Free Radical Biology and Medicine 104 (2017) 165–177

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

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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ARTICLE INFO

*Keywords:*
BCAA
Peripheral blood mononuclear cells
mTORC1
PI3K/Akt
Inflammation
Oxidative stress

ABSTRACT

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; **$O2^{\cdot -}$**, 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; **$\Delta\Psi m$**, mitochondrial membrane potential

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http://dx.doi.org/10.1016/j.freeradbiomed.2017.01.009
Received 4 July 2016; Received in revised form 23 December 2016; Accepted 6 January 2017
Available online 13 January 2017

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 $O_2^{\bullet -}$ 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

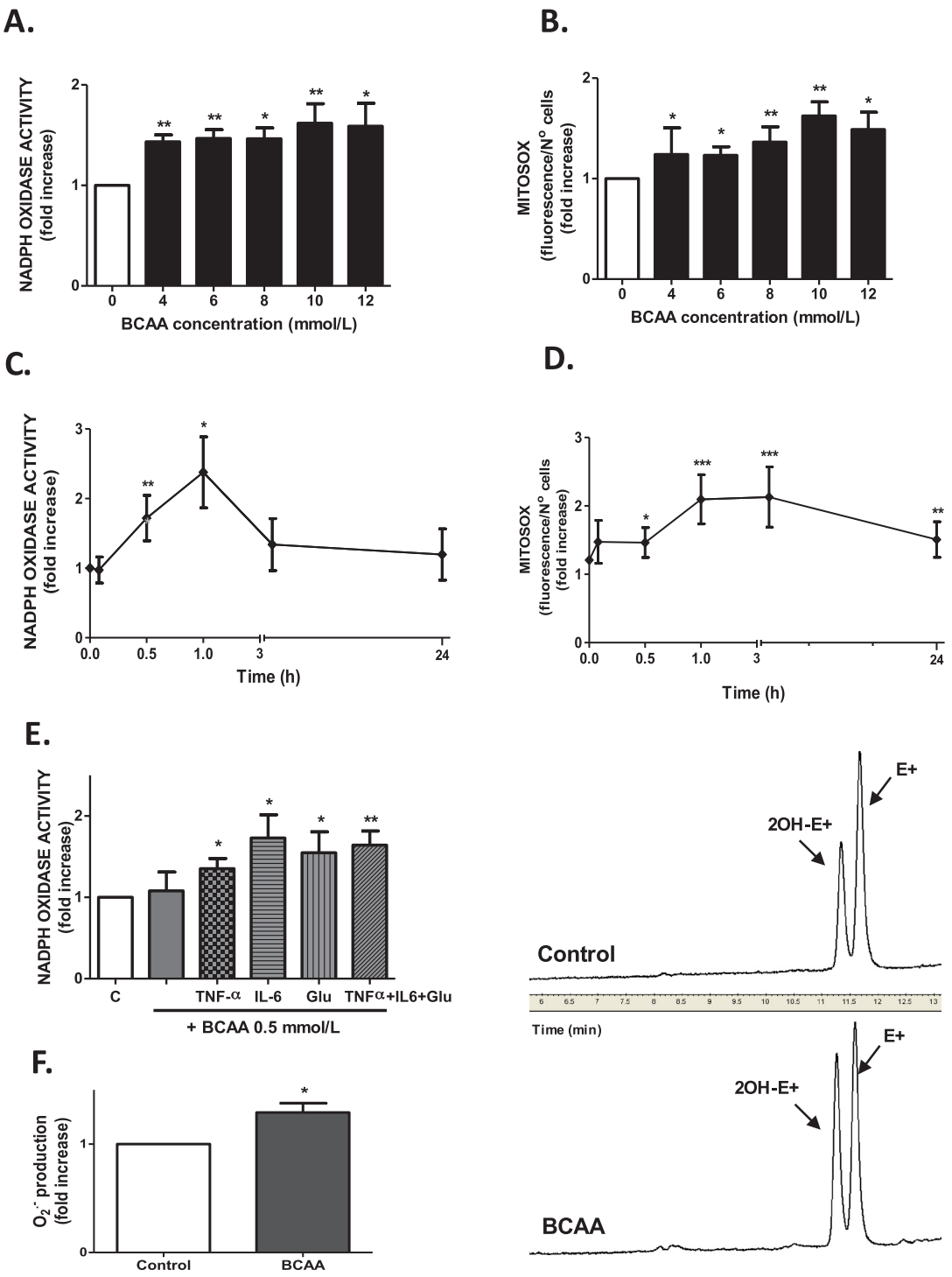


**Fig. 1.** BCAA activate oxidative stress in PBMCs. Cells were exposed to (A) (B) increasing concentrations of BCAA (4–12 mmol/L) for 1 h (C) (D) 10 mmol/L of BCAA for increasing time periods to determine NADPH oxidase activity which was measured in total cellular extracts by chemiluminescence assay and Mitosox by fluorescence was measured in plate luminometer (Enspire Perkin Elmer). (E) PBMCs were exposed to lower BCAA concentration (0.5 mmol/L) for 1 h with and without pro-inflammatory cytokines (IL-6, 102 U/ml or TNF-α, 30 ng/ml) and high glucose (Glu, 30 mmol/L) (F) Chromatogram of $O_2^{\bullet -}$ production by HPLC analysis. $O_2^{\bullet -}$ production was evaluated by an increase in 2-OH-$E^+$ generation by HPLC analysis of DHE fluorescence in PBMCs stimulated with BCAA. The levels were expressed as fold increase over control (considered 1). Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ vs Control. n = 6–8.

reaction was started by the addition of a lucigenin mixture 5 µmol/L) and NADPH (100 µmol/L) (Sigma-Aldrich) to the protein sample in a final volume of 250 µL. Chemiluminescence was determined every 2.4 s for 3 min in a microtiter plate luminometer (Enspire Perkin Elmer). Basal activity in the absence of NADPH was subtracted from each reading and normalized to protein concentration.

### *2.7. Assessment of intracellular mitochondrial superoxide production and membrane potential*

The mitochondrial membrane potential was measured using the fluorescent probe tetramethylrhodamine methyl ester (TMRM). PBMCs were incubated with 150 µmol/L TMRM (Life Technologies) at 37 °C for 10 min and then analysed by flow cytometry at 549 nm (FACScan; BD

Biosciences, San Jose, CA). For quantifying the production of mitochondrial superoxide, PBMCs were incubated with MitoSOX Red (0.5 μmol/L) for 30 min in the dark, and counterstained with DAPI (Sigma). The cells were then analysed by flow cytometry or visualized with a confocal microscope (Leica TCS SP2, 40X objective).

### 2.8. Measurement of $O_2^{\bullet -}$ production by high-performance liquid chromatography

Cell samples were homogenized in acetonitrile (300 μl), sonicated, centrifuged (12,000 rpm, 15 min at 4 °C), and the supernatant was collected and dried. Pellet was resuspended in Krebs-HEPES-DPTA 25 μmol/L, and 5 μl was used for protein determination. Samples (4 μg) were filtered (0.22 μm) and analysed by HPLC (Agilent Technologies 1200 series, Santa Clara, CA) using a 5 μm C-18 reverse-phase column (Kinetex 150 × 4.6 mm; Phenomenex, Torrance, CA) and a gradient of solutions A (pure acetonitrile) and B (water/10% acetonitrile/0.1% trifluoroacetic acid, v/v/v) at a flow rate of 0.4 ml/min and run. Ethidium and 2-OH-$E^+$ were monitored by fluorescence detection with excitation at 480 nm and emission at 580 nm. The 2-OH-$E^+$ peak reflects the amount of $O_2^{\bullet -}$ formed in the tissue during the incubation per microgram of protein. The increase of 2-OH-E+ peak was represented by an increase (n-fold) versus control. To optimize the HPLC analysis, 5 μmol/L DHE was incubated with xanthine/xanthine oxidase (0–50 μmol/L/0.1 U/ml) in KHS-HEPES containing 100 μmol/L diethylenetriamine pentaacetic acid (KHS-HEPES/DTPA) at 37 °C for 30 min.

