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Age associated decline in the conversion of leucine to β-Hydroxy-β-Methylbutyrate in rats☆,☆☆

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ABSTRACT

Background: The loss of muscle mass is considered to be a major factor contributing to strength decline during aging. β-Hydroxy-β-Methylbutyrate (HMB), a metabolite of leucine has been shown to enhance muscle protein synthesis and attenuate loss of muscle mass by multiple pathways. However, the production and regulation of endogenous levels of HMB over the lifespan have not been investigated.
Objective: The objective of the present study was to do a cross-sectional analysis of the basal plasma levels of HMB in male Sprague–Dawley rats of different ages and to compare the efficiency of conversion of leucine to HMB in young versus older rats.
Methods: Plasma levels of HMB and α-ketoisocaproate (KIC) were analyzed in rats of different age groups (3, 9, 12 and 24 months old, $n = 10$ per group). Levels of 4-HPPD, the enzyme involved in the conversion of KIC to HMB in the liver were determined by ELISA. The conversion efficiency of leucine to HMB was compared between 3 and 24 month rats after an oral bolus dose of leucine.
Results: Endogenous circulating levels of HMB were significantly reduced in older age rats compared to young rats (100 ± 3.7 vs 156 ± 10 (mean ± SEM), ng/mL, $p < 0.001$). A significant negative correlation was seen between HMB levels and age. The liver levels of 4-HPPD were found to be significantly lower in old versus young rats. Consistent with this, the conversion efficiency of leucine to HMB was significantly lower in the aged versus young cohorts.
Conclusions: In summary, this study depicts for the first time that the basal levels of HMB, a metabolite of amino acid leucine, declines with age, and that this decline is due to perturbations in the key enzyme 4-HPPD which catalyzes the conversion of KIC to HMB. As a consequence, the efficiency of conversion of leucine to HMB is diminished in older rats compared to younger rats.

1. Introduction

Aging is associated with a decline in lean mass in humans (Forbes and Halloran, 1976) and rodents (Holloszy et al., 1991) with adverse physiological consequences. An age-associated decrease in lean mass, specifically skeletal muscle mass is associated with an increase in falls, functional decline, weakness and mortality (Lord et al., 1994; Visser et al., 2002; Laukkanen et al., 1995). The mechanisms underlying these changes in body composition with age are unclear. However, age-associated alterations in the muscle anabolic response to nutritional stimuli and a decline in protein intake may be significant contributing factors (Fujita and Volpi, 2006).

There is a growing body of literature to demonstrate that HMB, a metabolite of branched chain amino acid L-leucine (Nissen et al., 2000), can attenuate skeletal muscle atrophy under various catabolic conditions. These include cancer cachexia (May et al., 2002; Smith et al., 2005), AIDS (Clark et al., 2000), sepsis (Kovarik et al., 2010) and aging (Vukovich et al., 2001). Several studies have also proposed that HMB can stimulate muscle anabolism (Nissen et al., 1996; Slater and Jenkins, 2000) through the activation of the mTOR pathway (Eley et al., 2007; Kornasio et al., 1793). Several animal and human studies have reported beneficial effects of HMB supplementation for disease conditions characterized by muscle loss. More recently, HMB was demonstrated to improve strength and muscle quality without resistance exercise in the elderly (Stout et al., 2013), and

Abbreviations: 4-HPPD, 4-hydroxyphenylpyruvate dioxygenase; AUC 0–t, area under the plasma concentration-time curve (from 0 to time t); HMB, β-Hydroxy-β-Methylbutyrate; KIC, α-ketoisocaproate; KICD, α-ketoisocaproate dioxygenase; mTOR, Mammalian Target of Rapamycin.

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prevented muscle mass loss during 10 days of bed rest in healthy older adults (Deutz et al., 2013).

The endogenous conversion of leucine to HMB is minimal (Van Koevering and Nissen, 1992). In vitro studies in rat and human liver homogenates (Sabourin and Bieber, 1982, 1983) and in vivo studies in pigs and sheep provide evidence that the endogenous conversion efficiency of L-leucine to HMB is ~2–10% (Van Koevering and Nissen, 1992). L-Leucine metabolism to HMB is a 2-step process starting with its transamination to α-ketoisocaproate (KIC) (Supplementary Fig. 1). KIC is then either metabolized to isovaleryl-CoA by the enzyme α-ketoacid dehydrogenase (Paxton and Harris, 1982) in the mitochondria, or to HMB by KIC dioxygenase (KICD), also known as 4-hydroxyphenylpyruvate dioxygenase (4-HPPD) in the cytosol (Van Koevering and Nissen, 1992).

The regulation of HMB synthesis pathway with respect to age is poorly understood. In this study we sought to determine the effect of aging on the conversion efficiency of leucine to HMB in male Sprague Dawley rats from 3 to 24 months old. We also explored the efficiency of conversion of leucine to HMB in older versus young rats.

2. Materials and methods

2.1. Chemicals and reagents

CaHMB (purity 98.5%) was obtained from Technical Sourcing International Inc. (TSI, Missoula, MT). L-Leucine (HPLC grade with >98% purity) was procured from Sigma (St. Louis, MO, USA). Acetonitrile was from Merck Fine Chemicals (Mumbai, India); HPLC grade methyl-t-butyl ether (MTBE) was procured from Rankem Chemicals (India). Ultra-pure water (18.2 MΩ cm) was prepared using Millipore Milli-Q water purification system. Drug-free K_2-EDTA was obtained from Biochemed Services, USA. 4-HPPD assay kit was from USCN life sciences Inc. (Wuhan, China). All other chemicals and reagents used were of analytical grade and supplied by Merck (Darmstadt, Germany).

