Nutrition 27 (2011) 92–99

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

β-Hydroxy-β-methylbutyrate modifies human peripheral blood mononuclear cell proliferation and cytokine production in vitro

Everson A. Nunes Ph.D. [a,*], Amy R. Lomax B.Sc. [b], Paul S. Noakes Ph.D. [b], Elizabeth A. Miles Ph.D. [b], Luiz C. Fernandes Ph.D. [a], Philip C. Calder Ph.D. [b]

[a] Department of Physiology, Biological Science Building, Federal University of Paraná, Curitiba-PR, Brazil
[b] Institute of Human Nutrition, School of Medicine, University of Southampton, Southampton, United Kingdom

ARTICLE INFO

Article history:
Received 7 May 2009
Accepted 26 December 2009

Keywords:
HMB
Cytokines
Lymphocyte
Proliferation
Immunomodulation
Cell cycle

ABSTRACT

Objective: The main objective was to investigate the potential immunomodulatory effects of β-hydroxy-β-methylbutyrate (HMB) in human cells.
Methods: Peripheral blood mononuclear cells were isolated from the blood of eight volunteers and assayed for proliferation, cell cycle progression, surface expression of CD25, intracellular expression of pERK1/2, and cytokine production after in vitro exposure to a range of HMB concentrations (0.1 to 10 mM).
Results: Above 1 mM, HMB decreased the extent of proliferation normally observed after stimulation by concanavalin A. The decrease was evident at 10 mM HMB, when the proliferation index was 50% reduced when compared with the absence of HMB. Cell cycle analysis demonstrated an increase in the proportion of cells at the G0-G1 phase at 10 mM HMB. CD25 and pERK1/2 expression were not related to the observed effect on proliferation. HMB affected the concentrations of all five cytokines measured following stimulation. Tumor necrosis factor-α concentration in the culture medium was reduced by ~35% at all HMB concentrations. Th1/Th2 cytokine production was modified toward a Th2 profile when HMB was at 1 or 10 mM. Thus, HMB at 10 mM impairs lymphocyte proliferation and progression through the cell cycle. The lowest concentration used here (0.1 mM) exerted some actions on cytokine production, including decreasing TNF-α production, but not on proliferation and cell cycle progression.
Conclusion: HMB may be a useful agent to consider for modulation of immune function in specific situations.

Introduction

The immune response is influenced by several factors, including nutritional status and nutrient balance [1-3]. Thus, use of nutritional strategies aiming to improve immune function and modify inflammatory processes in various clinical settings has increased in recent years [1]. The modulation of immune parameters seems to represent an effective way to control inflammatory status, prevent diseases, and increase lifespan [4].

The branched chain amino acid leucine modifies many cellular functions including those of immune cells [5]. However, some of the cellular effects promoted by exposure to leucine have been attributed to its metabolites rather than to leucine itself [6]. β-Hydroxy-β-methylbutyrate (HMB) is an intermediate of leucine metabolism that is formed by transamination to α-ketoisocaproate, in skeletal muscle, followed by oxidation of the α-ketoisocaproate in the cytosol of hepatic cells, and perhaps in other tissues [7]. Studies in fish showed that HMB improves the response to pathogenic challenges and to other stressors [8,9]. Such observations suggest that HMB might influence the immune response. In accordance with this suggestion, studies in fish showed that HMB can improve both innate and specific

Role of each author: Everson Nunes was responsible for the idealization of the project, cell cultures, data analysis, and manuscript writing; Amy Lomax did the CFDA assays and the respective data analysis; Paul Noakes recruited the volunteers and performed the blood sample collection and cytokine assays; Elizabeth Miles performed the cell cycle assays; Luiz Fernandes performed the critical evaluation of the data, statistical analysis, and manuscript writing; Philip Calder performed the project supervision, critical evaluation of the data, and manuscript writing.

* Corresponding author. Tel.: +55-41-3361-1784; fax: +55-41-3361-1784.
E-mail address: eanunes@ufpr.br (E. A. Nunes).