### 2.9. DNA binding assay

DNA binding assay was performed as described by Li et al. with minor modifications [27]. Oligonucleotides for NF-κB and Nrf2 (0.125 pmol/μl) and NF-κB and Nrf2 complementary sequences (50 nmol/L) were synthesized by Invitrogen. Primary antibodies were used for p65 (1/200, Cell Signalling, Boston, MA, USA) and Nrf2 (1/200, Biorbyt, United Kingdom) detection. A donkey anti rabbit Alexa 488 or 633 (1/2000, Life Technology) secondary antibody was used for p65 or p-Nrf2 detection, respectively, in a microtiter plate fluorimeter (Enspire, Perkin Elmer). Data were represented as fluorescence intensity (488 or 633 nm), respectively.

### 2.10. Cell migration assay

The migration of PBMCs was examined using a 6.5 mm transwell chamber with an 8 μm pore size (Corning Costar Inc., Corning, NY). Cells were allowed to migrate for 1 h after stimulation. Migration values were determined by counting three fields per chamber in a confocal Leica TCS SP2 (40× objective) and calculated as fold-increase over control.

### 2.11. Statistical analysis

Results are expressed as mean ± standard error (SEM). n means the number of blood samples from healthy donors. Statistical analysis was performed using Mann-Whitney statistical and multiple comparison by Kruskal-Wallis, with a significance level chosen at $p < 0.05$.

## 3. Results

### 3.1. BCAA promote time- and dose-dependent oxidative stress

We examined the impact of extracellular BCAA on two main cellular sources of superoxide anions ($O_2^{\cdot -}$) generation, such are NAPDH oxidase and the mitochondria. Firstly, PBMCs were exposed to increasing concentrations of BCAA (4–12 mmol/L), capable of inducing pathological effects in MSUD patients [13] and cultured cancer cells [28,29]. In PBMCs, BCAA significantly elicited NADPH oxidase activity and mitochondrial redox status (mitosox) with a maximal effect observed at 10 mmol/L after 1 h stimulation (Fig. 1A and B). Moreover, the time course experiments depicted an optimal time of 1 h for activation of both sources of superoxide by 10 mmol/L of BCAA (Fig. 1C and D).

In addition, we performed experiments in PBMCs to replicate the pro-inflammatory and hyperglycemic conditions which characterizes T2DM and other pathologies. For this purpose, PBMCs were exposed to a combination of high glucose (30 mmol/L) and pro-inflammatory cytokines (IL-6, $10^2$ U/ml or TNF-α, 30 ng/ml). Under these harmful conditions, BCAA at a lower concentration of 0.5 mmol/L was able to enhance NADPH oxidase activity (Fig. 1E).

To demonstrate more specifically the $O_2^{\bullet -}$ production, high-performance liquid chromatography (HPLC) measurements were performed since this technique detects 2-hydroxyethidium (2-OH-$E^+$), a specific product of DHE superoxide oxidation. The HPLC chromatogram of acetonitrile-extracted PBMCs showed both at the 2-OH-$E^+$ and ethidium peaks. In BCAA-treated cells, for 1 h, a significant increase was observed in the 2-OH-$E^+$ peak compared with controls (Fig. 1F). Treatment with the selective Nox1 and Nox2 pharmacological inhibitors ml-171 and gp91dstat, respectively, decreased BCAA-induced $O_2^{\bullet -}$ production (*data not shown*).

### 3.2. BCAA stimulate the PI3K/Akt-mTORC1 signalling pathway in PBMCs

Since very little is known about effects of BCAA on PBMCs, we next aimed to gain insight into the signalling pathways activated by BCAA upstream ROS formation. To evaluate whether BCAA could activate mTOR signalling, PBMCs were exposed to increasing concentrations of BCAA (0.2–12 mmol/L). mTOR activation was evaluated by the phosphorylation at Ser2448 (specific of mTORC1) (Fig. 2A). BCAA significantly induced mTORC1 phosphorylation in PBMCs with a maximal effect observed at 10 mmol/L after 1 h stimulation (Fig. 2A and C). However, when PBMCs were exposed to the pro-inflammatory and hyperglycemic conditions we noted that mTORC1 was phosphorylated at lower BCAA concentration (0.5 mmol/L) (Fig. 2B). Moreover, the time course experiments shown an optimal time of 1 h for mTORC1 phosphorylation by BCAA (10 mmol/L) (Fig. 2C), in accordance with previous reports in other type of cells [28,29].

Under these experimental conditions, BCAA-induced mTORC1 activation was similar to that achieved by a well-known inflammatory signal, LPS (1 μg/ml) (Fig. 2D). PI3K/Akt pathway activation has been described as an upstream mTORC1 activator [21,30]. In PBMCs, BCAA promoted Akt phosphorylation with maximal response at 1 h (Fig. 2C and D), an effect that was shared by LPS (Fig. 2D). The mTOR inhibitor rapamycin prevented the activation of Akt (Fig. 2D) and abrogated mTORC1 activation (Fig. 2D) by both BCAA and LPS, as expected. At the same time, the Akt inhibitor wortmannin prevented the activation of mTORC1 by BCAA (Fig. 2E), suggesting a positive cross-activation between Akt and mTORC1 in PBMCs exposed to BCAA.

### 3.3. BCAA promote AMPK activation in PBMCs

The AMP-activated protein kinase (AMPK) is a key cellular energy sensor that may in turn regulate nutritional sensing [31]. Since the effects of high BCAA concentrations on AMPK are not defined, we investigated this pathway in PBMCs. BCAA were capable to increase time-dependent phosphorylation of AMPK (Thr172) from 5 min to 24 h with a maximal effect at 1 h (Fig. 3A). As AMPK has two residues of phosphorylation with opposite function (Thr172 activation and Ser485/491 inhibition) [31–33], we also explored whether BCAA act on Ser485/491. That residue was not affected by BCAA (10 mmol/L) (Fig. 3B), but insulin (used as positive control of Ser485/491 phosphorylation), alone or in combination with AICAR, as expected, induced Ser485/491 phosphorylation (Fig. 3B). In contrast, when we determined the Thr172 phosphorylation of AMPK, we noted that both AICAR

