A specific amino acid formula prevents alcoholic liver disease in rodents

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ABSTRACT

Chronic alcohol consumption promotes mitochondrial dysfunction, oxidative stress, defective protein metabolism, and fat accumulation in hepatocytes (liver steatosis). Inadequate amino-acid metabolism is worsened by protein malnutrition, frequently present in alcohol-consuming patients, with reduced circulating branched-chain amino acids (BCAAs). Here we asked whether dietary supplementation with a specific amino-acid mixture, enriched in BCAAs (BCAAem) and able to promote mitochondrial function in muscle of middle-aged rodents, would prevent mitochondrial dysfunction and liver steatosis in Wistar rats fed on a Lieber-DeCarli ethanol (EtOH)-containing liquid diet. Supplementation of BCAAem, unlike a mixture based on the amino acid profile of casein, abrogated the EtOH-induced fat accumulation, mitochondrial impairment, and oxidative stress in liver. These effects of BCAAem were accompanied by normalization of leucine, arginine, and tryptophan levels, which were reduced in liver of EtOH-consuming rats. Moreover, while the EtOH exposure of HepG2 cells reduced mitochondrial DNA, mitochondrial transcription factors, and respiratory chain proteins, the BCAAem but not casein-derived amino acid supplementation halted this mitochondrial toxicity. Nicodinamide adenine dinucleotide levels and sirtuin 1 expression, as well as endothelial nitric oxide (eNOS) and mammalian/mechanistic target of rapamycin (mTOR) signaling pathways, were down-regulated in the EtOH-exposed HepG2 cells. BCAAem reverted these molecular defects and the mitochondrial dysfunction, suggesting that the mitochondrial integrity obtained with the amino acid supplementation could be mediated through a Sirt1-eNOS-mTOR pathway. Thus, a dietary activation of the mitochondrial biogenesis and function by a specific amino acid supplement protects against the EtOH toxicity and preserves the liver integrity in mammals.

NEW & NOTEWORTHY

Dietary supplementation of a specific amino acid formula prevents both fat accumulation and mitochondrial dysfunction in hepatocytes of alcohol-consuming rats. These effects are accompanied also by increased expression of anti-ROS genes. The amino-acid protective effects likely reflect activation of Sirt1-eNOS-mTOR pathway able to regulate the cellular energy balance of hepatocytes exposed to chronic, alcoholic damage.

KEYWORDS: alcoholic liver disease; branched-chain amino acids; endothelial nitric oxide synthase; mechanistic target of rapamycin; mitochondrial biogenesis.

INTRODUCTION

An excessive and chronic alcohol consumption may cause alcoholic liver disease (ALD), a major global health problem (68). ALD is a spectrum of liver pathologies mainly characterized by fat accumulation, and ranging from steatosis to steatohepatitis, fibrosis, and cirrhosis which can eventually progress to hepatocellular carcinoma (47). In particular, the early stages of the disease are associated with vesicular steatosis, caused by defective fatty acid oxidation (13). All of the alcohol-metabolizing enzymes, including alcohol dehydrogenase, catalase, NADPH-oxidase, xantine oxidase, and the microsomal alcohol oxidizing system, lead to acetaldehyde accumulation in liver. Acetaldehyde can be oxidized to acetate, mainly through aldehyde dehydrogenases, which are mitochondrial, nicotinamide dinucleotide (NAD^+)-dependent enzymes, so that the concentration of the reduced pyridine coenzyme NADH increases, and the NAD^+/NADH ratio decreases in hepatocytes. Such an imbalance has been classically proposed to explain several of the metabolic changes produced directly in liver cells by alcohol oxidation, including triglyceride accumulation (18). Similarly, sirtuin 1 (Sirt1), a NAD^+-dependent protein deacetylase that removes post-traslational acyl modifications from various cellular substrates to regulate a wide range of biological pathways (14), was found to be reduced in liver of alcohol-consuming animals (49). Sirt1 controls hepatic gluconeogenesis/glycolytic processes and mitochondrial biogenesis, through peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1α) (56, 80). Moreover, Lieber and co-workers have demonstrated that Sirt1 and PGC-1α, acting as a complex, can physiologically regulate each others' activity in liver of alcohol-consuming animals (49).

Although the adverse effects of excessive alcohol consumption on liver mitochondria are well known (11, 32), the molecular impairment impinging on the alcohol-dependent mitochondrial dysfunction remains to be defined. Incapacity to maintain adequate ATP concentrations, associated to reduced activities of all of the respiratory complexes (except complex II) (22), impaired mitochondrial protein synthesis (17), in addition to mitochondrial DNA (mtDNA) damage (12) and ribosomal defects (10), have been suggested to cause failing oxidative phosphorylation. These processes increase reactive oxygen species (ROS) production and oxidative damage in the steatotic liver, accompanied by a marked mitochondrial dysfunction (54).

Beyond their role as key building blocks for protein synthesis, amino acids, particularly the branched-chain amino acids (BCAAs), are also significant sources of sterol, ketone bodies, and glucose (9). Mammalian/mechanistic target of rapamycin (mTOR) complex 1 (mTORC1), which is acutely sensitive to rapamycin and amino acid availability, mediates some of these processes (84). We have recently shown that dietary supplementation with a BCAA-enriched mixture (BCAAem) activated endothelial nitric oxide synthase (eNOS) with increased NO production in cardiac and skeletal muscle of middle-aged mice (23). Notably, the BCAAem-induced NO promoted mTORC1-dependent mitochondrial biogenesis and function in muscle cells (23). Because altered amino acid metabolism is a hallmark of ALD, with low levels of circulating BCAAs (15), the present study aimed to investigate the effects of BCAAem supplementation on the mitochondrial damage in liver of chronically ethanol (EtOH)-consuming rats. Our results demonstrate that this specific amino acid supplementation was able to prevent both structural mitochondrial damage and mitochondrial dysfunction in liver of alcoholic rats. We found that anti-ROS defense

system, eNOS, and mTOR pathways likely play an important role in the protective effects of dietary BCAAem supplementation in hepatic cells. Together, our results support the potential usefulness of dietary supplementation with a specific amino acid formula to prevent the alcoholic liver disease in humans.

MATERIALS AND METHODS

Animals and Treatments

The experimental protocol was approved and conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), and the Italian Ministry of Heath, and complied with The National Animal Protection Guidelines. For experiment #1, a total of 26 male Wistar rats (3 months old) from Charles River (Calco, Como, Italy) were used. The animals were housed separately in clean polypropylene cages and divided into four groups: 1) the pair-fed group (pair-fed CTRL, n = 6), was fed with a control liquid diet, in which EtOH was replaced by isocaloric maltose dextran; 2) the EtOH group (EtOH, n = 7), was fed with a Lieber-DeCarli liquid diet containing EtOH *ad libitum* [gradually increasing amount of EtOH, reaching 36 % of caloric intake after 1 week, corresponding to a final concentration of 6.2 % (vol/vol)]; 3) the BCAAem group (BCAAem, n = 6), fed with a control liquid diet, in which EtOH was replaced by isocaloric maltose dextran, and supplemented with branched-chain amino acid mixture (10 g/L), that provided 1.5 g/kg/day BCAAem; and 4) the EtOH plus BCAAem group (EtOH + BCAAem, n = 7), fed with a Lieber-DeCarli liquid diet containing EtOH and BCAAem *ad libitum*. For experiment #2, a total of 30 male Wistar rats (3 months old) were divided into five groups: 1) the pair-fed group (CTRL, n = 6), 2) the EtOH group (EtOH, n = 6), and 3) the EtOH plus BCAAem group (n = 6) were fed as in Experiment #1; 4) the casein-amino acid group (CAA, n = 6), fed with a control liquid diet, in which EtOH was replaced by isocaloric maltose dextran, and supplemented with purified amino acid mixture based on the amino acid profile of casein (10 g/L) (which is the main protein source in rodent laboratory diet), that provided 1.5 g/kg/day CAA supplement; and 5) the EtOH plus CAA group (EtOH +

CAA, n = 6), fed with a Lieber-DeCarli liquid diet containing EtOH and CAA mixture *ad libitum*. All of the diets were isocaloric with each other, and they were obtained from Bio-Serv, Frenchtown, NJ, USA. The composition of each diet is described in Table 1. The amino acid concentrations of BCAAem used was previously found to be active in rodents and mimic the recommended daily dose for humans (23). Dietary treatments were prolonged for 8 weeks, in a 12 h light/12 h dark cycle at 22 °C, in a quiet, temperature- and humidity-controlled room. The BCAAem composition, amino acid relative percentage, and dietary intake of each amino acid have been reported in D'Antona et al. (24). Body weight and food intake were recorded twice a week. At the end, animals were sacrified by guillotine. Left lobe of liver was fixed for microscopy analysis, while the rest of the organ was quickly frozen in liquid nitrogen and stored at – 80 °C for subsequent analysis (see below).

