



Beta-hydroxy-beta-methylbutyrate Inhibits Lipopolysaccharide-induced Interleukin-6 Expression by Increasing Protein Phosphatase-1 α Expression

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To cite this article:

Mitsutaka Yakabe, Sumito Ogawa, Hidetaka Ota, Katsuya Iijima, Masato Eto, Yasuyoshi Ouchi, Masahiro Akishita.

Beta-hydroxy-beta-methylbutyrate Inhibits Lipopolysaccharide-induced Interleukin-6 Expression by Increasing Protein Phosphatase-1 α Expression. *Biomedical Sciences*. Vol. 1, No. 1, 2015, pp. 1-5. doi: 10.11648/j.bs.20150101.11

Abstract: Beta-hydroxy-beta-methylbutyrate (HMB), a leucine metabolite, has been suggested to inhibit inflammation. However, its mechanism is not fully understood. Here we show that HMB repressed LPS-induced interleukin-6 (IL-6) expression by inhibiting the NF- κ B and AP-1 pathways. When protein phosphatase-1 α (PP1 α) was knocked down, the effects of HMB were partly abrogated. We conclude that HMB might repress inflammation by modulating PP1 α and its downstream NF- κ B and AP-1 pathways.

Keywords: Interleukin-6 (IL-6), NF- κ B (Nuclear Factor- κ B), p38, c-Jun N-terminal Kinase (JNK), Beta-hydroxy-beta-methylbutyrate (HMB)

1. Introduction

Amino acids are supposed to inhibit inflammation. For example, taurine significantly inhibited the increase in the expression of a pro-inflammatory chemokine, macrophage inflammatory protein 2 (MIP-2) in an experimental colitis mouse model (1). Thiotaaurine, a taurine metabolite, inhibited caspase-3 activity and apoptosis in human leukocytes (2). Branched-chain amino acids (BCAAs), especially leucine, might counteract exercise-induced inflammatory cytokine expression in muscle (3). A previous study showed that leucine attenuated the increased phosphorylation of double-stranded-RNA-dependent protein kinase (PKR) in skeletal muscle of mice bearing the MAC16 tumor by inducing PP1 α expression (4).

HMB is a metabolite of leucine, and is reported to possess some beneficial effects in humans. For example, it increased muscle size and strength when administered to healthy people, and attenuated muscle loss in the elderly and clinical patients (with AIDS, cancers etc.) (5). It increased the phosphorylation of mTOR, improved protein anabolism in muscles of an in vivo model of cancer cachexia (6), and attenuated dexamethasone-induced protein degradation (7).

HMB has also been suggested to possess anti-inflammatory effects. It reduced TNF α (tumor necrosis factor- α) production by human mononuclear cells in vitro (8). Chronic obstructive pulmonary disease (COPD) patients supplemented with HMB showed significantly lower white blood cell count and plasma C-reactive protein level (9). The combination of HMB, L-glutamine, and L-arginine has been reported to ameliorate radiation-induced acute inflammation and mucosal atrophy in rats (10). However, few studies have elucidated the mechanisms by which HMB inhibits inflammation.

Lipopolysaccharide (LPS) is commonly used as a stimulant of inflammation. It binds to Toll-like receptor 4 (TLR4) (11), increases expression of inflammatory cytokines by activating the NF- κ B and activator protein 1 (AP-1) pathways, and initiates immune responses (12). NF- κ B is localized in the cytoplasm, bound to its inhibitory subunit I κ B α (13). LPS phosphorylates and degrades I κ B α , then NF- κ B moves into the nucleus and binds to the κ B binding sites in the promoter and enhancer regions of various genes. The AP-1 pathway is regulated by MAPKs - c-Jun N-terminal kinase 1/2 (JNK1/2) and p38. AP-1 consists of c-Jun and c-Fos. c-Jun is phosphorylated and activated

mainly by JNK1/2 (14). The transactivation domain of c-Fos is phosphorylated and activated by p38 (15). MAPKs are activated by their upstream mitogen-activated protein kinase kinases (MKKs); JNK1/2 by MKK4, and p38 by MKK3/6 (16, 17). By blocking these pathways, inflammatory cytokines, including IL-6, TNF α and IL-1 β were inhibited (18-20).