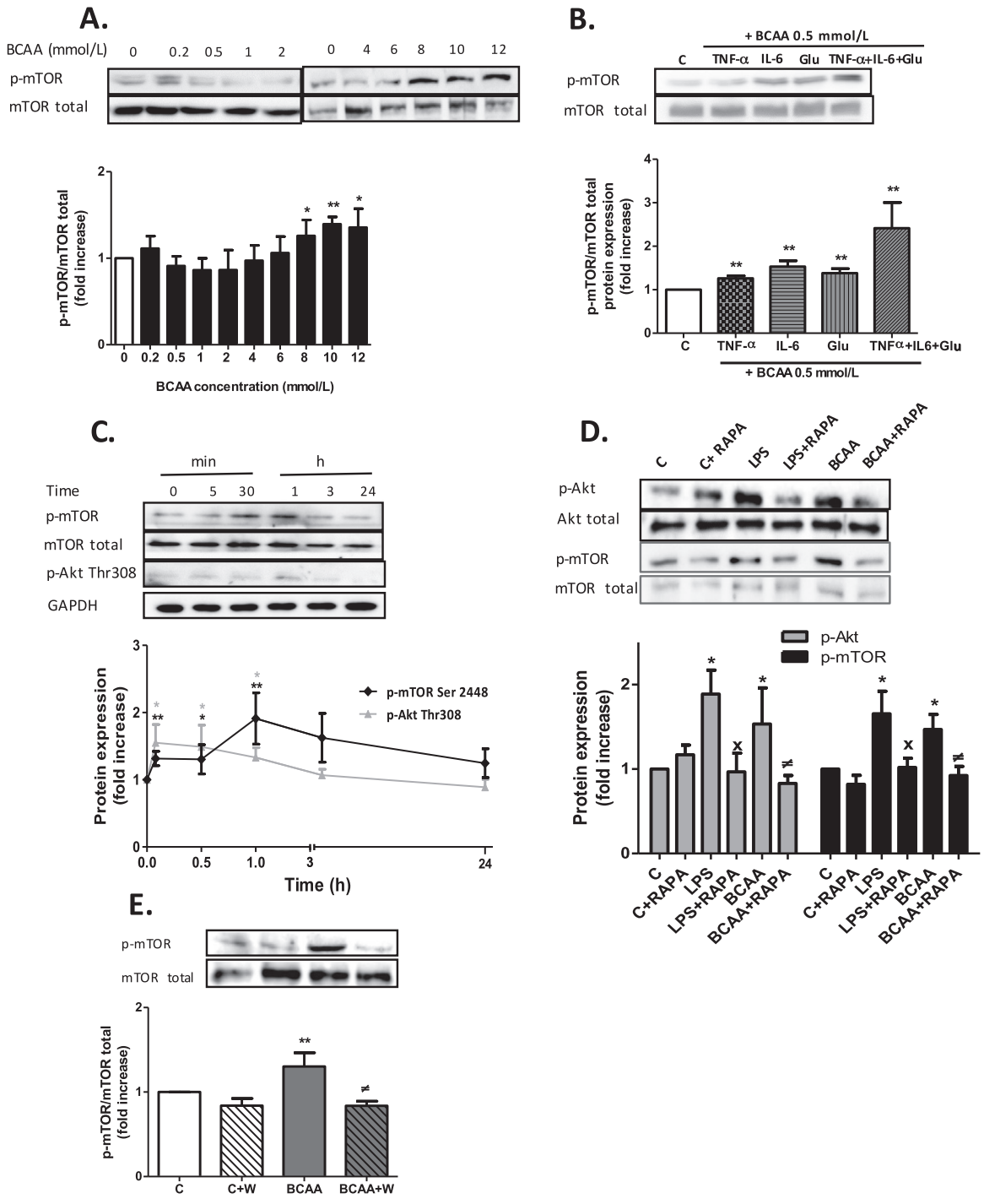


**Fig. 2.** BCAA activate the PI3K/Akt-mTORC1 axis in PBMCs. PBMCs were exposed to (A) increasing concentrations of BCAA (0.2–12 mmol/L) for 1 h (B) lower BCAA concentration (0.5 mmol/L) for 1 h with and without pro-inflammatory cytokines (IL-6, $10^2$ U/ml or TNF-α, 30 ng/ml) and high glucose (30 mmol/L) (C) 10 mmol/L BCAA for increasing time periods (C) pre-incubated 30 min with mTORC1 inhibitor (rapamycin, 100 nmol/L) before stimulation with BCAA (10 mmol/L) or LPS (1 μg/ml) for 1 h (D) pre-incubated 30 min with Akt inhibitor (wortmannin, 1 μmol/L) before stimulation with BCAA (10 mmol/L, 1 h). mTOR, Akt or AMPK phosphorylated levels (p-mTOR or p-Akt respectively) were determined by western blot. p-mTOR, and p-Akt levels were obtained from densitometric analysis, as ratios versus corresponding total mTOR or Akt values, expressed as fold increase over control (considered 1). For each panel, representative blots are shown on the top. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs Control. $^{\neq}P < 0.05$; $^{\neq\neq}P < 0,01$ vs BCAA $^{X}P < 0.05$ vs LPS. n = 4–7.

and BCAA, separately or in combination, induced that phosphorylation and therefore AMPK activation. The insulin did not change the Thr172 phosphorylation (Fig. 3B).

BCAA-induced AMPK activation was similar to that performed by AICAR (Figs. 3B and 3C). The obtained results confirm the differential phosphorylation of AMPK depending of residue on which act different stimuli.

Moreover, AICAR prevented the activation of mTORC1 and Akt elicited by BCAA (Fig. 3D), suggesting that the pharmacological over-activation of AMPK could act as an upstream negative regulator of the PI3K/Akt-mTORC1 axis activation. Contrary to that observed for Akt, the activation of AMPK by BCAA was rapamycin-insensitive and thus independent of mTORC1 (Fig. 3C).