Sample Preparation

Liver (n = 4 animals/group) was weighted, homogenized in cold methanol:water (v/v, 1:1), and extracted according to Want et al. (76). The vacuum dried samples were suspended in 120 µl/50 mg tissue of methanol:1 mM TDFHA = 1:1 and centrifuged at 16,000 g for 10 minutes at 4 °C. Two µl of surnatant were directly loaded onto the UPLC-mass spectrometer and analyzed as reported below. Four technical replicates for each sample were run using the three different methods.

Chromatography and amino acid quantification in liver

Standard amino acids were purchased from Sigma (Milan, Italy). Each amino acid stock solutions were prepared at 1 mg/ml in water, diluted to the final concentration

of 3 pmol/µl, and directly infused by syringe at 10 µl/min into the TripleTOF 5600^{+} mass spectrometer (AB Sciex, Milan, Italy). Thus, declustering potential (DP) and collision energy (CE) were optimized for each amino acid. Next, three mixtures of amino acids were prepared on the basis of DP and CE values: MIX 1, containing threonine, asparagine, tyrosine, and serine, and analyzed with DP: 30 V, CE: 15 V; MIX 2, containing glycine, alanine, leucine, isoleucine, valine, proline, histidine, methionine, aspartic acid, glutamine, and phenylalanine, and analyzed with DP: 40 V, CE: 15 V; and MIX 3, containing glutamic acid, lysine, arginine, and tryptophan, and analyzed with DP: 80 V, CE: 18 V. All of the amino acids were acquired in the positive polarity, in both TOF MS and Product Ion mode, according to the *m/z* values reported in Table 2. The source parameters were: gas 1: 33 psi, gas 2: 58 psi, curtain gas: 25 psi, temperature: 500 °C, and ISVF (IonSpray Voltage Floating): 5500 V. In order to obtain calibration curves, technical quadruplicates of different amounts (10, 33, 50, 100, 200, 400 pmol) of the three mixtures were injected into the mass spectrometer upon UPLC separation, using the UPLC 1290 (Agilent Technologies Italia, Cernusco sul Naviglio, Milan, Italy). The chromatographic column was from Waters, Acquity HSS T3 C18 2.1 x 100 mm, 1.7 µm, while the mobile phase was A: 1 mM TDFHA (tridecafluoroheptanoic acid) in water; B: 1 mM TDFHA in acetonitrile. A gradient of B from 12.5 % to 26.5 % in 4 min, followed by a ramp from 26.5 to 92 % in 3.5 min was used to separate all of the amino acids, with a flow rate of 0.35 ml/min and a column temperature of 65°C as described (43). The autosampler was set at 4 °C. Calibration curves were plotted using chromatographic peak areas and a weighted regression (1/x for all compounds except asparagine, tyrosine, valine, and glutamic acid, which were fit to $1/x^2$) by means of MultiQuant software version 2.1 (SCIEX). Quantitative values for each

amino acid (pmol) in the rat liver samples were obtained by relating chromatographic peak areas to those derived from externally run calibration standards and normalized to tissue (mg).

Liver Histopathological Analysis

Liver was cut in 5-mm-thick slices and fixed in 4 % paraformaldehyde for 24 h and processed for paraffin embedding. Sections were stained with haematoxylin and eosin (H&E).

Transmission Electron Microscopy Analysis

Liver was removed, fixed with 2.5 % glutaraldehyde in cacodylate buffer (pH 7.4, 0.2M), and post-fixed for 1 h with 1 % OsO_4 in the same buffer. The samples were processed with standard procedures for embedding in Araldite (Sigma-Aldrich Chemical Co, Milan, Italy) and polymerized at 60 °C for 72 h. Thick sections (about 1 µm) were stained with Epoxy Tissue Stain (#14950, Electron Microscopy Sciences, PA, USA). Ultrathin sections (70 nm) were stained with a saturated aqueous solution of uranyl acetate and lead citrate and examined with a Philips CM10 electron microscope (Royal Philips Electronics, Amsterdam, the Netherlands) at 80 KV. On thin sections, ultrastructural data on mitochondria from perivenous central area were collected from 18 randomly selected areas from each sample at a final enlargement of x5000 using electron microscope film (Kodak electron film 4489, 6.5x9 cm, New York, USA). Five different section levels of each sample were examined. The total area examined was about 140,000.00 μm^2 in each group. We have previously shown that cytochrome c oxidase and peroxisome staining were markedly reduced, while markers of endoplasmic reticulum stress and inflammation were markedly increased

mainly in the perivenous central hepatocytes of the EtOH-consuming rats (20). For this reason we have focused the present analysis on the perivenous central hepatocytes. All measurements were obtained using standard morphometric techniques, as previously described (20, 77). Cytoplasmic (A*cyt*) and mitochondrial (A*mit*) area, the A*mit* to A*cyt* ratio (A*mit*/A*cyt*), and the number of mitochondria over 100 μm^2 of cytoplasm (i.e., the mitochondrial density; N*mit*/100 μm^2) were measured.

Cell Culture and Treatment

Human HCC HepG2 cells were purchased from the American Type Culture Collection (HB-8065; ATCC, Manassas, VA). Cells were routinely cultured in RPMI-1640 medium, supplemented with 10 % fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), in an atmosphere with 5 % CO_2 at 37 °C. Two million HepG2 cells were seeded per 75 cm^2 flask (Corning Inc., Corning, NY). Six h after seeding, 75 mM (0.34 %) EtOH and 1 % BCAAem (or CAA mixture) were added, alone or in combination. Untreated cells were plated as controls. Every 24 h, media were replaced in both control and treatment flasks, with fresh media, with or without EtOH and BCAAem, respectively. Four days after seeding, cells were trypsinized and seeded into new flasks, at 2 million viable cells per flask, with daily media changes, as described before (64). Five days after the split process (a total of 9 days with or without EtOH, BCAAem, CAA mixture, or EtOH plus BCAAem or CAA mixture), the cells were harvested as reported below for the different assays.

Moreover, HepG2 cells were seeded in 6-well plates and treated each day with 100 nM rapamycin, a macrolide compound that inhibits mTOR signaling, or with 5 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the soluble guanylyl cyclase

inhibitor, 1 h before BCAAem (1 %) addition, and 6 h before 100 mM EtOH treatment (48 h) (28). Vehicle-treated cells were exposed to 0.02 % dimethyl sulfoxide (DMSO) for 48 h. Furthermore, eNOS knockdown was obtained with transient transfection of small interference RNA (siRNA). HepG2 cells were seeded in 6-well plates and transfected with 100 nmol/l eNOS siRNA SMARTpool (Dharmacon, Lafayette, CO) or siCONTROL nontargeting siRNA using Dharmafect transfection reagent. After 48 h transfection, the cells were then treated with 1 % BCAAem and 100 mM EtOH for 48 h. Efficacy of transfection was determined using siGLO-RISC-free nontargeting siRNA and estimation of siRNA uptake by fluorescence detection (absorbance/emission 557/570). Proteins were extracted for Western blotting analysis.

Oil Red O Staining

HepG2 cells were washed twice with DPBS and fixed with 10 % formalin for 1 h. Then, they were stained with 0.3 % Oil Red O in 60 % isopropanol for 2 h at room temperature. The cells were subsequently washed three times with distilled water. Fat droplets were dissolved with 100 % propanol, and quantified by measuring the optical absorbance at a wavelength of 510 nm, using a Biorad Model 680 microplate reader (66).