0899-9007/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.nut.2009.12.008

immune function [10,11], while in vitro studies [8,9,12] showed that HMB can increase the response of piscine T and B lymphocytes and macrophages. Three studies have investigated the effect of HMB on mammalian immune responses [6,13,14]. However, as far as we are aware, there are no studies of HMB on human immune cell function besides proliferation [6].

In humans, HMB has been used to prevent body weight loss and skeletal muscle damage, and to increase skeletal muscle mass and strength in healthy and ill individuals [15]. The complete mechanism of action is unclear, but it is assumed that HMB acts like an anti-catabolic agent by interfering in specific wasting-linked cell signaling pathways [16,17]. Kuhls et al. [4] recently showed that critically ill trauma patients receiving HMB or a mix of HMB, arginine, and glutamine presented a reduction of the Systemic Inflammatory Response Syndrome (SIRS) incidence at day 3 and 7 after trauma when compared with a placebo. These findings indicate that HMB can potentially be used as a dietary immunomodulator, modifying the immune response to trauma leading to prevention of some adverse events triggered by its exacerbation. Immune responses involve and are regulated by several factors including extracellular cytokines and surface and/or intracellular protein expression. There is a lack of information about the effects of HMB on immune-related molecules, like cytokines. Recently it was shown that muscle cell lines exposed to HMB presented different expression and activation of the MAPK/ERK pathway [18]. This pathway is also an important regulator of immune cell activation, e.g., in lymphocytes [19]. Thus, effects of HMB on specific protein expression and/or activation might help to explain some of the actions reported in the experimental models referred to above.

Because there is little information on the effects of HMB on human immune function, this study set out to identify whether HMB influences the responses of human immune cells in vitro: outcomes studied were lymphocyte proliferation and cell cycle progression, cytokine production, and expression of cell surface and intracellular proteins. We hypothesized that HMB would modulate lymphocyte proliferation and T-cell-derived cytokine production in vitro and that these effects would be dose-dependent and related to modifications in cell surface expression of CD25 and intracellular expression of extracellular signal-regulated kinase 1 and 2 (ERK1/2).

Materials and methods

Chemicals and reagents

All chemicals were purchased from Sigma (Poole, UK) unless stated otherwise. HMB was purchased as the acid form (3-hydroxy-3-methyl butyric acid, 98%) from Alfa Aesar (Heysham, UK). Stock HMB solutions (100 mM) were prepared in RPMI culture medium, the pH set to 7.3, with 10 M NaOH, and then filtered through a 0.2 μM Millipore filter for further use in cell cultures. Fluorescein isothiocyanate conjugated mouse anti-human CD25 (IL-2Rα) monoclonal antibody (mAb), the isotype-matched non-reactive mouse IgG1 antibody, the phycoerythrin (PE) conjugated mouse anti-phospho-ERK1/2 antibody, and the negative control anti-human anti-phospho-ERK1/2 (T202/Y204) non-labeled antibody were purchased from BD Biosciences (Abingdon, UK).

Blood collection and peripheral blood mononuclear cells (PBMC) isolation

Venous blood was collected from eight healthy volunteers aged 23–45 y (three male and five female) with informed consent. Whole blood was collected into Vacutainer tubes (BD Biosciences, Oxford, UK) containing sodium heparin as anti-coagulant and was then layered over Histopaque-1077 (Sigma) density gradient separation solution and centrifuged at 300 × *g* for 20 min at room temperature. The mononuclear cell (PBMC) layer was removed and washed twice in RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. Cell viability and cell counts were assessed by Trypan blue exclusion.