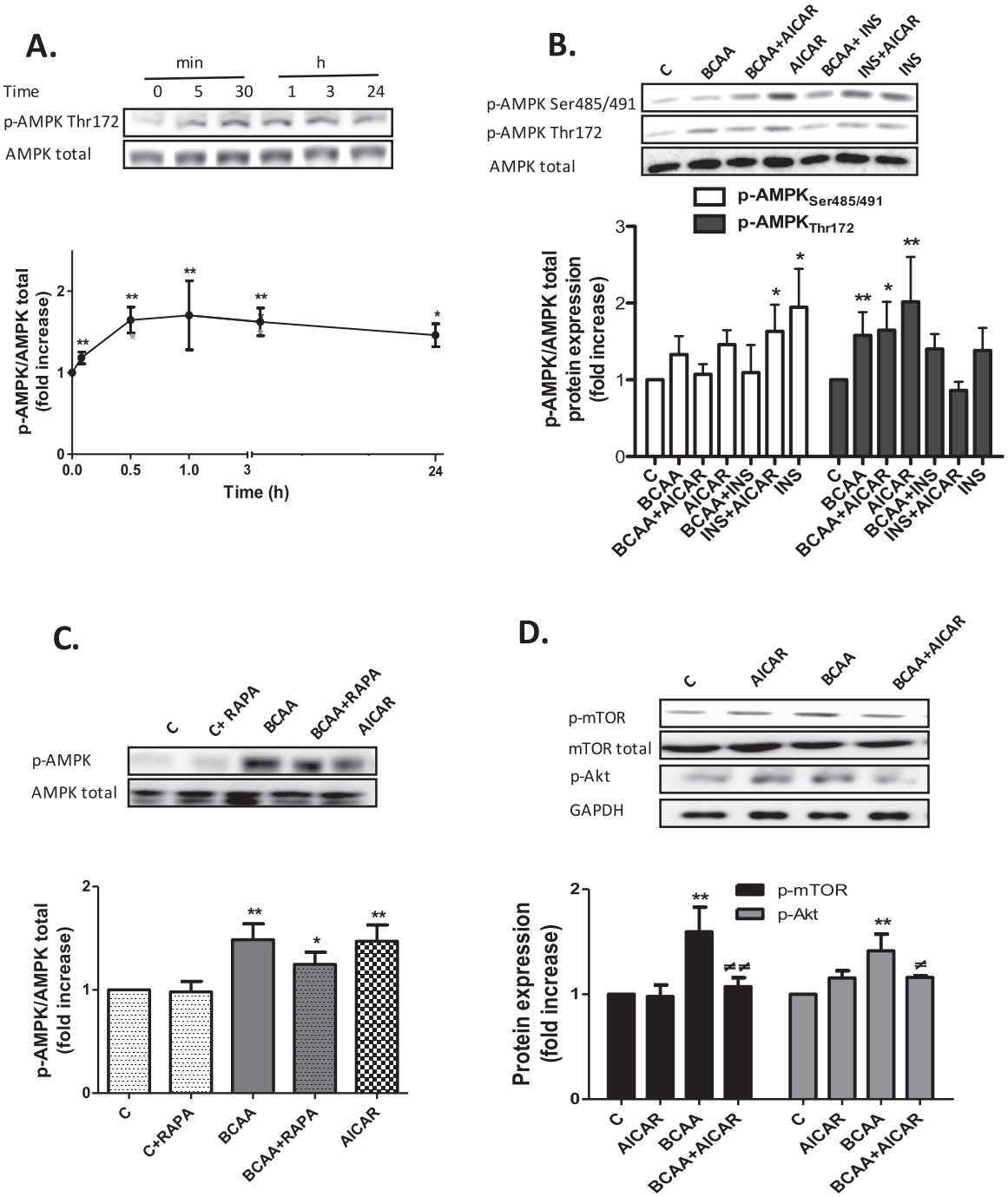


**Fig. 3.** BCAA activate AMPK axis and its inducer modulate Akt/TORC1 activation. PBMCs were incubated with (A) BCAA (10 mmol/L) for increasing time periods (B) BCAA (10 mmol/L) with or without AICAR (0.5 mmol/L) or insulin (1 nmol/l) for 1 h. (C) BCAA (10 mmol/L) with or without rapamycin (100 nmol/L) or AICAR (0.5 mmol/L) ) for 1 h. mTOR, Akt or AMPK phosphorylated levels were determined by western blot. p-mTOR, p-AMPK and p-Akt levels were obtained from densitometric analysis, as ratios versus corresponding total mTOR, AMPK or GAPDH values, expressed as fold increase over control (considered 1). For each panel, representative blots are shown on the top. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs Control. $^{\neq}P < 0.05$; $^{\neq\neq}P < 0.01$ vs BCAA. n = 4–7.

### 3.4. BCAA promote oxidative stress via PI3K/Akt-mTORC1

Next we investigated whether PIK/Akt-mTOR and AMPK pathways could be implicated in the increased ROS production by BCAA. We observed that BCAA-elicited ROS were reduced by the mTORC1 inhibitor (rapamycin), the Akt inhibitor (wortmannin) and the AMPK activator (AICAR), suggesting that these pathways are upstream of ROS production (Fig. 4A, B and C). As NADPH oxidase was activated by BCAA we performed additional experiments to test which of its catalytic subunits participated in ROS production. The inhibition of NOX-1 and NOX-2 subunits (both expressed in PBMCs) by their specific inhibitors (ML171 and gp91dstat, respectively) abrogated BCAA activated NADPH oxidase (Fig. 4A).

We tested whether additional signalling pathways were regulating the production of superoxide anions induced by BCAA in PBMCs. The activation of Nrf2 by sulforaphane allowed to eliminate both cellular sources of superoxide anions generation elicited by BCAA (Fig. 4B), similarly to that observed in the presence of ROS scavenger mito-TEMPO used as control (Fig. 4B).

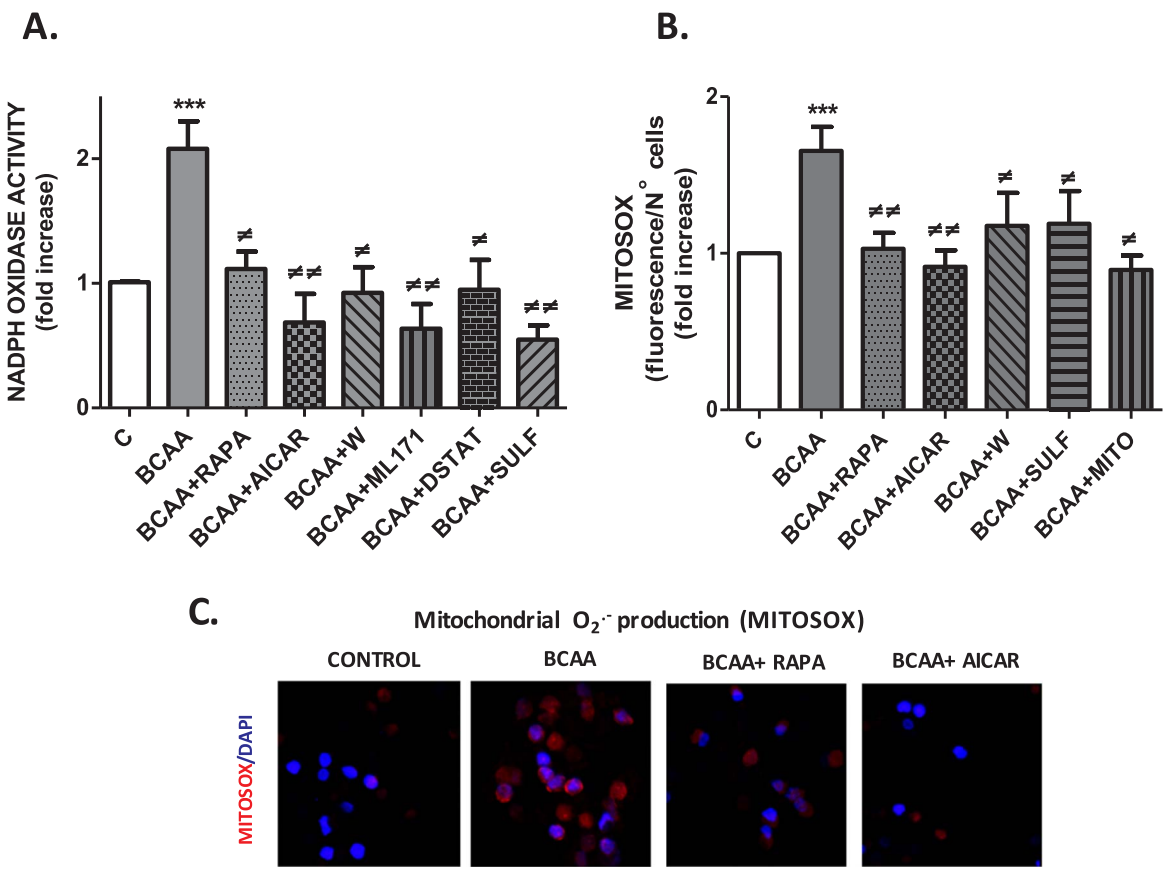


**Fig. 4.** BCAA induce ROS production via PI3K/Akt-mTORC1. Cells were pre-incubated different inhibitors rapamicyn (100 nmol/L), AICAR (0.5 mmol/L), wortmannin (1 μmol/L), ML171 (0.5 μmol/L), gp91dstat (5 μmol/L), mito-TEMPO (0.5 μmol/L) and sulforaphane (20 μmol/L) for 30 min and effect of BCAA (10 mmol/L, 1 h) were investigated (A) on NADPH oxidase activity (B) mitochondrial $O^{2\cdot-}$ was measured by fluorescence by plate luminometer. (C) Confocal microscopy showing $O^{2\cdot-}$ production by Mitosox (red) and nuclei (blue). Data represent Z-stack of confocal images. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ $^{***}P < 0.0005$ vs Control. $^{\neq}P < 0.05$ $^{\neq\neq}P < 0.005$ vs BCAA. n = 5–7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

### 3.5. BCAA cause mitochondrial dysfunction

Mitochondria is a major producer of ROS in mammalian cells [34], but also a main target for oxidative stress damage that may impair either the assembly or the function of the respiratory chain [34]. Such impairment allows further accumulation of ROS, thus resulting in a vicious cycle potentially leading to energy depletion and ultimately to cell death [35].