Quantitative RT-PCR Analysis

Quantitative RT-PCR reactions were performed as described (73) and run with the iQ SybrGreenI SuperMix (Bio-Rad; Segrate, Italy) on an iCycler iQ Real-Time PCR detection system (Bio-Rad). Briefly, RNA was isolated from tissue using the RNeasy® Tissue Mini Kit (Qiagen, Milan, Italy). cDNA was synthesized using

iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Segrate, Italy). Primers were designed using Beacon Designer 2.6 software from Premier Biosoft International (see Tables 3 and 4). The cycle number at which the various transcripts were detectable (threshold cycle, CT) was compared to that of TBP, referred to as ΔCT. The gene relative levels were expressed as $2^{-(\Delta\Delta CT)}$, in which ΔΔCT equals ΔCT of EtOH- or BCAAem- or CAA mixture-treated rat (or treated HepG2 cells) minus ΔCT of the control rat (or untreated HepG2 cells).

Western Blot Analysis

Protein extracts were obtained from liver with T-PER Mammalian Protein Extraction Reagent (Pierce, ThermoScientific, Rockford, USA) as described by the manufacturer, in the presence of protease and phosphatase inhibitors cocktail (Sigma Aldrich, Milan, Italy). Protein content was measured by the bicinchoninic acid protein assay (BCA, Pierce, Euroclone, Milan, Italy), and 50 µg of proteins were run on SDS-PAGE under reducing conditions. The separated proteins were then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Segrate, Italy). Proteins of interest were revealed with specific antibodies: anti-COX IV (cytochrome c oxidase subunit IV), anti-Cyt c (cytochrome complex), anti-p-eNOS (Ser1177-phospho-eNOS), anti-acetyl p53, anti-p-Akt (Ser473-phospho-Akt), anti-Akt, anti-p-p70 S6 kinase (Thr389-phospho-p70S6kinase), anti-p70 S6 Kinase, anti-Sirt1, anti-β-actin (all from Cell Signaling, Euroclone, Milan, Italy), anti-eNOS (Santa Cruz, CA, USA), anti-p53 (from GeneSpin, Milan, Italy), anti-SOD1 (superoxide dismutase [Cu-Zn], from Santa Cruz, CA, USA), anti-catalase (from Santa Cruz, CA, USA), at 1:1000 dilution each one. The immunostaining was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse

290 immunoglobulin for 1 h at room temperature. After the visualization of p-eNOS,
291 acetyl p53, p-Akt, and p-p70 S6 kinase, filters were stripped with the Restore^{TM}
292 Western Blot Stripping Buffer (Euroclone, Milan, Italy) and further used for the
293 visualization of total eNOS, total p53, total Akt, or total p70 S6 kinase. The amount
294 of protein was measured using SuperSignal Substrate (Pierce, Euroclone, Milan,
295 Italy), and quantified by densitometry with IMAGEJ software image analyser.

296

297 *Mitochondrial DNA Measurement*

298 For mtDNA analysis, total DNA was extracted with QIAamp DNA extraction kit
299 (Qiagen). The content of mtDNA was calculated using Real-Time quantitative PCR
300 by measuring the threshold cycle ratio (ΔCT) of a mitochondrial encoded gene
301 NADH dehydrogenase subunit 1 (ND1) *vs.* a nuclear encoded gene (β-Globin) in
302 liver of EtOH-, BCAAem-, or CAA mixture-treated and control (CTRL) rats, as
303 described (27).

304 *Citrate Synthase Activity Measurement*

305 The citrate synthase activity was measured spectrophotometrically at 412 nm at 30 °C
306 in liver tissue extracts (51). Liver samples were added to a buffer containing 0.1 mM
307 5,5−dithio−bis−(2−nitrobenzoic) acid, 0.5 mM oxaloacetate, 50 μM EDTA, 0.31 mM
308 acetyl CoA, 5 mM triethanolamine hydrochloride, and 0.1 M Tris-HCl, pH 8.1.
309 Citrate synthase activity was expressed as nmol citrate produced per min per mg of
310 protein. The data were normalized to total protein content, determined as reported
311 above.

312

NAD^+ and NADH Measurement

NAD^+ and NADH levels were measured by enzymatic NADH recycling assay, using the NAD^+/NADH Quantification kit from Biovision (Vinci-Biochem, Florence, Italy), according to the manufacturer's recommendations. Liver samples were homogenized in 400 µl of NAD^+/NADH extraction buffer, and filtered using Microcon YM-10 (GE Healthcare, Euroclone, Milan, Italy). Then, the samples were split into two sets, one of which was used to carry out the thermal decomposition of NAD^+, followed by the cycling assay for the determination of NADH content. The other set was used to measure the total NADH plus NAD^+ content, by performing the cycling assay without the thermal decomposition. Then, the NAD^+/NADH ratio was calculated. The data were normalized by total protein content (see above).

Mitochondrial Oxidative Stress

To measure the oxidative damage of DNA, the highly sensitive 8-hydroxy-2'-deoxyguanosine (8-OHdG) Check ELISA Kit (JalCA) was used (24). Measurements were carried out in accordance with the manufacturer's protocol. Total DNA was extracted using QIampDNAMini Kit (Qiagen), and digested with nuclease P1 and alkaline phosphatase (Sigma-Aldrich, Milan, Italy). Quality and quantity of DNA were confirmed by a NanoDrop ND-1000 spectrophotometry analysis. Absorbance of the ELISA reaction product was determined spectrophotometrically using 450 nm as the primary wave.

Statistical Analysis and Data Presentation

Statistical analysis was performed with a one-way ANOVA followed by Student-Newman-Keuls' test, or Student's t-test. Data were presented as the means ± standard

deviation (SD), unless otherwise specified. A statistically significant difference was accepted at $P < 0.05$.

RESULTS

BCAAem prevents liver steatosis in rats

Exposure of male Wistar rats to the Lieber DeCarli liquid diet containing 6.2 % EtOH for 8 weeks resulted in a significant increase of the liver weight, together with fat accumulation, compared to the pair-fed controls as previously reported (Fig. 1, *A* and *C*) (6, 61). However, experiment #1 demonstrated that dietary supplementation with BCAAem, although unable to change liver weight *per se*, prevented both liver growth and fat accumulation due to alcohol diet, confirming our previous results (Fig. 1*A* and *C*) (21). Conversely, experiment #2 showed that CAA diet, *i.e.* the Lieber DeCarli liquid diet containing purified amino acid mixture based on the amino acid profile of casein, was unable to prevent the EtOH-dependent liver growth (Fig. 1*A*). Moreover, the EtOH-fed rats gained less body weight than pair-fed animals, yet in a not statistically different manner (Fig. 1*B*), while the body weight of the EtOH-consuming rats treated with BCAAem, unlike with CAA diet, was comparable to that of pair-fed rats (Fig. 1*B*). BCAAem and CAA diet were ineffective on body weight when supplemented alone (Fig. 1*B*). No statistically significant difference was evident in food intake among the groups (data not shown) (21). Next, free amino acid levels were measured in liver tissue. As reported in Table 5, arginine, leucine, and tryptophan concentrations were reduced by EtOH consumption. While ineffective when supplemented alone, BCAAem prevented reduction of these three amino acids. Also, isoleucine, serine, tyrosine, and valine concentrations were lower in liver of mice exposed to EtOH-containing diet, yet BCAAem supplementation was unable to prevent their decline. Concentrations of the remaining amino acids were not statistically different among the groups. Notably, while hepatocytes of the EtOH-consuming rats accumulated many lipid droplets, fat accumulation was prevented by

the BCAAem supplement (Fig. 1*C*). This result prompted us to hypothesize that the specific BCAAem amino acid supplementation could improve the EtOH-induced mitochondrial dysfunction in hepatocytes.

