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Original article

# High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation

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A R T I C L E I N F O

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A B S T R A C T

Leucine, isoleucine and valine are essential aminoacids termed branched-chain amino acids (BCAA) due to its aliphatic side-chain. In several pathological and physiological conditions increased BCAA plasma concentrations have been described. Elevated BCAA levels predict insulin resistance development. Moreover, BCAA levels higher than 2 mmol/L are neurotoxic by inducing microglial activation in maple syrup urine disease. However, there are no studies about the direct effects of BCAA in circulating cells. We have explored whether BCAA could promote oxidative stress and pro-inflammatory status in peripheral blood mononuclear cells (PBMCs) obtained from healthy donors. In cultured PBMCs, 10 mmol/L BCAA increased the production of reactive oxygen species (ROS) via both NADPH oxidase and the mitochondria, and activated Akt-mTOR signalling. By using several inhibitors and activators of these molecular pathways we have described that mTOR activation by BCAA is linked to ROS production and mitochondrial dysfunction. BCAA stimulated the activation of the redox-sensitive transcription factor NF-κB, which resulted in the release of pro-inflammatory molecules, such as interleukin-6, tumor necrosis factor-α, intracellular adhesion molecule-1 or CD40L, and the migration of PBMCs. In conclusion, elevated BCAA blood levels can promote the activation of circulating PBMCs, by a mechanism that involving ROS production and NF-κB pathway activation. These data suggest that high concentrations of BCAA could exert deleterious effects on circulating blood cells and therefore contribute to the pro-inflammatory and oxidative status observed in several pathophysiological conditions.

## 1. Introduction

Branched-chain aminoacids (BCAA: leucine, isoleucine and valine) are essential aminoacids. The intricate cellular balance of amino acid influx and efflux is maintained by A- and L-system of protein transporters which are regulated by hormones and amino acid starvation [1–3]. Unlike most amino acids, only a minor fraction of the dietary BCAA are metabolized by the liver; while the largest part of them enter to the systemic circulation to reach their main metabolism sites, including skeletal muscles, adipose tissue and brain [4,5].

*Abbreviations:* **AICAR**, 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; **AMPK**, AMP-activated protein kinase; **AP**, alkaline phosphatase; **BCAA**, branched-chain amino acids; **BCKDC**, branched-chain alpha-ketoacid dehydrogenase complex; **BSA/PBS**, bovine serum albumin/phosphate-buffered saline; **CD40L**, CD40 ligand; **DAPI**, 4',6-diamidino-2-phenylindole dihydrochloride; **DPI**, Diphenyleneiodonium chloride; **GAPDH**, Glyceraldehyde 3-phosphate dehydrogenase; **HRP**, Horseradish peroxidase; **ICAM-1**, intercellular adhesion molecule 1; **IL-6**, Interleukin-6; **LPS**, lipopolysaccharide; **MAPK**, Mitogen-activated protein kinase; **Mito-TEMPO**, 2,2,6,6-tetramethyl-4-[[2-(triphenylphosphonio)acetyl]amino]-1-piperidinyloxy, monochloride, monohydrate; **mTORC**, mammalian target of rapamycin complex; **NFκB**, nuclear transcription factor-κB; **Nrf2 or NFE2L2**, Nuclear factor (erythroid-derived 2)-like 2; **$O_2^{\bullet -}$**, Superoxide anion radical; **PBMC**, peripheral blood mononuclear cells; **p-NPP**, p-Nitrophenyl Phosphate; **ROS**, Reactive Oxygen Species; **RT-PCR**, Reverse transcription polymerase chain reaction; **PI3K/Akt**, phosphatydilinositol (3,4,5)-triphosphate; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **TMRM**, Tetramethyl rhodamine methyl ester; **TNFα**, Tumor necrosis factor alpha; **UCP-2**, uncoupling protein 2; **ΔΨm**, mitochondrial membrane potential

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In several pathological and physiological conditions increased BCAA plasma concentrations have been found. More than 50 years ago, slight but significant elevation of BCAA levels, between 0.38 and 0,67 mmol/L, were reported in obese subjects [6,7] as compared to 0.28–0.5 mmol/L in healthy population [8,9]. Later on, different metabolomics studies found out a negative association between plasma BCAA concentrations and insulin sensitivity in overweight and obese patients [10,11], suggesting that BCAA could be involved in insulin-related disorders. Genetic deficiency of BCAA catabolism leads to metabolic diseases, such as the maple syrup urine disease (MSUD) which is caused by a deficiency of branched-chain alpha-ketoacid dehydrogenase complex (BCKDC). MSUD patients present highly elevated BCAA concentrations in a range between 1 and 4 mmol/L, which are responsible of several neurological damage [12,13]. However, the mechanisms involved in this pathological process are poorly understood. Some studies suggested that BCAA are neurotoxic *per se* and enhance excitotoxicity in cortical neuronal cells through mechanisms that require the presence of astrocytes [14]. In addition, recent studies have reported that BCAA modulate the immune properties of microglial cells [15] and increased the inflammatory profile of MSUD patients [13].

The deficient mice in branched chain aminotransferase (BCATm KO), the first BCAA catabolic enzyme presented elevated plasma and tissue BCAA levels associated to heart, kidney and spleen hypertrophy [16]. However, there are no information about the potential direct effects of BCAA in circulating blood cells.