In the present study, we stimulated RAW264.7 cells, a murine macrophage cell line, with LPS, and examined effects of HMB on inflammation. We investigated how HMB affected the NF- κ B pathway, the AP-1 pathway and LPS-induced cytokine expressions.

2. Materials and Methods

Materials. Fetal bovine serum (FBS) was purchased from Biowest (France). Dulbecco's modified essential medium (DMEM) was from Nikken Bio Med Lab (Japan). Penicillin-streptomycin was from Life Technologies Inc (NY, USA). HMB (as the calcium salt) was from Alfa Aesar (MA, USA). LPS was from Sigma Aldrich Japan. The mouse macrophage cell line RAW264.7 was obtained from the American Type Culture Collection (ATCC).

Cell Culture. RAW264.7 cells were cultured at 37°C in a humidified atmosphere (5% CO₂/95% air) in DMEM supplemented with 10% FBS, 100U/ml penicillin, and 100 μ g/ml streptomycin. When RAW264.7 cells reached confluence, they were subjected to experiments.

RNA analysis. RNA was extracted from treated cells using an RNeasy Mini Kit (Qiagen, CA, USA) according to the manufacturer's protocol. Approximately 1 μ g of total RNA was treated with DNase I (Fisher Scientific, PA, USA). cDNA synthesis from RNA was performed using an Omniscript RT Kit (Qiagen, CA, USA) and Oligo (dT) primers (Invitrogen, CA, USA). Quantitative real-time PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems, CA, USA). Reactions were performed in a 20 μ l mixture containing cDNA, specific primers for each gene and the SYBR Green Mastermix (Applied Biosystems, CA, USA). Specific PCR products were detected by measuring the fluorescence of SYBR Green, a double-stranded DNA-binding dye. After the real-time PCR reaction, the dissociation (melting) curve was generated to check the specificity of the PCR reaction. The relative mRNA expression level was calculated from the threshold cycle value of each PCR product and normalized to that of 36B4 by using the comparative threshold cycle method. All real-time PCR experiments were performed in triplicates. Primer sequences were as follows.

36B4 control: 5'-GTTTCAGCATGTTTCAGCAGTGTG - 3' (forward) and 5'-AATCTCCAGAGGCACCATTTGA -3' (reverse);

IL-6: 5'-TAGTCCTTCCTACCCCAATTTCC- 3' (forward) and 5'-TTGGTCCTTAGCCACTCCTTC-3' (reverse);

PPP1CA: 5'-ATGTCCGACAGCGAGAAGC-3' (forward) and 5'-ACAGCCGTAGAAGGTCATAGT -3' (reverse)

Luciferase assay and siRNA transfection. RAW264.7 cells were transfected with Lipofectamine LTX (Invitrogen, CA, USA) according to the manufacturer's protocol. AP-1-Luc plasmid (Stratagene, CA, USA) was transfected, and at the same time, a constant amount of pRL-Sv40-Luc plasmid (Promega, WC, USA) was transfected as control for transfection efficiency. RAW264.7 cells were pretreated with HMB (5mM) for 12h, and treated with LPS (1ng/ml) for an additional 12h. The cells were harvested with passive lysis buffer, then luciferase activity was measured with a DLReady Luminometer (Berthold Technologies, Germany). Luciferase activity was normalized by dividing AP-1-Luc activity by pRL-Sv40-Luc activity. These assays were conducted in triplicate.

For siRNA experiments, 50nM PP1 α siRNA (Santa Cruz, CA, USA) and control siRNA (Santa Cruz, CA, USA) were transfected with Lipofectamine LTX. After 48h of transfection, cells were subjected to western blotting and RNA analysis.