BCAAem prevents mitochondrial damage in rat hepatocytes

To this end, electron microscopy analysis was performed on livers from the diverse groups. Mitochondrial number was significantly reduced in hepatocytes of the EtOH-consuming group relative to pair-fed control rats (Fig. 2 and Table 6). On the other hand, the mitochondrial mean area was higher, while the mitochondrial area to cytoplasmic area (A*mit*/A*cyt*) ratio was lower in the EtOH-consuming group compared to the pair-fed group (Fig. 2 and Table 6). Moreover, smooth endoplasmic reticulum tubules were dilated, and the rough endoplasmic reticulum was disorganized in hepatocytes of alcohol-consuming animals (Fig. 2). BCAAem supplementation of EtOH-fed rats markedly increased mitochondria number, in addition to the mean area and A*mit*/A*cyt* ratio (Fig. 2 and Table 6). In animals fed on EtOH plus BCAAem, smooth and rough endoplasmic reticulum also appeared of normal size and well organized, comparable to those found in hepatocytes of pair-fed rats, with abundant glycogen deposits and no macroautophagic vacuoles (data not shown). BCAAem supplementation alone was unable to change both number and mean area of mitochondria (Fig. 2 and Table 6). These results were consistent with a healthy effect of amino acids, suggesting that BCAAem could prevent the EtOH-induced mitochondrial damage by promoting mitochondrial biogenesis in hepatocytes.

BCAAem restores hepatic mitochondrial biogenesis and function impaired by EtOH consumption

Thus, we evaluated the capacity of BCAAem supplementation to ameliorate impaired mitochondrial biogenesis and function due to the EtOH consumption. As expected, the hepatic mRNA levels of proliferator-activated receptor γ coactivator 1α (PGC-1α), nuclear respiratory factor-1 (NRF-1), and mitochondrial DNA transcription factor A (Tfam) were all lowered by EtOH consumption when compared to pair-fed control animals. However, these markers were renormalized in livers of rats in which BCAAem was supplemented together with EtOH (Fig. 3*A*). BCAAem was ineffective when supplemented alone. Moreover, in line with electron microscopy results, EtOH reduced mtDNA amount by 35 ± 1.5 % compared to pair-fed rats, whereas its consumption with BCAAem increased mtDNA amount by 55 ± 2.6 % and 138 ± 4.0 % compared to pair-fed and EtOH group, respectively (Fig. 3*B*). In accord to liver weight results (Fig. 1*A*), CAA diet was unable to affect mtDNA amount either when supplemented with EtOH or alone (Fig. 3*B*). While protein levels of both COX IV and Cyt c, as well as citrate synthase activity, were reduced in liver of EtOH-consuming rats compared to pair-fed control animals, they were all renormalized in liver of rats exposed to alcoholic diet supplemented with BCAAem (Fig. 3*C* and *D*). Again, BCAAem supplementation alone did not induce any change in mtDNA, mitochondrial protein levels, and citrate synthase activity. Collectively these findings suggest that BCAAem supplementation, by promoting mitochondrial biogenesis and function, opposes to the hepatic liver mitochondrial damage induced by EtOH consumption.

Since eNOS-dependent NO production was found to promote mitochondrial biogenesis in different cells, including hepatocytes (30, 57), we analysed eNOS mRNA and protein levels in livers of both pair-fed controls and EtOH-consuming rats, supplemented with or without BCAAem. A reduction by 38 ± 2.1 % of eNOS mRNA level was evident in the EtOH group compared to the pair-fed group, while the BCAAem supplementation counteracted the effect of EtOH, without an effect *per se* (Fig. 4*A*). Although EtOH did not decrease eNOS protein level compared to pair-fed control animals, the BCAAem-supplemented alcohol-consuming animals showed instead a relevant increase of eNOS protein levels (Fig. 4*A*). Furthermore, differently from mRNA data, BCAAem supplementation alone induced a significant increase of eNOS protein. Thus, these results support the hypothesis that eNOS may contribute to the recovery induced by the amino acid mixture of the EtOH-impaired mitochondrial biogenesis.

Given that Sirt1 expression was found to be increased by the eNOS-dependent NO (57), and Sirt1 in turn deacetylates and activates eNOS and PGC-1α (55), we investigated this cross-talk in our model. Although we confirmed that EtOH consumption reduced Sirt1 mRNA levels in liver as previously reported (81, 82), no changes of Sirt1 protein levels were evident (Fig. 4*B*). Similarly, BCAAem supplementation renormalized Sirt1 mRNA without effect on Sirt1 protein level of EtOH-consuming rats (Fig. 4*B*). Although it is not uncommon for mRNA and protein measurements to not be fully concordant (50), we investigated further this point. Because Sirt1 deacetylase activity is NAD^+-dependent (35), and the NAD^+/NADH ratio is usually decreased in EtOH-consuming animals (31, 34, 78), the effect of BCAAem supplementation on NAD^+/NADH levels was studied. As shown in Fig.

4*C*, livers of the EtOH-consuming rats displayed a ~50 % reduction in NAD^+/NADH ratio, which was however restored by the BCAAem, unlike CAA (data not shown), supplementation. In line with this, the acetylated form of p53, a well-known target of Sirt1 (52), was higher in liver of the EtOH-consuming than control rats, indicating that the deacetylation activity of Sirt1 was reduced in EtOH group (Fig. 4*D*). Conversely, acetyl-p53 was decreased by 70 % in liver of rats supplemented with BCAAem, alone or with EtOH, confirming that the amino acid formula was able to activate Sirt1 (Fig. 4*D*). CAA diet was ineffective on Sirt1 activity in all groups (data not shown). Furthermore, the drop in hepatic Sirt1 activity in EtOH-treated animals occured in the presence of low eNOS mRNA levels (Fig. 4*A*) and this, therefore, strengthens the hypothesis of a cross-talk between Sirt1 and eNOS.

BCAAem protects from the oxidative damage induced by EtOH

Alcohol metabolism generates reactive oxygen species (ROS), thus leading to oxidative stress in hepatocytes (4) which is associated with a well-documented impairment of antioxidant defense system. To verify whether the protection by BCAAem supplementation against EtOH-induced liver damage also involves antioxidant effects, we investigated the expression of the anti-ROS enzymes in liver of EtOH-consuming rats, supplemented or not with the amino acid formula. As expected, glutathione peroxidase 1 (GPX1), catalase (Cat), and superoxide dismutase [Cu-Zn], also known as superoxide dismutase 1 (SOD1) mRNA levels were reduced in EtOH group compared to the pair-fed group (Fig. 5*A*). However, BCAAem supplementation increased them by 10-fold (GPX1) and 3-fold (Cat and SOD1), respectively (Fig. 5*A*). In addition, the SOD1 protein levels were reduced, although without a statistical significance, in liver of EtOH rats, while the BCAAem

supplementation markedly raised Cat and SOD1 (Fig. 5*B*). These findings suggested that ROS production was reduced after amino acid supplementation as previously seen in skeletal muscle of middle-aged mice exposed to BCAAem (23). Accordingly, while the amount of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, was dramatically increased in liver of EtOH-fed rats, this effect was totally reverted by consumption of BCAAem, which was ineffective when administered alone (Fig. 5*C*).

BCAAem improves mitochondrial function and ROS defense system in EtOH-exposed HepG2 cells

In order to investigate more deeply the molecular mechanisms involved in the effects of BCAAem, we extended our findings by using an *in vitro* model of hepatic EtOH toxicity. To this end, hepatic HepG2 cells were treated with EtOH, with or without either BCAAem or CAA mixture. First, we analyzed the mitochondrial biogenesis markers to confirm the effects of alcohol and amino acids observed *in vivo*. PGC-1α and Tfam mRNA levels were unchanged or slightly lower in HepG2 cells exposed to 75 mM EtOH for 9 days, than in untreated control cells (Fig. 6*A*). However, in line with *in vivo* results, 1% BCAAem, unlike CAA mixture, supplementation for 9 days increased PGC-1α and Tfam mRNA levels with respect to both untreated and EtOH treated cells (Fig. 6, *A* and *B*). Sirt1 mRNA was also increased in HepG2 treated with BCAAem, but not with CAA mixture, alone or in combination with EtOH (Fig. 6, *C* and *D*). No significant differences were found in Sirt1 protein levels after both EtOH and BCAAem, as already observed in liver tissue (Fig. 6*C*). Acetyl-p53 levels were not significantly different in EtOH-treated compared to untreated HepG2 cells (data not shown). Accordingly, the NAD^+/NADH ratio was reduced by only 10 % in

alcohol-treated compared to untreated cells (Fig. 6*E*). BCAAem treatment, however, markedly increased the NAD^+/NADH ratio in both EtOH-treated and untreated cells (Fig. 6*E*). In line with this, BCAAem also lowered acetyl-p53 levels, confirming that the amino acid mixture was able to activate Sirt1 (data not shown).