Flow cytometry analysis of CD25 surface expression

Flow cytometric analysis was performed for CD25 expression on the surface of PBMC, after 24 h exposure to 0, 0.1, 1, and 10 mM HMB. One hundred sixty microliters of cell suspension (10^6 cells/mL) was cultured in 96-well plates with either 20 μL (final concentration, 5 μg/mL) ConA (stimulated) or RPMI (unstimulated). The total volume was made up to 200 μL with 20 μL HMB to give the desired final concentration. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. After 24 h culture, cells were transferred to flow cytometry tubes and washed twice with Pharmingen Stain Buffer (BD Biosciences). All samples were then fixed using BD Cytofix Fixation Buffer (BD Biosciences) according to the manufacturer's instructions and kept in Stain Buffer for further analysis. Subsequently, cells were maintained at 4°C for 30 min with 15 μL fluorescein isothiocyanate-labeled mouse anti-human anti-CD25 (IL-2Rα) mAb. Negative controls were incubated with isotype-matched non-reactive IgG1 antibody. Finally, the cells were washed twice in Stain Buffer and analyzed by flow cytometry using a FACScalibur and CellQuest-Pro software (BD Biosciences). Data were collected on 10°000 cells and were then analyzed using Weasel software v2.6.1.

Flow cytometry analysis of phospho-ERK1/2 intracellular expression

Intracellular phospho-ERK1/2 (pERK1/2) expression in PBMC was assayed by flow cytometry after 24 h exposure to 0, 0.1, 1, and 10 mM HMB. Cell cultures were set up and incubated for 24 h as described above. After culture, cells were transferred to flow cytometry tubes and washed twice with Pharmingen Stain Buffer. All samples were then fixed using BD Cytofix Fixation Buffer according to the manufacturer's instructions. Once fixed, cells were permeabilized using BD Phosflow–Perm Buffer III (BD Biosciences) following the manufacturer's instructions. For intracellular labeling, cells were maintained at 4°C for 30 min with 20 μL of PE-labeled mouse anti-human anti-pERK1/2 (T202/Y204) mAb. Negative controls were incubated with anti-human anti-pERK1/2 non-labeled antibody. Finally, the cells were washed twice in Stain Buffer and the data were acquired by flow cytometry using a FACScalibur and CellQuest-Pro software. Data were collected on 10°000 cells and were analyzed using Weasel software v.2.6.1.

5-Carboxyfluorescein diacetate labeling of PBMC

5-Carboxyfluorescein diacetate (CFDA) was stored frozen (−20°C) in a 5 mg/mL "stock solution." This was diluted eight-fold to prepare the working solution for each experiment. Ten microliters of the working solution was added for each mL of PBMC cell suspension (10^6 cells/mL) in prewarmed RPMI without fetal calf serum (FCS). After 10 min in the dark at 37°C, the cells were diluted 1:1 with 10% FCS in ice cold RMPI and incubated on ice in the dark for 5 min. Afterward, cells were washed twice and resuspended in complete medium (RPMI-1640, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin, 10% FCS) to yield a final cell concentration of 10^6 cells/mL. One hundred sixty microliters of cell

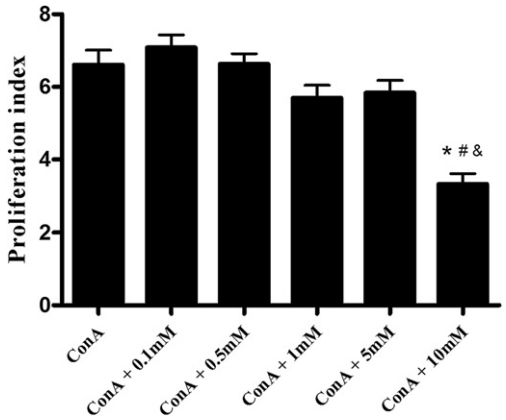

Fig. 1. Effect of β-hydroxy-β-methylbutyrate (HMB) on the proliferation index of cultured human peripheral blood mononuclear cells (PBMC). PBMC were loaded with 5-carboxyfluorescein diacetate (CFDA) and cultured in the presence of HMB (0, 0.1, 0.5, 1, 5, and 10 mM) and ConA (5 μg/mL) for 96 h. CFDA fluorescence was analyzed by flow cytometry. Proliferation index was calculated as the sum of cells in all generations divided by the calculated original parental cells. Data are presented as mean ± SEM of data for PBMC from eight volunteers. $^*P < 0.05$ compared with ConA alone. # $P < 0.05$ compared with ConA + 0.1 mM HMB. & $P < 0.05$ compared with ConA + 0.5 mM HMB.