2.2. Animals

Male Sprague Dawley rats of different age groups (3, 9, 12 and 24 months old, $n = 10$ per group) were housed in standard polycarbonate cages under controlled environmental conditions (22 ± 3 °C temperature, 50 ± 20% humidity, a light/dark cycle of 12 h each and 15–20 fresh air changes per hour). Animals were housed group-wise (2 to 3 animals per cage) and were fed a, ad libitum, with a certified irradiated laboratory rodent diet (Nutrilab rodent diet, Product # F00NUA00003, containing 20% protein, 5.5% fat ether extract, 5% crude fibre, 51.5% nitrogen free extract, 3395 kcal/kg metabolizable energy and 7% ash) from Nutrilab brand, Tetragon Chemie, Bangalore, India. All the animals allowed to acclimatize for a period of about 7 days before initiation of the experiment. Animals had free access to water at all time.

2.3. Sample collection

The animals were fasted overnight and blood was collected from all the animals by tail-vein bleeding. Plasma samples were separated by centrifugation using micro17R refrigerated centrifuge (Thermo Scientific, Germany) at 8.9 × *g* for 5 min at 4 °C and stored at −80 °C for estimation of endogenous KIC and HMB levels. After blood collection, all the animals were euthanized by CO_2 asphyxiation. Liver samples were collected (~250–300 mg), flash frozen in liquid nitrogen and stored at −80 °C until further analysis for HPPD levels.

2.4. Ethics statement

This study was conducted in accordance with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) compliance. The 'Form B' for carrying out animal experimentation was reviewed and approved by the Institutional Animal Ethics Committee (SYNGENE/IAEC/286/03-2012). All aspects of the protocol and experimental design, including animal number, anesthesia and euthanasia methodologies were provided.

2.5. Quantitation of 4-HPPD levels in liver samples

4-HPPD levels were determined using ELISA Sandwich enzyme immunoassay according to the manufacturer's instructions. Briefly, frozen liver tissue samples weighing ~50 mg were homogenized in ice cold PBS in a total volume of 500 μL using bullet blender homogenizer (Next Advance, USA). The homogenates were then subjected to brief sonication followed by two cycles of freeze thaw to lyse the cells. The samples were centrifuged at 13,000 rpm for 20 min using Sorval T21 bench top centrifuge (Sorvall, USA) and supernatants were collected and stored at −80 °C until analysis. Samples and standards were added to pre-coated 96-well microplates and incubated at 37 °C for 2 h. 100 μL of detection reagent-A was added to all the wells and incubated for further 1 h at 37 °C. Plates were then washed and developed using TMB substrate followed by termination of reaction by adding stop solution provided in the kit. The color intensity was measured using a spectrophotometer at a wavelength of 450 nm. The concentration of 4-HPPD in the tissue samples was then determined by plotting the standard graph with concentration on x-axis and OD values on y-axis and performing a 4-PL fit to determine the unknown concentrations using GraphPad Prism 6.

2.6. Pharmacokinetic study

All animals were fed, ad libitum, with a certified irradiated laboratory rodent diet. Rats of different age groups (3, 9, 12 and 24 months old) ($n = 10$ per group) as described above were feed-deprived overnight (15 h), and had free access to drinking water. L-Leucine was administered at an oral dose of 1.35 g/kg body weight as a suspension in 0.25% carboxymethyl cellulose containing 0.1% Tween 80. About 0.25 mL of blood sample was collected from each animal into Eppendorf tubes with K_2EDTA at 0 h (pre-dose sample), 0.5, 1, 2, 3, 4, 6, 8 and 10 h post-dosing, and centrifuged using micro17R refrigerated centrifuge (Thermo Scientific, Germany) at 8.9 × *g* for 5 min to separate plasma. The plasma samples were stored at −80 °C until further analysis. The plasma concentration–time data were analyzed using a non-compartmental method with WinNonlin version 5.3 (Pharsight Corporation, Mountain View, CA, USA). The observed C_{max} and Tmax were obtained by visual inspection of the experimental data. The AUC was calculated using linear trapezoidal method. Baseline levels of KIC and HMB were not taken into account in the calculation to determine the pharmacokinetic parameters.

2.7. Quantitation of HMB and KIC in rat plasma by LC–MS/MS

The levels of HMB and KIC in plasma samples were determined using LC–MS/MS method using labelled HMB as internal standard (IS). A protein precipitation technique was employed to isolate the active analytes and IS from matrix. The chromatography was achieved using Restek column (Allure organic acid, 5 μ, 150 mm × 3.0 mm with flow rate at 700 μL/min of mobile phase containing water, acetonitrile, and methanol with formic acid). The mobile phase ratio consists of water:acetonitrile:methanol:formic acid (85:7.5:7.5:0.1). The working spiking solutions for calibration curve were prepared in methanol and water with formic acid (15:85:0.1) (diluent). An aqueous calibration curve standard was prepared in water by the addition of analytes and IS with 1 mL of diluent and injected into LC–MS/MS. The plasma samples (QC samples) were prepared by using 10 μL of sample volume, 5 μL of IS and 200 μL of diluent. The Q1 → Q3 transitions of