We then investigated the effect of BCAAem treatment on ROS defense system in cultured cells. SOD1 mRNA and protein levels were reduced by ~10 % and 30 %, respectively, in HepG2 exposed to EtOH compared to untreated cells (Fig. 6*F*). However, when HepG2 cells were supplemented with EtOH together with BCAAem, but not CAA mixture, SOD1 mRNA and protein levels returned to levels similar to those seen in the untreated cells, while both their protein and mRNA levels increased by ~30 % compared to untreated cells when HepG2 cells were treated with BCAAem alone (Fig. 6*F* and data not shown). These *in vitro* findings, as well as *in vivo* results, suggest that the specific BCAAem formula is able to counteract the toxic effects of EtOH on mitochondrial function, and to reduce the EtOH-induced oxidative stress by specifically acting on hepatic tissue and in a cell-autonomous manner,

BCAAem improves fat oxidation in EtOH-exposed HepG2 cells

Since it is well known that oxidative stress and mitochondrial dysfunction lead to fat accumulation in hepatocytes, we studied the efficacy of amino acid supplementation to counteract the EtOH-dependent impairment of fat β-oxidation of fatty acids. Expression of carnitine palmitoyltransferase 1 (CPT1), also known as carnitine acylpalmitoyltransferase 1, the key enzyme in the regulation of β-oxidation of long-chain fatty acids, was in fact reduced by alcohol as compared to untreated HepG2

(Fig. 7*A*). On the contrary, BCAAem up-regulated CPT1 mRNA when administered alone or with EtOH (Fig. 7*A*).

Moreover, to assess the possible effect of BCAAem supplementation on the EtOH-induced excessive fat storage, the intracellular lipid accumulation was analyzed by Oil Red O staining in HepG2 cells treated with 100 nM EtOH and 1 % BCAAem. Following 48 h treatment, the lipid content was significantly increased when HepG2 cells were exposed to EtOH, whereas it was reduced when the cells were exposed to BCAAem compared to untreated cells (Fig. 7*B*). In addition, BCAAem supplementation was able to renormalize the lipid content in the EtOH-treated HepG2 cells (Fig. 7*B*).

EtOH and BCAAem control multiple signaling pathways in HepG2 cells

Given that BCAAem was found to promote eNOS-dependent NO production, and NO is also known to control β-oxidation in muscle (23, 44), we analyzed the expression of eNOS and its activity in HepG2 treated with EtOH, with or without BCAAem. A slight decrease of eNOS mRNA was observed in cells treated with EtOH. BCAAem, however, increased eNOS mRNA, both supplemented alone or together with EtOH, compared to the untreated cells (Fig. 8*A*) Yet, eNOS activity, measured as Ser1177-eNOS phosphorylation (19), was markedly reduced in EtOH treated cells (Fig. 8*A*). Although ineffective on eNOS activity when supplemented alone, BCAAem completely restored the EtOH-induced decrease of eNOS-phosphorylation (Fig. 8*B*). To strengthen further the role of eNOS on mitochondrial protection by BCAAem supplementation, HepG2 cells were transfected with small interference RNA (siRNA) against eNOS or nontargeting siRNA as a negative control. We verified that eNOS

siRNA reduced eNOS protein levels by 70 % (Fig. 8*C*). While HepG2 treated with EtOH showed decreased PGC-1α and COXIV protein levels, and BCAAem supplementation promoted a statistically significant recovery of both proteins, eNOS knockdown blocked the BCAAem effects in the presence or not of EtOH (Fig. 8*C*). Importantly, the eNOS silencing *per se* did not modify PGC-1α and COXIV levels. Thus, our present results suggest that the effects of BCAAem on mitochondrial parameters are at least in part mediated by eNOS in hepatic cells. We had previously demonstrated that the eNOS-derived NO promotes mitochondrial biogenesis in various cell types through guanosine 3',5'-monophosphate (cyclic GMP, or cGMP) (57). Thus, we examined the effect of the selective guanylate cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Co-incubation of BCAAem with ODQ (5 μM) for 2 days reversed the positive effect of amino acid supplementation on reduction of PGC-1α and COX IV protein induced by EtOH, whereas ODQ alone had no effect (Fig. 8*D*). Thus, the eNOS-produced NO through activation of cGMP-dependent signal transduction pathway may promote the healthy effects of BCAAem on mitochondria of hepatic cells.

Protein kinase B (PKB), also known as Akt, is a serine/threonine-protein kinase that plays a key role in multiple cellular processes, such as glucose metabolism, cell cycle, and angiogenesis, in addition to regulate, among others, eNOS activity (53). On these basis, we investigated Akt function, measured as phosphorylation in Ser473, in untreated or ethanol-treated HepG2 cells, with or without BCAAem. We observed a marked decrease of p-Akt normalized to total Akt in HepG2 cells treated with EtOH, which was completely restored by BCAAem (Fig. 8*B*). BCAAem increased Akt phosphorylation also when supplemented alone compared to untreated cells (Fig. 8*B*).

Since eNOS-dependent NO production is known to regulate mTOR system in different cell types (7, 23, 63, 83), we also investigated the mTOR pathway in our experimental model. Phosphorylation levels of ribosomal protein p70S6 kinase, a major downstream target of mTOR complex 1 (TORC1), was measured in HepG2 cells by immunoblot analysis. We observed a 40 % decrease of phospho-p70S6 kinase, normalized to total p70S6 kinase, after EtOH exposure compared to untreated cells, which was completely rescued by BCAAem supplementation (Fig. 8*B, D* and *E*). This effect was antagonized by rapamycin, a macrolide compound that inhibits mTORC1 signaling (Fig. 8*E*), thus confirming the direct involvement of mTORC1 in the mechanism of action of BCAAem. Notably, the rescue of defective mitochondrial biogenesis markers by BCAAem was consistently antagonized by rapamycin in HepG2 exposed to EtOH (Fig. 8*F*). Moreover, ODQ fully antagonized the effects of BCAAem on phospho-p70S6 kinase in hepatic cells treated or not with EtOH (Fig. 8*D*), supporting the role of NO-dependent cGMP on mTORC1 activity. Thus, our results suggest that the healthy effects of BCAAem on mitochondrial function in the EtOH-treated HepG2 cells could be due to a Sirt1-eNOS-Akt-mTORC1 signaling pathway.

DISCUSSION

In this paper, we showed that dietary supplementation of BCAAem protected rats against alcoholic fatty liver and mitochondrial dysfunction, by reversing most of the EtOH-induced metabolic impairments. Several studies have demonstrated that chronic alcohol consumption leads to morphological and functional changes of mitochondria in different organs, including liver (37), in both animals and humans (62). Mitochondrial enlargement and swelling, with uncommon shapes and giant mitochondria (38, 39), characterized by high matrix density (33, 42, 48), mitochondrial cristae shortening and disorganization, decreased mitochondrial number, or absence of matrix granules and intramitochondrial crystalline inclusions have been described in hepatocytes of alcoholics. Consequently, these changes affect mitochondrial functions, leading to reduced respiratory rates and ATP synthesis (2), in addition to increased ROS production (69). Thus, mitochondrial dysfunction contributes largely to initiation and progression of the alcohol-induced liver damage, also because the liver deficit in maintaining energy production precedes inadequate adaptive organ repairing mechanisms (5).