In this context, we determined the impact of BCAA on the mitochondrial membrane potential (ΔΨm), used as a marker of mitochondrial dysfunction. BCAA induced depolarization of the mitochondrial membrane either in the whole PBMCs population or in each of the two PBMCs sub-populations, lymphocytes and monocytes (Fig. 5A and 5B). In all cases, the depolarization elicited by BCAA was prevented by the mTOR inhibitor (rapamycin) or by the AMPK activator (AICAR) (Fig. 5A and B). Moreover, BCAA reduced the levels of the uncoupling protein (UCP)-2, an inner membrane protein reported to limit the production of mitochondrial ROS [36] (Fig. 5C). This effect of BCAA on UCP-2 levels were equally prevented by both rapamycin and AICAR (Fig. 5C).

### 3.6. BCAA promote NF-κB activation and the expression of pro-inflammatory genes

A positive relationship between oxidative stress generation and the activation of the pro-inflammatory NF-κB pathway has been described in different clinical conditions [37]. One of the earliest events in NF-κB pathway activation is the phosphorylation of p65 subunit [38]. We performed time course experiments of p65 phosphorylation in response to 10 mmol/L of BCAA. We noted a time-dependent activation of p-p65 after 5 min which remained stable for up to 3 h being the optimal time at 1 h (Fig. 6A). As observed with LPS, BCAA augmented the phosphorylation of the p65 component of NF-κB, as well as its nuclear translocation (Figs. 6B and C), without significantly affecting the p50 component (*data not shown*). To further investigate whether the activated and located into the nucleus p65 could directly interact with DNA, we performed a DNA binding assay. As shown the Fig. 6D, BCAA-treated cell presented, higher fluorescence intensity than untreated cells suggesting that BCAA could increase p65 DNA binding activity. The inhibition of mTORC1 by rapamycin and the activation of AMPK by AICAR prevented the effects of BCAA on p65 activation (Fig. 6B, C and 6D). Furthermore, the increased p65 expression elicited by BCAA was dependent on the generation of ROS since it was abolished in the presence of mito-TEMPO and DPI (NADPH oxidase inhibitor) (Fig. 6E).

Next we evaluated several pro-inflammatory genes regulated by the NF-κB such as IL-6 and TNF-α (Fig. 7A and B), as well as membrane receptors such as CD40L and ICAM-1 that facilitate leukocyte adhesion and migration [39] (Fig. 7C and D).

The expression of these pro-inflammatory factors in response to BCAA was blocked by rapamycin and AICAR suggesting mTORC1 and AMPK pathway participation in that process (Fig. 7A to D).

### 3.7. BCAA promote PBMCs migration

To investigate the functional consequences of BCAA on PBMCs, we performed an *in vitro* chemotaxis assay. Incubation with BCAA for 1 h significantly stimulated PBMCs chemotaxis in comparison with control conditions, similarly to that observed in the presence of LPS (Fig. 7E).

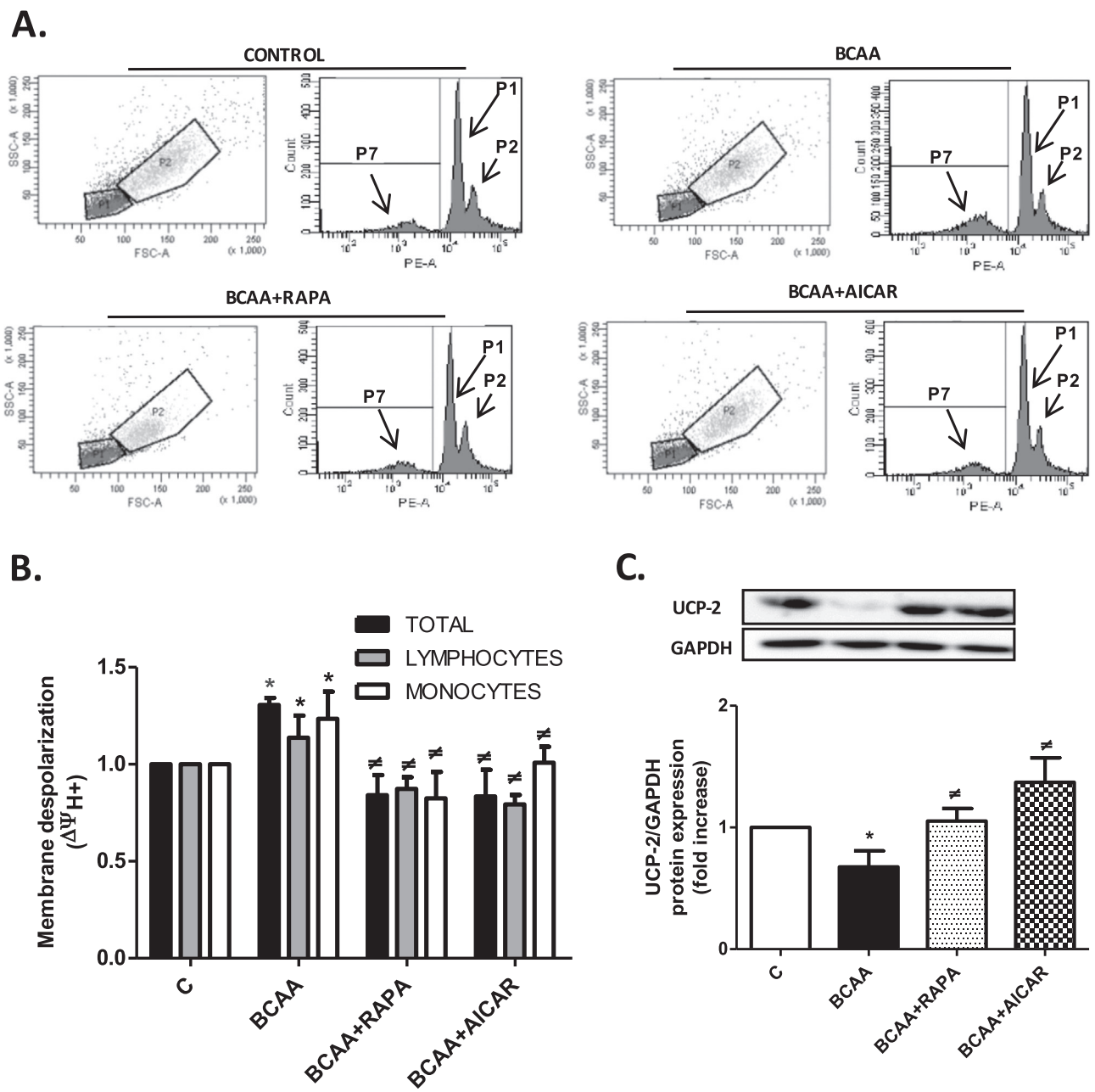


**Fig. 5.** Mitochondrial membrane potential measured by TMRM. PBMCs were incubated for 1 h with BCAA (10 mmol/L) and pre-incubated 30 min before with rapamycin (100 nmol/L) and AICAR (0.5 mmol/L) (A) One representative dot-plot and histograms is shown. P1 are lymphocytes, P2 monocytes are and P7 are died cells. (B) a summarized bar graph showing depolarization of mitochondrial membrane (C) UCP-2 and GAPDH relative protein expression. The representative blots are shown on the top. The values were expressed as fold increase over control (considered 1). Data are expressed as mean ± SEM. $^{*}P < 0.05$; vs Control. $^{\neq}P < 0.05$ vs BCAA. n = 5–6.