Western blotting. After subjected to treatments, RAW264.7 cells were washed with ice-cold PBS and then lysed in RIPA buffer with a protease inhibitor cocktail, cComplete Mini (Roche Applied Science, Germany) and a phosphatase inhibitor cocktail, PhosSTOP (Roche Applied Science, Germany), and subjected to SDS-PAGE, and then transferred to PVDF membranes. The membranes were blocked with TBS/Tween buffer containing 5% Phosphoblocker (Cell Biolabs, CA, USA) or non-fat milk, washed, and immunoblotted with antibodies. Primary antibodies against P-I κ B α , I κ B α , P-JNK1/2, total JNK1/2, P-p38, total p38, P-MKK4, P-MKK3/6 were from Cell Signaling (MA, USA), the antibody against PP1 α was from Santa Cruz (CA, USA), and the antibody against β -actin was from Sigma Aldrich Japan. Anti-rabbit IgG and anti-mouse IgG (GE Healthcare, UK) were used as the second antibody. After immunoblotting, the proteins were visualized by means of chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech, France). All western blotting experiments were performed at least three times.

3. Results

First, we examined the expression of LPS-induced inflammatory cytokines and the effects of HMB on them in RAW264.7 cells. LPS treatment increased IL-6 expression, and it was significantly downregulated by HMB (Fig. 1A). HMB also inhibited the LPS-induced TNF α , IL-1 β and iNOS expressions (data not shown).

We investigated whether HMB downregulated IL-6 through the NF- κ B pathway. After LPS treatment, I κ B α was phosphorylated and degraded. HMB inhibited the LPS-induced surge of P-I κ B α /I κ B α ratio by reducing P-I κ B α and increasing I κ B α (Fig. 1B). Therefore, HMB was suggested to prevent NF- κ B translocation into the nucleus and reduce cytokine expressions.

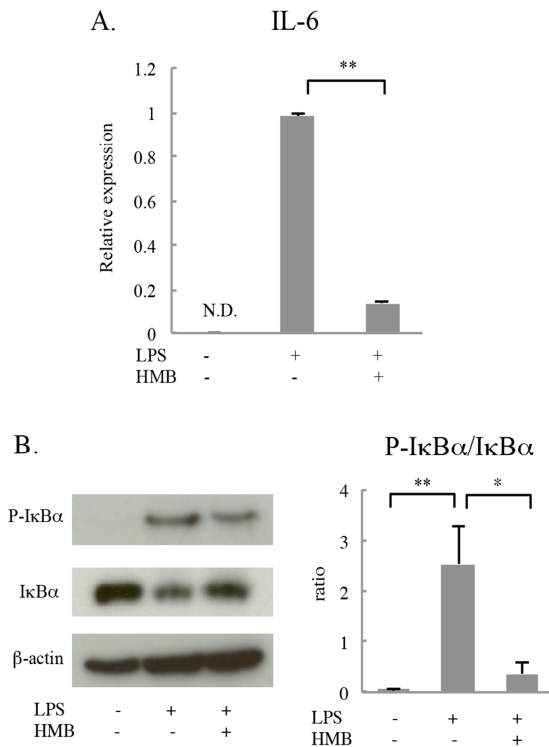


Figure 1. (A) After pretreatment with 5mM HMB for 12h, cells were treated for 2h with 1ng/ml LPS. (B) After pretreatment with 5mM HMB for 6h, cells were treated for 30min with 1ng/ml LPS. The β -actin loading control is shown below. The results are expressed as the phospho-I κ B α /I κ B α ratio.