Our findings that dietary BCAAem supplementation was able to prevent the decline in mitochondrial biogenesis markers (*i.e.*, PGC-1α, NRF-1, and Tfam) and mtDNA, as well as respiratory-chain proteins COX IV and Cyt c, and mitochondrial citrate synthase activity, that occurs in the liver of EtOH-consuming rats indicate, therefore, that the mitochondrial stimulating activity of BCAAem mixture impinges on its protective effect against ALD. Restoring mitochondrial function by BCAAem improves the metabolic derangement caused by EtOH ingestion. Reduced fat accumulation was in fact observed in livers of EtOH-fed rats supplemented with

605 BCAAem. Accordingly, BCAAem was also able to upregulate CPT1 expression, the
606 key enzyme in mitochondrial β-oxidation of fatty acids, whose expression was
607 decreased in EtOH-fed animals. Impaired fatty acid oxidation in alcohol-consuming
608 rats, and its recovery by BCAAem supplementation, could therefore underlie the
609 mechanism involved in the EtOH damage and in the protective effect of the amino
610 acid supplement, respectively. Although the stimulatory effect of BCAAem
611 supplement on CPT1 expression might be linked to the ketogenic potential of
612 leucine and lysine (59), our experimental conditions seem to exclude this hypothesis
613 since ketogenesis is strictly linked to a shortage of carbohydrate, while HepG2 cells
614 were maintained in 2 g/L glucose culture medium. Moreover, the increased
615 expression of SOD1, GPX1, and catalase observed in BCAAem-fed rats also
616 indicates a protective action of the amino acid supplement against the EtOH-induced
617 oxidative stress (45), which was after all confirmed by the ability of BCAAem to
618 reduce 8-OHdG amount in liver DNA of the alcoholic animals.
619
620 Hepatic ethanol metabolism occurs mainly via oxidation by means of alcohol
621 dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Both ADH and
622 ALDH use NAD^+ as an enzymatic cofactor, which is reduced to NADH.
623 Consequently, during ethanol oxidation the NAD^+/NADH ratio is significantly
624 decreased, altering the cellular redox state and triggering several adverse events
625 (60). These include inhibition of tricarboxylic acid (TCA) cycle and reduction of
626 fatty acid oxidation, which may lead to hepatic steatosis (75). Moreover, changes of
627 NAD^+/NADH ratio may be also linked to a decreased Sirt1 deacetylase activity,
628 being Sirt1 a NAD^+-dependent enzyme whose activity improves mitochondrial
629 function (46). Accordingly, the liver NAD^+/NADH ratio was decreased in rats after

EtOH consumption and, most importantly, dietary supplementation with BCAAem restored this ratio with increase of Sirt1 activity.

Reduction of Sirt1 and PGC-1α, that causes mitochondrial dysfunction in liver of the alcohol-consuming animals (49), was accompanied by reduced expression and function of eNOS. This seems to be relevant, because we have previously demonstrated that the eNOS-dependent NO production promotes both Sirt1 expression in different tissues, including liver (58), and mTOR activity in skeletal and cardiac muscle cells (23). Moreover, BCAAem was found to activate both eNOS and mTOR signaling pathways in a feed-forward manner in muscle (23). Notably, our present findings highlight that the amino acid supplementation, although unable to change the free amino acid levels in liver when supplemented alone, normalized the liver concentrations of free arginine, leucine, and tryptophan that were reduced by alcohol consumption. Arginine, a substrate of eNOS, produces NO for signaling purposes and citrulline as a byproduct. It is a conditionally essential amino acid in both humans and rodents, as it may be required depending on the health status or life cycle of the individual (8). Arginine has been proposed as therapeutic supplement in patients with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome, which has lower concentrations of NO metabolites (nitrite and nitrate) during stroke-like episodes (40). A few studies investigated the efficacy of high doses of arginine for attenuation of ethanol withdrawal signs or in hepatic encephalopathy and hyperammonemia (1, 67, 74). Also, leucine, an essential branched-chain amino acid whose breakdown products are acetyl-CoA and acetoacetate, is the most important ketogenic amino acid in humans (8). Great advances are currently in progress toward

654 a molecular definition of leucine-dependent mTORC1 activation to coordinate
655 eukaryotic cell growth and metabolism with environmental inputs, including
656 nutrients and growth factors (79). In particular, research has established a central
657 role for mTOR in regulating numerous essential cell processes – from protein
658 synthesis to autophagy – and mTOR signaling dysfunction is implicated in cancer
659 and diabetes development as well as in aging (70). Leucine supplementation has
660 beneficial effects in malnourished elderly people with skeletal muscle dysfunction
661 (*i.e.*, sarcopenia) and in other disorders. Moreover, leucine promotes mitochondrial
662 biogenesis, in addition to increase protein synthesis (26). Malnutrition and its major
663 component sarcopenia are known to be primarily responsible for the adverse clinical
664 consequences in patients with liver disease (25). EtOH and its metabolites act on
665 skeletal muscle, and the consequences of liver disease result in disturbed
666 proteostasis (*i.e.*, protein homeostasis) and sarcopenia. Leucine supplementation and
667 mitochondrial biogenesis promoting agents are currently in active evaluation to
668 prevent and reverse sarcopenia in patients with ALD and cirrhosis (25). Finally,
669 tryptophan catabolism in mammals – whose first step is mediated by tryptophan
670 dioxygenase, an enzyme mainly confined to the liver - is known to be implicated in
671 synthesis of the nicotinamide ring of NAD^+ (8). A greater liver tryptophan
672 availability would therefore lead to an increase in activity of hepatic tryptophan
673 dioxygenase and, thus, NAD^+ synthesis. This is consistent with our present results
674 on the BCAAem efficacy to normalize the reduced NAD^+/NADH ratio and Sirt1
675 activity in liver of EtOH-consuming rats. Plasma levels of tryptophan are reduced in
676 patients with alcoholic liver cirrhosis (65), and clinical studies have confirmed that
677 tryptophan protects liver from non-alcoholic fatty liver disease (NAFLD) and
678 preserves the organ during partial resection surgery (16). Thus, our results on free

amino acid levels in liver suggest that a selective amino acid mixture may influence healthily metabolism of specific amino acids affected by the EtOH assumption.

Because numerous studies have demonstrated that the alcohol effects on liver are dependent on complex systemic regulatory molecules, including for example sex hormones (72), the mechanistic relationships among metabolic sensors and signaling systems were investigated in cultured HepG2 cells. In particular, eNOS knockdown by means of selective eNOS siRNA in HepG2 cells significantly blocked the beneficial effects of amino acid supplementation on mitochondrial dysfunction induced by EtOH. This suggested the relevance of eNOS-dependent NO in mediating these effects. In fact, ODQ - the selective inhibitor of NO-dependent guanylate cyclase - blocked the BCAAem action on mitochondrial markers, strengthening that the NO protective activity is a cGMP-dependent process. In line with this, the levels of phospho-Akt and phospho-p70S6K showed the same regulation, with reduced levels after EtOH-treatment and recovered levels after EtOH plus BCAAem treatment. Exposure of HepG2 cells to BCAAem also counteracted the mitochondrial dysfunction induced by EtOH, and this protective effect was blocked by rapamycin treatment, suggesting the involvement of mTORC1 signaling in the mitochondrial protective effect of BCAAem. Recent studies have identified Akt, mTORC1, and p70S6K as positive mediators in promoting *de novo* lipogenesis (70). All of these kinases converge on and activate sterol regulatory element-binding protein 1c (SREBP1c), the master transcription factor coordinating the expression of enzymes involved in lipid synthesis (71). Experiments in genetically modified livers highlight the central role of Akt in promoting lipogenesis. Kenerson et al. (36) have recently investigated the molecular

mechanisms involved in NAFLD induced in mice by 8-week consumption of an high-fat diet. By using *in vivo* single and combined genetic deletions (*Tsc1*, *S6K1*, and *Pten* null-mutant mice), they reported that mTORC1 activation promoted protection of liver from the HFD-induced lipid accumulation through p70S6K, independent of Akt suppression (36). Their results suggest that a number of "compensatory" mechanisms may provide protection against steatosis when Akt and mTORC1 are co-activated. Consistently, our present results show that the dietary activation of both mTORC1 and Akt seem to be related to the protective effects of amino acid supplementation in the alcohol-induced fatty liver. Compensatory expression of CPT1 and fat oxidation have been indeed described in our as in Kenerson et al.'s paper (36).