BCAA were known to exert several cell signalling responses mainly via the activation of the mammalian target of rapamycin (mTORC1) axis, which can result in hypertrophy [16], proliferation and migration in cancer cells [17] and in insulin resistance [11,18]. The conserved serine/threonine kinase mTOR is a downstream effector of phosphatidylinositol (3,4,5)-trisphosphate kinase (PI3K/AKT) which can form two distinct multiprotein complexes, mTORC1 and mTORC2. mTORC1 but not the mTORC2 is activated by diverse stimuli, such as growth factors, nutrients, energy and stress signals, via PI3K, MAPK or AMPK, in order to regulate cell growth, proliferation and survival [19,20]. Only mTORC1, but not mTORC2 is sensitive to rapamycin inhibition [21]. In cancer cells, the activation of mTOR signalling has also been linked to the generation of oxidative stress and the release of pro-inflammatory cytokines, mediated by the activation of the nuclear transcription factor-κB (NF-κB ) [22].

Despite the established association between elevated circulating BCAA and their deleterious effects, little is known about the capacity of BCAA to directly contribute to the pro-inflammatory and pro-oxidant status. The redox-sensitive nuclear transcription factor-κB (NF-κB) is a major player in inflammation-related responses in cardiovascular disease [23], but there are not studies about BCAA effects in this signalling pathway.

In the present study, we have explored whether extracellular BCAA could exert deleterious effects on circulating blood cells (PBMCs), the major cell type involved in the pathogenesis of inflammatory diseases) by the induction of oxidative processes and the up-regulation of pro-inflammatory factors. Moreover, the study aimed to gain insight into the signalling mechanisms activated by BCAA with particular emphasis on NF-κB pathway.

## 2. Materials and methods

### *2.1. Materials*

BCAA were prepared as a mixture of leucine, isoleucine and valine at 0.2–12 mmol/L from Sigma Aldrich (Sigma Chemical Co., St. Louis, MO, USA), lipopolysacharide (LPS; 1 μg/ml), glucose (30 mmol/l), insulin (1 nmol/L), rapamycin (100 nmol/L), wortmannin (1 μmol/L), diphenyliodonium chloride (DPI; 10 μmol/L), and sulforaphane (20 μmol/L) were obtained from Sigma Aldrich. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 0.5 mmol/L) was purchased from Toronto Research Chemicals, while BAY-11–7082 (1 mmol/L) and ML171 (0.5 μmol/L) were from Calbiochem (La Jolla, CA), mito-TEMPO (0.5 μmol/L) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and gp91dstat (5 μmol/L) was from Anaspec (Fremont, CA). IL-6 ($10^2$ U/ml) and TNF-α (30 ng/ml) were purchased from Preprotech (Preprotech, London UK). Medium RPMI and fetal bovine serum (FBS) were from Sigma Aldrich.

### *2.2. Cell culture*

Primary cultures of peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained at the Blood bank from Fundación Jiménez Díaz (FJD) after written informed consent. The procedure was approved by the Research Ethics Committee of Instituto de Investigaciones Sanitarias FJD. PBMCs were isolated by density centrifugation in Lymphoprep separation medium (MP Biomedicals, Ilikrich, France), and cultured in medium RMPI containing 5.5 mmol/L glucose and supplemented with 1% FBS, as described earlier [24].

### *2.3. Western blot*

Whole cell lysates were harvested in lysis buffer [25]. Lysates (30–50 μg per lane) were separated by 10% SDS-PAGE, transferred to nitrocellulose membranes (BioRad), and incubated with primary antibodies against p-mTOR (Ser2448), mTOR, p-Akt (Thr308), Akt, Nrf2, UCP-2 (C-terminal) (1/500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p-AMPK (Thr172 or Ser485/491) and AMPK, p-p65 (1/500; Cell Signalling, Boston, MA, USA), GAPDH (1/1000; Merck-Millipore). Appropriate HRP-labelled anti-mouse (1/5000, DAKO Cytomation) or anti-rabbit (1/5000, Santa Cruz Biotechnology) secondary antibodies were subsequently used for 1 h at room temperature. The signal was detected using Luminata Forte (Millipore Corporation, Billerica, MA, USA) with a ImageQuant LAS 4000 gel documentation system (GE Healthcare) and normalized to GAPDH.

### *2.4. RNA analysis*

Cells were harvested in TRIzol (Life Technologies Inc., Gaithersburg, MD, USA) to obtain total RNA, which was reverse transcribed using a high capacity cDNA RT kit (Applied Biosystems). Quantitative PCR (qPCR) was performed in 7500 Fast ABI System (Life Technologies Inc.) using commercial human Taqman assays: IL-6: Hs00174131_m1; TNFα: Hs00174128_m1; ICAM-1: Hs00164932_m1; CD40L: Hs00163934_m1; 18S rRNA: 4310893E.

### *2.5. Indirect immunofluorescence*

PBMCs were fixed using phosphate buffered 4% paraformaldehyde and permeabilised with 0.02% Triton X-100 for 10 min at RT. After blockade in 3% bovine serum albumin/phosphate-buffered saline (BSA/PBS), PBMCs were incubated with primary antibodies against p-p65 antibody (1/200, NF-κB-p65 C-20, Santa Cruz) or p-Nrf2 (1/200, Biorbyt, United Kingdom) overnight at 4 °C, followed by incubation with a secondary Alexa 488-conjugated anti-rabbit antibody (1/200; Life Technology) for 1 h at RT. For nuclear counterstaining 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI;1/5000, Sigma Aldrich) was used and the cells were visualized with a confocal microscope (Leica TCS SP2 with a 40× objective).

### *2.6. NADPH oxidase activity*

The $O_2^{\cdot -}$ production generated by NADPH oxidase activity was determined by a chemiluminescence assay, as described [26]. Briefly, PBMCs were rinsed with PBS and harvested in phosphate buffer pH 7.4 (50 mmol/L $KH_2PO_4$, 1 mmol/L EGTA, 150 mmol/L sucrose). The

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