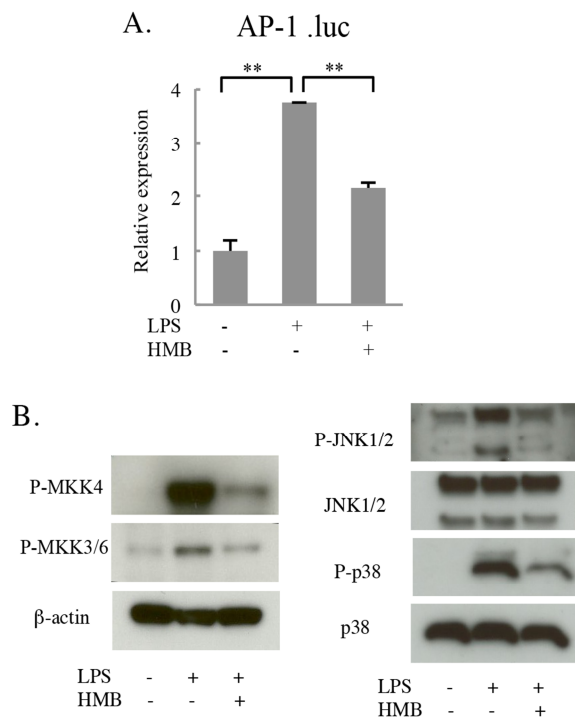


Figure 2. (A) After pretreatment with 5mM HMB for 12h, cells were treated for 12h with 1ng/ml LPS. (B) After pretreatment with 5mM HMB for 6h, cells were treated for 15min with 1ng/ml LPS. Phosphorylation of MKK4, phosphorylation of MKK3/6, phosphorylation of JNK1/2 and phosphorylation of p38 were examined, respectively. For P-MKK4 and P-MKK3/6, the β -actin loading control is shown.

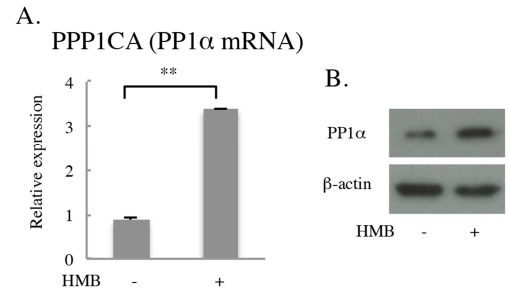


Figure 3. (A) Cells were treated with 5mM HMB for 3h and PP1 α mRNA was measured by realtime PCR. (B) Cells were treated with 5mM HMB for 6h and PP1 α protein was measured by Western blotting.

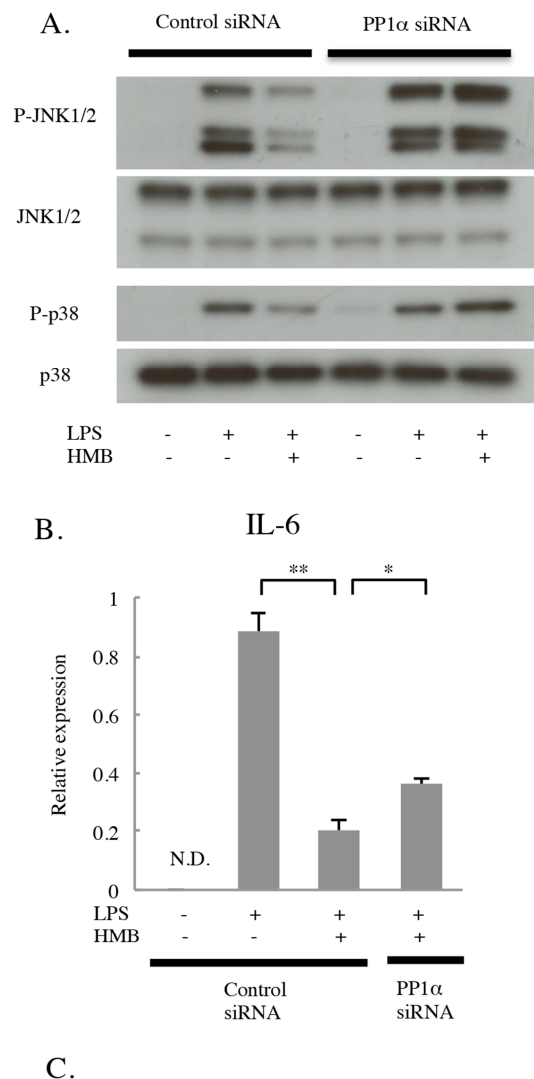


Figure 4. Effects of HMB on the LPS-induced phosphorylation of MAPKs (A), and IL-6 expression (B). PP1 α protein was partially knocked down by siRNA (C).

Next, we performed an AP-1 luciferase assay in order to examine whether HMB also had inhibitory effects on the AP-1 pathway. AP-1 luciferase activity was upregulated by LPS, and it was downregulated by HMB (Fig. 2A). We investigated the phosphorylation of MAPKs (JNK1/2, p38) that regulated AP-1 activity. JNK1/2 and p38 were phosphorylated by LPS, and HMB inhibited this phosphorylation. We examined the phosphorylation of MKK4 and MKK3/6, which phosphorylate and activate JNK1/2 and p38, respectively, and they showed a similar tendency (Fig. 2B).