Finally, casein-amino acids, differently of BCAAem, were unable to both prevent liver growth and ameliorate the EtOH-impaired mitochondrial biogenesis under *in vivo* and *in vitro* experimental conditions, suggesting that only specific amino acid formulas may have benefic effects on selective liver diseases. Nutritional supplements with BCAAs have been assessed as a treatment option for cirrhosis and hepatic encephalopathy (in which the proportion of patients with alcoholic liver disease is usually very high), and multiple systematic reviews have analyzed randomized controlled trials on BCAAs compared with no intervention, placebo, or other (3, 41). A systematic review with meta-analyses of randomized controlled trials has found that oral BCAA supplements improved the manifestations of recurrent hepatic encephalopathy in patients with cirrhosis, without effects on mortality, nutrition, or adverse events (29). Clinical trials testing BCAA supplementation in humans with various other liver diseases have been published

729 with mixed, not conclusive results. Mixed results can be due to the complex
730 mechanisms involved and the clinical differences between patients with acute or
731 recurrent disorders. Moreover, the mode of administration, *i.e.* oral and parenteral
732 administration, inclusion criteria, additional patient and intervention characteristics
733 may influence the intervention benefits. Our present *in vivo* and *in vitro* results may
734 suggest that patients with diverse liver diseases need specific amino acid formulas.
735 Moreover, mitochondrial dysfunction may be considered as a target of beneficial
736 effect of BCAAem, strengthening the hypothesis that focusing on mitochondrial
737 function to screen molecules and/or nutrients for liver disease therapy is a promising
738 strategy. Similarly, the Sirt1-eNOS-Akt-mTORC1 signaling pathway may be
739 considered a therapeutic target of liver diseases. This result suggests that NO donors
740 and/or Sirt1 agonists, in addition to mTOR modulators, might prevent the EtOH-
741 induced hepatic steatosis. In summary, our findings therefore support the hypothesis
742 that only some specific amino acid mixtures are able to protect liver against alcohol
743 damage acting on mitochondria, possibly through a Sirt1-eNOS-Akt-mTORC1
744 cross-talk signaling pathway. Thus, if that were true also in humans the dietary
745 supplementation of the present or improved formula might represent a promising
746 strategy for prevention and treatment of alcoholic liver disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.T., G.C., C.R., M.R., and F.R. performed experiments; L.T. and C.R. analyzed data; L.T. and C.R. prepared figures; L.T., G.C., C.R., M.R., F.R., M.O.C., A.V., and E.N. approved final version of manuscript; L.T., A.V., and E.N. conception and design of research; L.T., A.V., and E.N. interpreted results of experiments; L.T. drafted manuscript; L.T., A.V., and E.N. edited and revised manuscript.

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Figure Legends

Figure 1. BCAAem supplementation normalizes liver and body weight of EtOH-consuming rats. (A) Liver and (B) body weight of pair-fed rats (pair-fed CTRL, n = 6), consuming a control liquid diet in which EtOH was replaced by isocaloric maltose dextran, and rats fed with a Lieber-DeCarli liquid diet containing EtOH or BCAAem/CAAem or EtOH plus BCAAem/CAAem (n = 6-7). Data in (A) and (B) represent mean ± SD ($^{*}P < 0.05$ *vs* CTRL rats). (C) Hematoxylin and eosin liver staining of CTRL, BCAAem, EtOH, and EtOH plus BCAAem fed rats. Scale bar, 100 µm. Analyses were performed in two animals per group. Representative staining images from at least three independent experiments are reported.

Figure 2. Electron miscrocopy analysis shows that BCAAem supplementation ameliorated the liver mitochondrial damage induced by alcohol. The EtOH-consuming rats had fewer mitochondria than pair-fed animals, whereas the BCAAem supplementation renormalized their density. N, nucleus; scale bar, 0.1 µm (magnification x5,200). Analyses were performed in two animals per group. Representative images from at least three independent experiments are reported.

Figure 3. Mitochondrial biogenesis and function markers are decreased in liver of EtOH-consuming rats, whereas supplementation with BCAAem unlike CAA diet restores them to control levels. (A) PGC1-α, NRF-1, and Tfam mRNA levels were

analyzed by means of quantitative RT-PCR. Relative expression values of the CTRL rats were taken as 1.0 (B) mtDNA amount was analyzed by means of quantitative RT-PCR. Relative units were expressed in comparison to those of the CTRL rats taken as 1.0. (C) COX IV and Cyt c protein levels were detected by immunoblot analysis. The relative values were measured by densitometric analysis relative to β-actin levels. Values in CTRL rats were taken as 1.0 (D) Citrate synthase activity of liver. The values were normalized to protein content (n = 5 experiments). All data represent mean ± SD. $^{*}P < 0.05$ *vs.* CTRL; $^{\#}P < 0.05$ *vs.* EtOH-consuming rats.

Figure 4. eNOS and Sirt1 expression in liver, in addition to NAD^{+}/NADH ratio and Sirt1 activity, are reduced by EtOH consumption, while BCAAem supplementation renormalizes them to control levels. (A) eNOS and (B) Sirt1 mRNA levels were analyzed by means of quantitative RT-PCR and Western blot, respectively. Relative expression values in CTRL rats were taken as 1.0. (C) NAD^{+} to NADH ratio was measured by means of a NAD^{+}/NADH quantification kit. The values were normalized to protein content. (D) Acetyl-p53 protein levels were measured by immunoblot analysis, and the relative values detected by densitometric analysis relative to the total p53. Values of CTRL were taken as 1.0 (n = 5 experiments). All data represent mean ± SD. $^{*}P < 0.05$ *vs.* CTRL; $^{\#}P < 0.05$ *vs.* EtOH-consuming rats.

Figure 5. EtOH consumption induces oxidative damage in liver, while BCAAem supplementation coordinated an antioxidant effect. (A) Glutathione peroxidase 1 (GPX1), catalase (Cat), and superoxide dismutase 1 (SOD1) mRNA levels were analyzed by means of quantitative RT-PCR, and the relative expression values of untreated rats (CTRL) were taken as 1.0 (n = 3 experiments). (B) Cat and SOD1

protein levels were detected by immunoblot analysis. The relative values were detected by densitometric analysis, relative to β-actin levels; values of CTRL were taken as 1.0. (C) Total DNA oxidative damage measured as 8-OHdG production in liver of EtOH and BCAAem-treated mice (n = 3 experiments). $^{*}P < 0.05$ *vs.* CTRL rats; $^{\#}P < 0.05$ *vs.* EtOH-consuming rats.

Figure 6. Mitochondrial biogenesis markers, in addition to Sirt1 expression and NAD^{+}/NADH ratio, are decreased in HepG2 cells exposed to EtOH, whereas supplementation with BCAAem restores them to control levels. (A, B, C, and D) PGC1-α, Tfam, and Sirt1 mRNA levels were analyzed by means of quantitative RT-PCR. Relative expression values in the untreated cells were taken as 1.0. Sirt1 protein level was detected by immunoblot analysis (C, right). The relative values were detected by densitometric analysis relative to β-actin. The untreated cell values were taken as 1.0. (E) NAD^{+} to NADH ratio in HepG2 cells was measured by means of a NAD^{+}/NADH quantification kit. The values were normalized to protein content. (F) SOD1 mRNA levels were analyzed in HepG2 cells by means of quantitative RT-PCR. Relative expression values of the untreated cells were taken as 1.0. SOD1 protein level was detected by immunoblot analysis. The relative values were detected by densitometric analysis, relative to β-actin levels. Values of untreated cells were taken as 1.0. All data represent mean ± SD (n = 5 experiments). $^{*}P < 0.05$ *vs.* untreated cells; $^{\#}P < 0.05$ *vs.* EtOH-treated cells.

Figure 7. BCAAem reduces fat accumulation in HepG2 cells. (A) Carnitine palmitoyltransferase I (CPT1) mRNA levels were analyzed by means of quantitative RT-PCR. Relative expression values of the untreated cells were taken as 1.0. (B)

Lipid droplets were measured by Oil Red O staining, then dissolved in isopropanol and quantified by reading the absorbance at 510 nm wavelength. All data represented mean ± SD (n = 5 experiments). $^{*}P < 0.05$ *vs.* untreated cells; $^{\#}P < 0.05$ *vs.* EtOH-treated cells.

