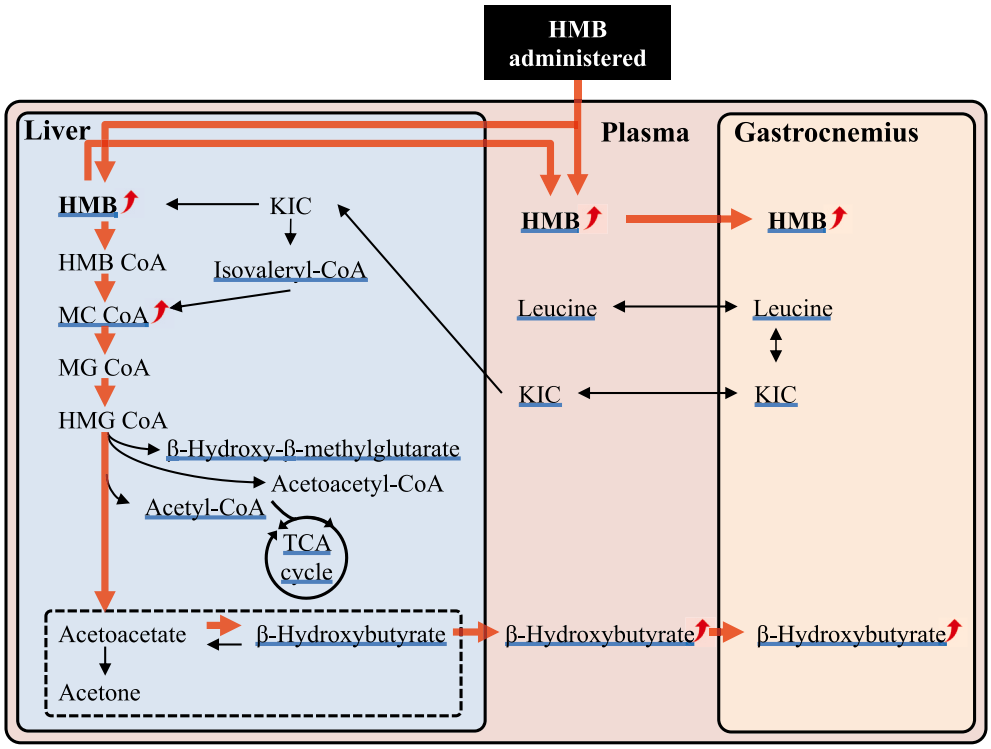

Fig. 4. Schematic models of leucine and HMB metabolic pathways. Underlines indicate detected metabolites; ↑, significant increased by HMB administration.

downstream metabolites. Focusing on HMB itself, orally administered HMB was rapidly metabolized in the liver, and could not be detected in the liver, gastrocnemius muscle, or plasma within 6 h after HMB administration (Figs. 1 and 2). The MC CoA ratio in the liver was significantly enhanced by HMB treatment (Fig. 1). However, isovaleryl-CoA in the liver was not affected by HMB treatment (Fig. 1). Thus, enhanced MC CoA was likely due to the HMB minor pathway through HMB CoA (not detected). Levels of acetyl-CoA (Fig. 1) and TCA metabolites (Supplementary Fig. 1) were not altered by HMB treatment. Among BCAA metabolites, no significant changes in β-hydroxybutyrate were observed in the liver (Fig. 1), but it is possible that β-hydroxybutyrate was rapidly released from the liver (Fig. 4). A previous report showed that ketone bodies were rapidly released from hepatocytes and were detected in the culture medium *in vitro* [15]. In fact, β-hydroxybutyrate in the gastrocnemius and plasma was significantly enhanced and remained high throughout the experiment (Fig. 2). Even though HMB disappeared within 6 h, the values for β-hydroxybutyrate were maintained for a long time. The half-life of β-hydroxybutyrate is approximately 0.8–3.1 h when ketone monoesters are administered [22]. It was reported that a single injection of β-hydroxybutyrate was rapidly excreted in the urine within 1 h and not detected after 3 h [23]. Based on the half-life of β-hydroxybutyrate, it was difficult to explain the long-term high values in plasma. When mice were given a ketogenic paste diet *ad libitum* (fat:protein ratio = 4:1) as a sole food source, they excreted high levels of β-hydroxybutyrate in the urine for more than 12 h [23]. The function of HMB for β-hydroxybutyrate production was similar to that of the ketogenic paste diet. Furthermore, plasma glucose was not changed by HMB administration (Supplementary Fig. 4). Therefore, it was suggested that the increase in β-hydroxybutyrate by HMB administration was not due to endogenous production associated with a decrease in glucose level, but rather HMB served as a substrate. However, the precise mechanism of HMB in β-hydroxybutyrate production is unclear at present. Further studies are necessary to clarify this mechanism.

The ketone bodies produced are transported to the brain, skeletal muscles, and other organs to provide an alternative energy supply. In addition to this role, recent studies have reported various other functions, such as anti-oxidation, anti-inflammation, and regulation of energy metabolism via receptor ligands and histone acetylation [24]. In particular, the function of GPR41 in noradrenaline release may explain the results of feeding inhibition (Fig. 3). The reason for feeding suppression by β-hydroxybutyrate may be due to inhibition of the sympathetic nervous system and suppression of noradrenaline secretion, since β-hydroxybutyrate contributes to these responses by antagonizing SCFA-GPR41 signaling [25]. Moreover, in healthy subjects who ingested exogenous ketone bodies, the levels of ghrelin, appetite, and food intake decreased [26]. Then decrease in food intake was also observed after intraperitoneal administration of β-hydroxybutyrate via blood circulation and intracerebroventricular administration, although the method of administration was different from the present study [27]. These results suggested that the decrease in food intake after 10 d of oral HMB administration was caused by an increase in β-hydroxybutyrate. In addition, β-hydroxybutyrate suppresses skeletal muscle catabolism caused by acute inflammation induced by lipopolysaccharide [28]. β-Hydroxybutyrate may also play a role in the skeletal muscle anti-catabolic effect reported for HMB. However, this study was a relative comparison of these substances and did not measure their concentrations. Therefore, it is still unclear whether the concentration of β-hydroxybutyrate is sufficient to activate GPR41. In addition, it is not clear whether HMB is a ligand for GPR41 or competes with β-hydroxybutyrate. Further investigation is needed to elucidate the mechanism of action.

In conclusion, this study evaluated the effects of oral administration of HMB on metabolites. The results showed that oral administration of HMB increased β-hydroxybutyrate levels in the plasma and gastrocnemius muscle. This suggested that the decrease in food intake and heavier gastrocnemius after 10 d of oral administration of HMB was due to the increase in β-hydroxybutyrate in mice.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101097.

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