The blockade of the Akt-mTORC1 axis by wortmannin and rapamycin as well as AMPK activation with AICAR abrogated the migration triggered by BCAA. Moreover, the inhibition of oxidative stress sources by DPI and mito-TEMPO or NF-κB activation by BAY-11–7082 also abrogated BCAA-activated cell migration (Fig. 7E), suggesting the participation of oxidative stress and NF-κB in this process.

### *3.8. BCAA induce the expression of antioxidant and anti-inflammatory defence proteins*

Nrf2 is a transcription factor expressed in response to oxidant and inflammatory insults that maintains a tight cross-talk with other redox regulators of oxidative stress and inflammation, such as NF-κB itself [40]. In PBMCs, we investigated whether BCAA did affect Nrf2 phosphorylation and its nuclear localization. BCAA increased Nrf2 protein phosphorylation (Fig. 8A), nuclear translocation (Fig. 8B) and binding to its DNA consensus sequence (Fig. 8C). Again, those effects of BCAA were dependent on mTORC1 and could be blunted through AMPK activation (Fig. 8A, B and C).

## 4. Discussion

The main finding of our work is that BCAA can directly trigger a mechanism that involves oxidative stress and NF-κB activation in circulating PBMCs, and therefore these aminoacids could contribute to the inflammatory process observed in different pathological conditions.

Recent studies suggest that MSUD patients present, besides neurological damage, sustained inflammation and activation of the immune system [13], including elevated serum levels of IL-1β, IL-6 and INF-ɣ.

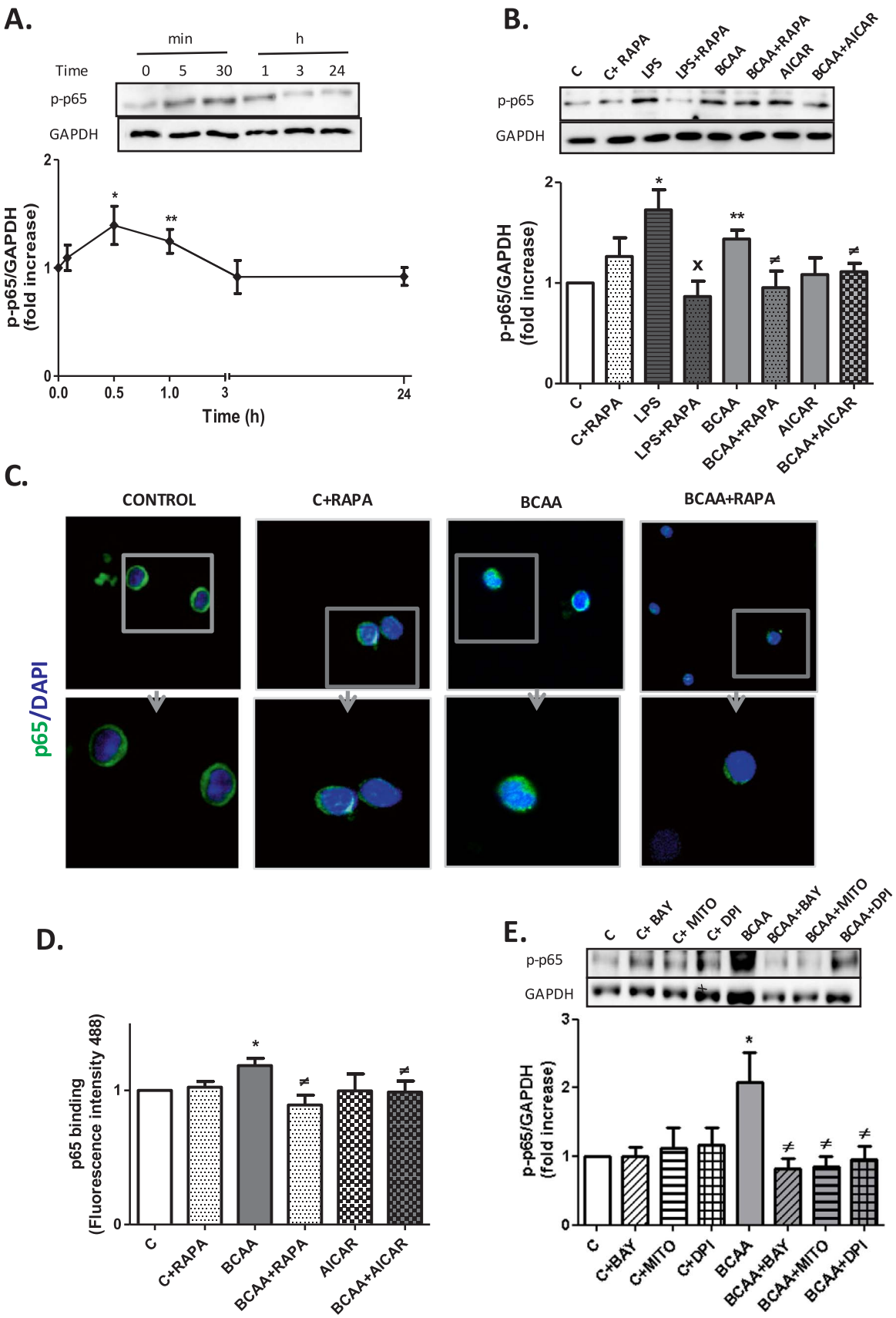


**Fig. 6.** BCAA activate NF-κB pathway in PBMCs via mTORC1. Effect of BCAA 10 mmol/L on (A) p65 phosphorylation for increasing time periods (B) p65 phosphorylation for 1 h pre-incubated 30 min with rapamycin (100 nmol/L) and AICAR (0.5 mmol/L) (E) BAY-11–7082 (1 mmol/L), DPI (10 μmol/L) and mito-tempo (0.5 μmol/L) determined by Western blot. Representative blots are shown. (C) Immunocytochemical images revealed localization of p65 in nucleus (D) and increased DNA-binding activity of p65. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs Control. $^{\neq}P < 0.05$ vs BCAA $^{X}P < 0.05$ vs LPS used as positive control. n = 4–7.