Because leucine increases the PP1 α expression, we examined whether HMB also increased the expression of the gene. Treatment of RAW264.7 cells with HMB for 3h increased the expression of PPP1CA, which encodes PP1 α (Fig. 3A), and HMB treatment for 6h increased PP1 α protein (Fig. 3B). We hypothesized that PP1 α was involved in the effects of HMB. Therefore, we transfected PP1 α siRNA into RAW264.7 cells with Lipofectamine LTX, and investigated the effects of HMB on MAPK phosphorylation. PP1 α was partially knocked down (Fig. 4C). HMB inhibited the LPS-induced phosphorylation of p38 and JNK1/2, but PP1 α knockdown abrogated the effects of HMB (Fig. 4A). PP1 α knockdown partially but significantly abrogated the inhibitory effect of HMB on LPS-induced IL-6 expression (Fig. 4B).

4. Discussion

Inflammatory cytokines are implicated in various diseases. For example, release of IL-1, TNF α and IL-6 from rheumatoid synovium might induce local inflammation in rheumatoid arthritis (21). A theory called inflamm-aging (inflammation+aging) proposes that, as humans age, inflammatory cytokines are one of the causes of various diseases such as atherosclerosis, dementia, type 2 diabetes and osteoporosis (22, 23). Repressing inflammation could counteract symptoms of the diseases.

It has been suggested that HMB has various beneficial effects, such as stimulating protein synthesis by mTOR activation (6) and repressing inflammation, but the mechanisms are not fully understood. Leucine enters the cell through SLC7A5 amino acid transporter and activates mTOR (24). HMB is a leucine metabolite and activates mTOR (25), so HMB might also pass through the same transporter.

In our data, HMB downregulated LPS-induced expression of IL-6 (Fig. 1A) and other cytokines (data not shown). These data suggest anti-inflammatory effects of HMB. HMB inhibited the degradation of I κ B α (Fig. 2B), suggesting that HMB inhibited the translocation of NF- κ B into the nucleus and had transrepressive function. When administered to tumor-bearing rats, HMB increased the expression of I κ B α and reduced the expression of p65 in tumor cells (26). Taken together, our data support an inhibitory role of HMB in the NF- κ B pathway. HMB also repressed AP-1 luciferase activity (Fig. 2A, B). In a previous study, LPS-induced p38 phosphorylation was inhibited by HMB in C2C12 myotubes (27). In our data, JNK1/2 was also inhibited. HMB increased

PP1 α expression (Fig. 3A, B), as leucine did *in vivo* (4). PP1 α knockdown abrogated the effects of HMB on JNK1/2 and p38 phosphorylation (Fig. 4A), suggesting that PP1 α is essential in these effects. PP1 α knockdown also abrogated the inhibitory effect of HMB on LPS-induced IL-6 expression, but only partially (Fig. 4B). The catalytic subunit of PP1 α binds to PKR and inactivates it (28). PKR is essential in LPS-induced IL-6 expression, and efficiently phosphorylates its downstream MKK3/6 and MKK4 (29). Therefore, our data suggest that PP1 α might be a target of HMB. On the other hand, PP1 α knockdown did not affect the effects of HMB on LPS-induced I κ B α degradation (data not shown), so HMB might affect the NF- κ B pathway by other molecular mechanisms.

The human IL-6 promoter has both a κ B binding site and an AP-1 binding site, and when either of them is deleted, the promoter activation is inhibited to some degree (30). Similarly, TNF α and IL-1 β promoters are considered to have κ B binding sites and AP-1 binding sites (19, 20). Our data suggest that HMB blocks both of the pathways in IL-6 transrepression, and TNF α and IL-1 β might share the similar mechanisms.

5. Conclusion

In summary, our data suggest that HMB has anti-inflammatory effects and that the effects are, in some part, due to the increased PP1 α expression and the reduced NF- κ B and AP-1 activity.

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