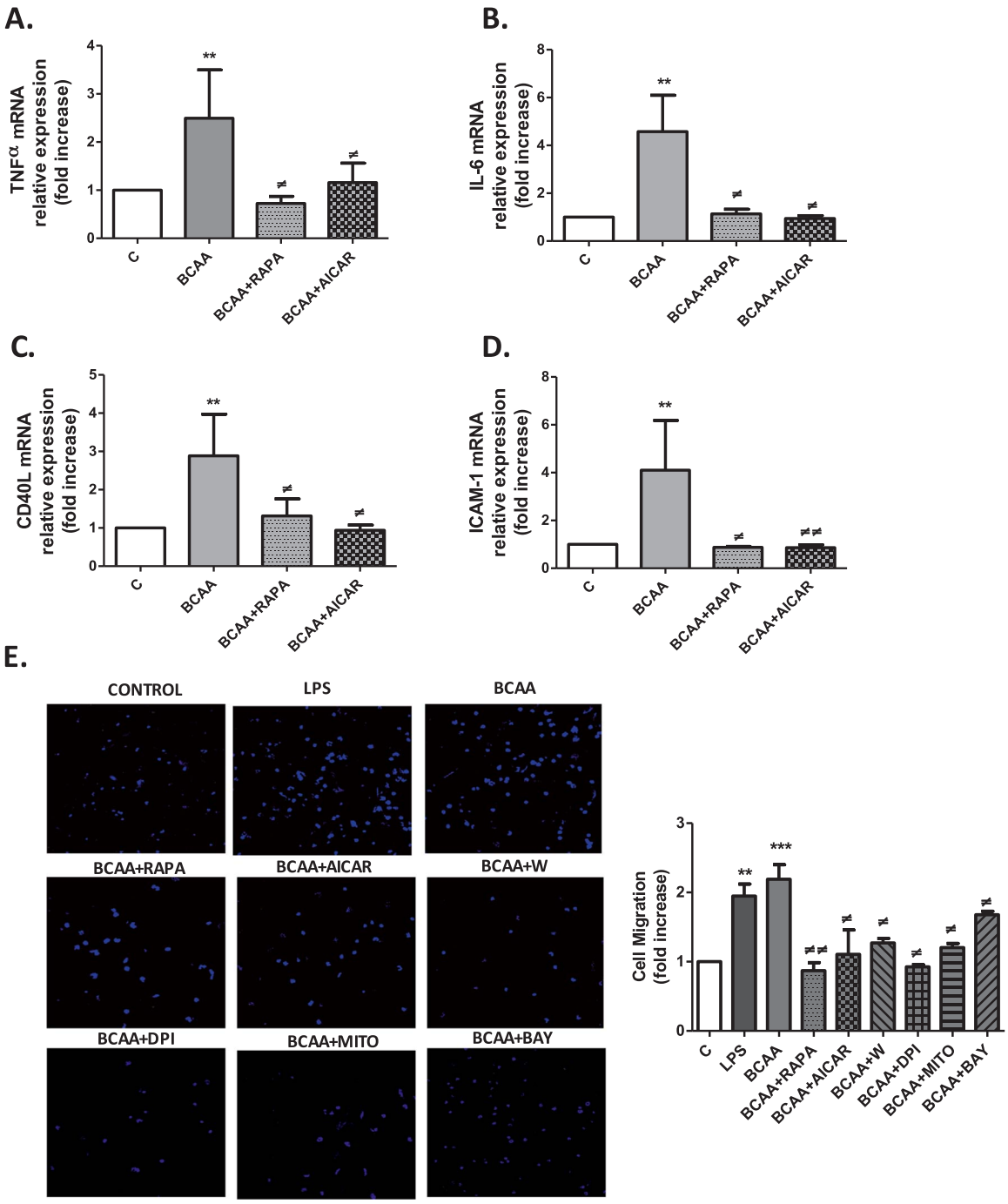


**Fig. 7.** BCAA induce NFκB-dependent inflammatory genes and T cell activation and cell migration. Effect of BCAA (10 mmol/L, 1 h) with and without rapamycin and AICAR measured by RNAm levels in human PBMCs. (A) IL-6 (B) TNFα (C) CD40L and (D) ICAM-1. (E) BCAA chemotactic effect on PBMCs by transwell migration assay. Unstimulated cell (Control) or stimulated with LPS (as positive control) and BCAA for 1 h with or without rapamicyn (RAPA), AICAR, wormannin (W), diphenyliodonium chloride (DPI), mito-TEMPO (MITO) and BAY-11-7082 (BAY). Data are expressed as mean ± SEM. *P < 0.05; **P < 0.01 or ***P < 0.005 vs Control. ≠P < 0.05≠ ≠P < 0.005 vs BCAA. n = 5–7.

Our results show that 10 mmol/L BCAA, at similar levels to those found in MSUD patients, could activate PBMCs inducing ROS production, activation of NF-κB and related pro-inflammatory genes. These results could help to explain the changes observed in those patients.

Unbalanced ROS production is one of the most important mechanisms involved in different pathological conditions [41–43]. Among the major ROS sources are NADPH oxidase and mitochondria. In this report we reveal that BCAA trigged the formation of ROS in PBMCs through the activation of two catalytic subunits of NADPH oxidase (NOX-1 and NOX-2), whose expression is particularly important in immune cells [43]. In addition, BCAA augmented the production of mitochondria-derived ROS, with a subsequent increase in oxidative damage and mitochondrial dysfunction. This mitochondrial dysfunction has been identified as a relevant mechanism in cardio-metabolic diseases, underlying cardiovascular risk factors such as diabetes, hypertension and atherosclerosis [44]. In PBMCs challenged with BCAA, the Nrf2-antioxidant response element signalling pathway became activated, probably as a mechanism to protect the cells against the excess ROS production [40].

Importantly, in this paper we have described for the first time to the best of our knowledge that BCAA activates the transcription factor NF-κB in cells of the immune system. BCAA-induced NF-κB activation was

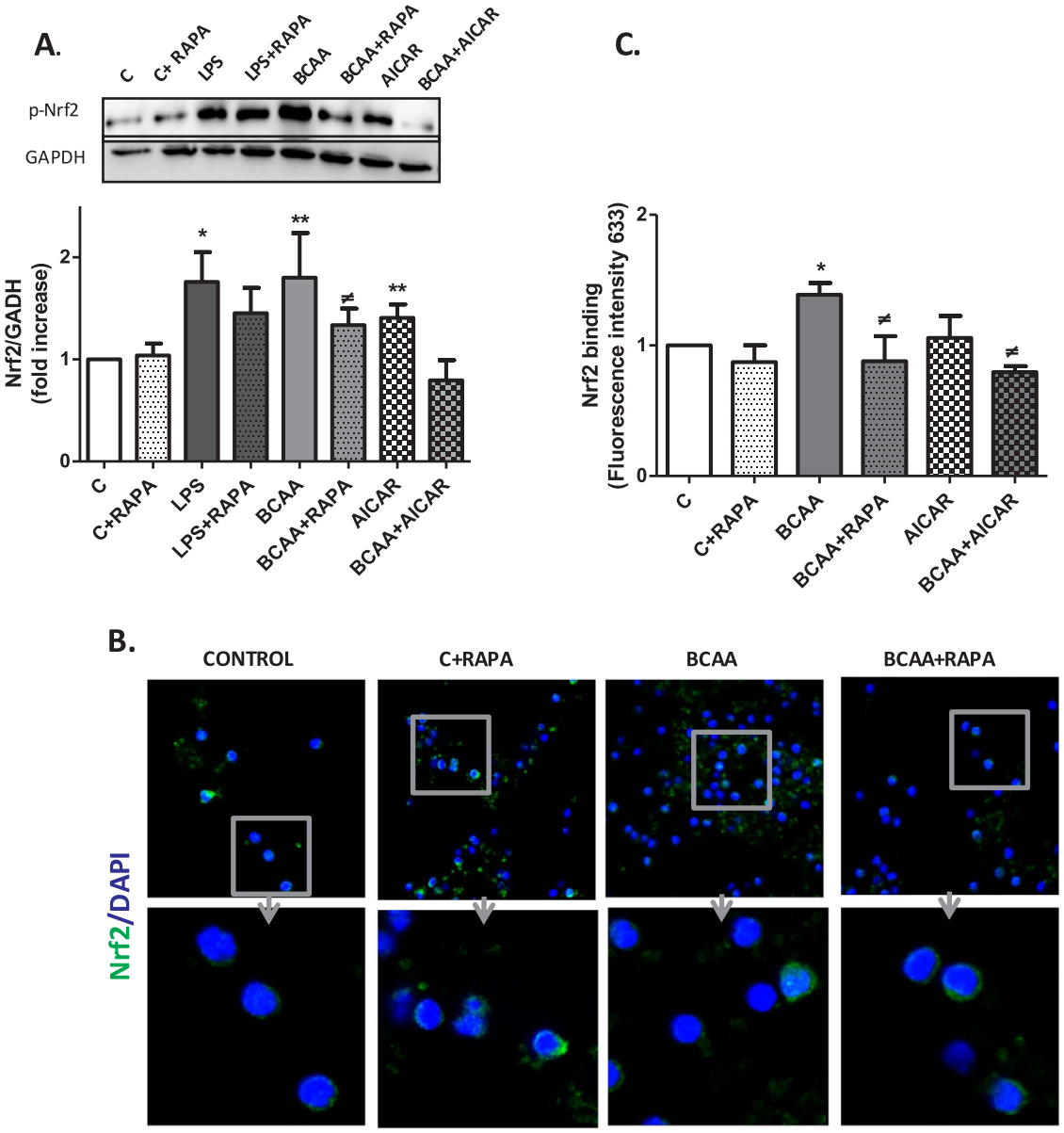


**Fig. 8.** BCAA induce Nrf2 independent of via mTORC1 activation and partially dependent AMPK activation. Effect of BCAA with and without rapamycin and AICAR in (A) protein expression of p-NRf2 and GAPDH as loading control are also shown (B) Immunocytochemical images revealed localization of Nrf2 in nucleus. (C) and increased DNA-binding activity of Nrf2. Data are expressed as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ vs Control. $^{\neq}P < 0.05$ vs BCAA $^{X}P < 0.05$ vs LPS used as positive control.

paralleled to over-expression of the pro-inflammatory cytokines, such as IL-6 or TNF-α, whose circulating levels are found elevated in patients suffering MSUD [13]. BCAA also favoured the expression of CD40L and ICAM-1, which are proteins involved in the activation and the migration of PBMCs [39]. Functionally, the activation triggered by BCAA conferred a higher migration capacity of PBMCs. NF-κB is a redox-sensitive transcription factor [45]. Accordingly, the blockade of ROS generation prevented the activation of NF-κB in PMBCs exposed to BCAA.

These oxidant and inflammatory BCAA effects were observed at concentrations that could be reached in MSUD [12,13] or in daily BCAA supplementation in sportmen [46], but higher than those found in patients suffering from obesity or diabetes [6,7,10,11]. However, chronic exposure to moderately elevated BCAA levels added to hyperglycaemia and pro-inflammatory conditions could decrease the threshold of mTOR phosphorylation and increase ROS formation. Thus, the potential harmful impact of BCAA may be particularly relevant in the context of a series of diseases characterized by oxidative stress and inflammation.

In fact, metabolomics studies has rekindled BCAA as a main metabolic signature associated with obesity in different populations [10,11,47]. BCAA positively correlate with body mass index in adult and pediatric individuals, while weight-reduction improves the BCAA profile [48–50]. Moreover, BCAA have been suggested as a major predictor of future diabetes [48] and blood levels correlated with cardio-metabolic complications, including insulin resistance [6,11].

In cancer cells, BCAA have proven to act as strong nutrient signals mainly acting via mTORC1 activation, a nutritional sensor that plays a key role in regulating cell growth, proliferation and migration [19–21]. We found that BCAA in PBMCs promoted the phosphorylation of mTOR at Ser2448. This effect was dependent on Akt, in line with that observed in other cell types [28,30]. Once activated at Ser2448, mTOR binds to Raptor, and other proteins to form the active enzyme complex mTORC1 which signals through the phosphorylation of downstream targets [21]. Reciprocally, we found that mTORC1 was able to modulate Akt activation, which suggests the existence of a cross-talk between both signalling molecules which needs further understanding. We also identified the pharmacological upstream activation of AMPK as a tool

to limit the formation of mTORC1 in response to BCAA. This is in line with previous studies in other cell types showing that AMPK can prevent mTORC1 activation [51]. Importantly, the effects of BCAA on oxidative stress generation, NF-κB activation, inflammation-related protein expression and migration of PBMCs were blunted by rapamycin, which inhibits mTORC1 via binding to the FKBP12 domain [21]. This high lights the pivotal role of mTORC1 in mediating pro-oxidant and pro-inflammatory activation of PBMCs by BCAA. The over-activation of mTORC1 has been associated with the progression of the metabolic syndrome, future development of type 2 diabetes and different associated complications [52].

Besides the above described pathological conditions linked to BCAA increased levels, those aminoacids are commonly used as nutritional supplements potentially to improve mental and physical performance and with the purpose of muscle building [53]. Of interest, daily BCAA supplementation could reach elevated blood levels around 3–6 mmol/L concentrations as those used in our *in vitro* studies. The potential clinical implications of chronic BCAA supplementation is unknown. In non-obese healthy individuals a high BCAA intake at short term is well-tolerated, because of the BCKDC reserve capacity in the body and the fact that BCKDC is activated by excess substrate under normal conditions [54]. Surprisingly, only some acute toxicity studies have been carried out [55,56] and to our knowledge no toxicity effects at long term have been described in humans so far. Our studies point out on the importance of future studies about the oxidative and inflammatory effects of uncontrolled BCAA intake among sportsmen.

## Ethics statement

The procedure was approved by the Research Ethics Committee of Instituto de Investigaciones Sanitarias Fundación Jiménez Díaz.

## Funding information

This work was supported by the Spanish Ministry of Economy and Competitiveness/Health research fund FIS-FEDERPI14/00386. The laboratory of Renal, Vascular and Diabetic Research Laboratory is also supported also by grants from FIS-FEDER (PIE13/00051; PI14/0041); FP7 grant e-PREDICE and from the Fundación Renal Iñigo Alvarez de Toledo (FRIAT)/Instituto Reina Sofia de Investigación Nefrológica.

## Author's contributions

O.Z., M.R.O and S.M. conceived the experiments and analysed data. O.Z., C.P., M.R.O and S.M. wrote the manuscript. O.Z. and E.C. performed the experiments and analysed data. M.S.S., C.V. and J.E. contributed to the discussion and reviewed and edited the manuscript.

## Competing interests

No conflicts of interest relevant to this article were reported.

## Acknowledgements

Olha Zhenyukh was the recipient of a fellowship from Fundación Conchita Rábago. The authors thank Enrique Bosch-Panadero, Mª de Mar Parreño and nurses from Blood bank of FJD for their technical assistance.

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