Figure 8. eNOS and mTORC1 signaling are involved in the protective effect of BCAAem supplementation in HepG2 cells. (A) eNOS mRNA was analyzed by means of quantitative RT-PCR. Relative expression value of untreated cells was taken as 1.0. (B) Phosphorylation of eNOS, Akt, and p70S6 kinase protein was detected by immunoblot analysis. The relative values were detected by densitometric analysis and normalized to total eNOS, Akt, and p70S6 kinase proteins. (C) eNOS, PGC-1α, and COX-IV protein levels were measured by immunoblot analysis in HepG2 cells transfected with either siRNA against eNOS or nontargeting siRNA, and treated with EtOH or BCAAem alone, or with EtOH in combination with BCAAem. (D) PGC-1α and COX-IV protein expression, and phosphorylation of p70S6 kinase were detected by immunoblot analysis in HepG2, treated with EtOH or BCAAem alone, or with EtOH plus BCAAem coincubated with or without 5 μM ODQ for 2 days. (E) Phosphorylation of p70S6 kinase and (F) Cyt c and COX-IV protein levels were detected by immunoblot analysis in HepG2 with or without 100 nM rapamycin. Values of untreated or vehicle-treated cells were taken as 1.0. Representative immunoblots of five reproducible ones are shown. All data represent mean ± SD (n = 5 experiments). $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* untreated or vehicle-treated cells; $^{\#}P < 0.05$ *vs.* EtOH-treated cells; $^{\dagger}P < 0.01$ *vs.* BCAAem plus EtOH-treated cells.

Figure 1

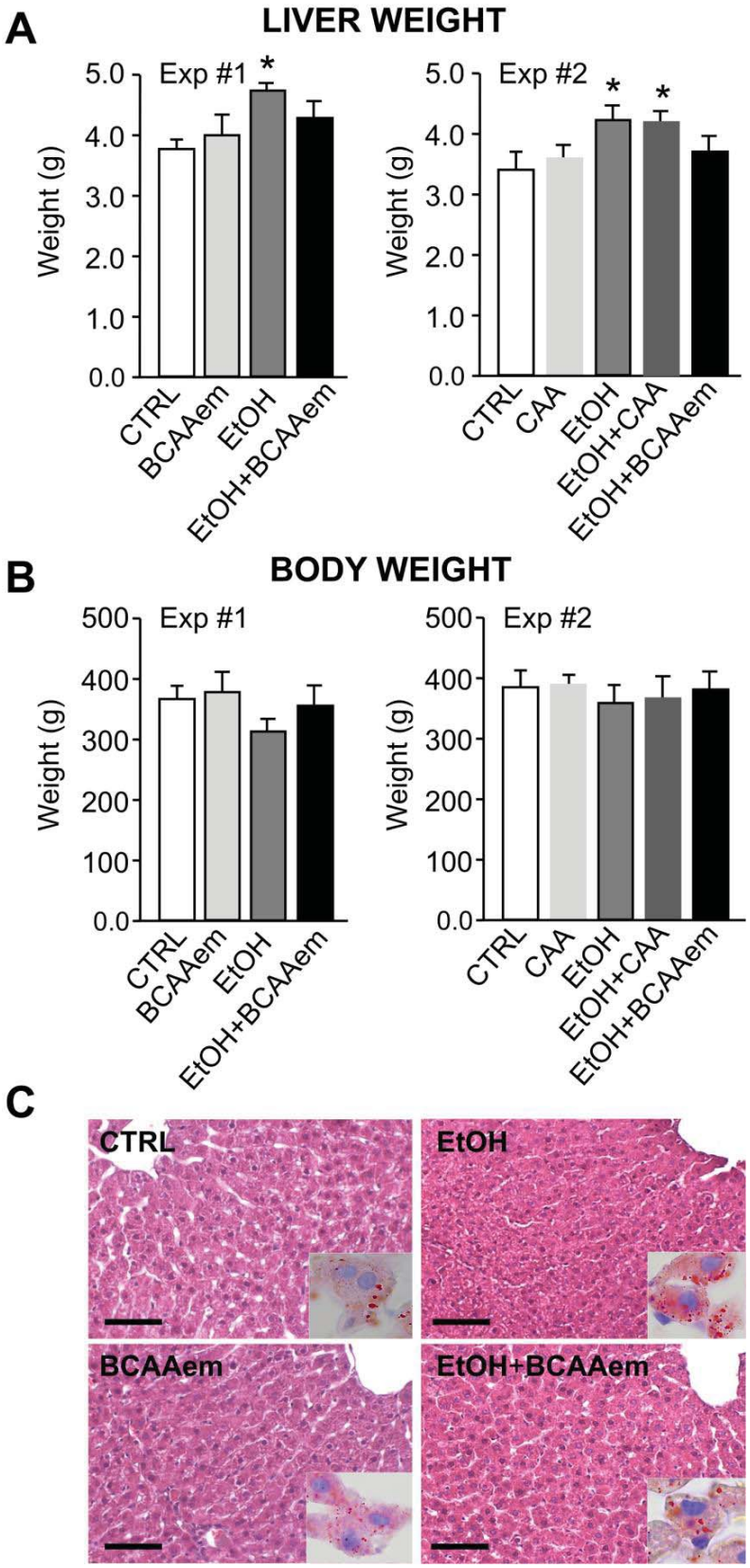

Figure 2

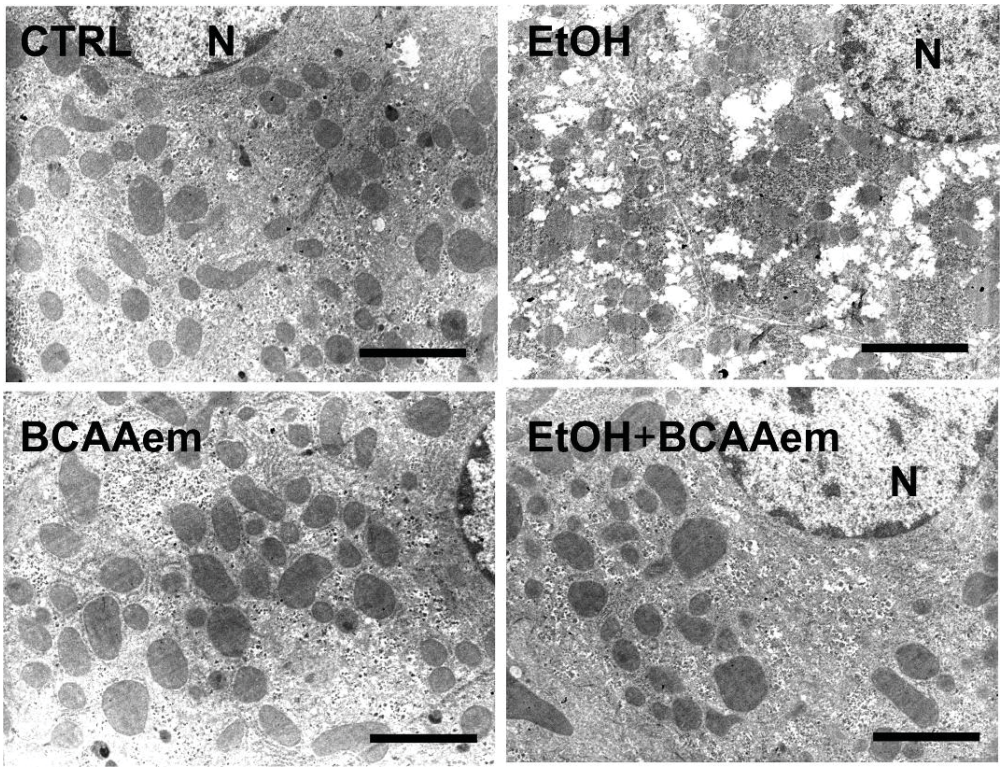

Figure 3

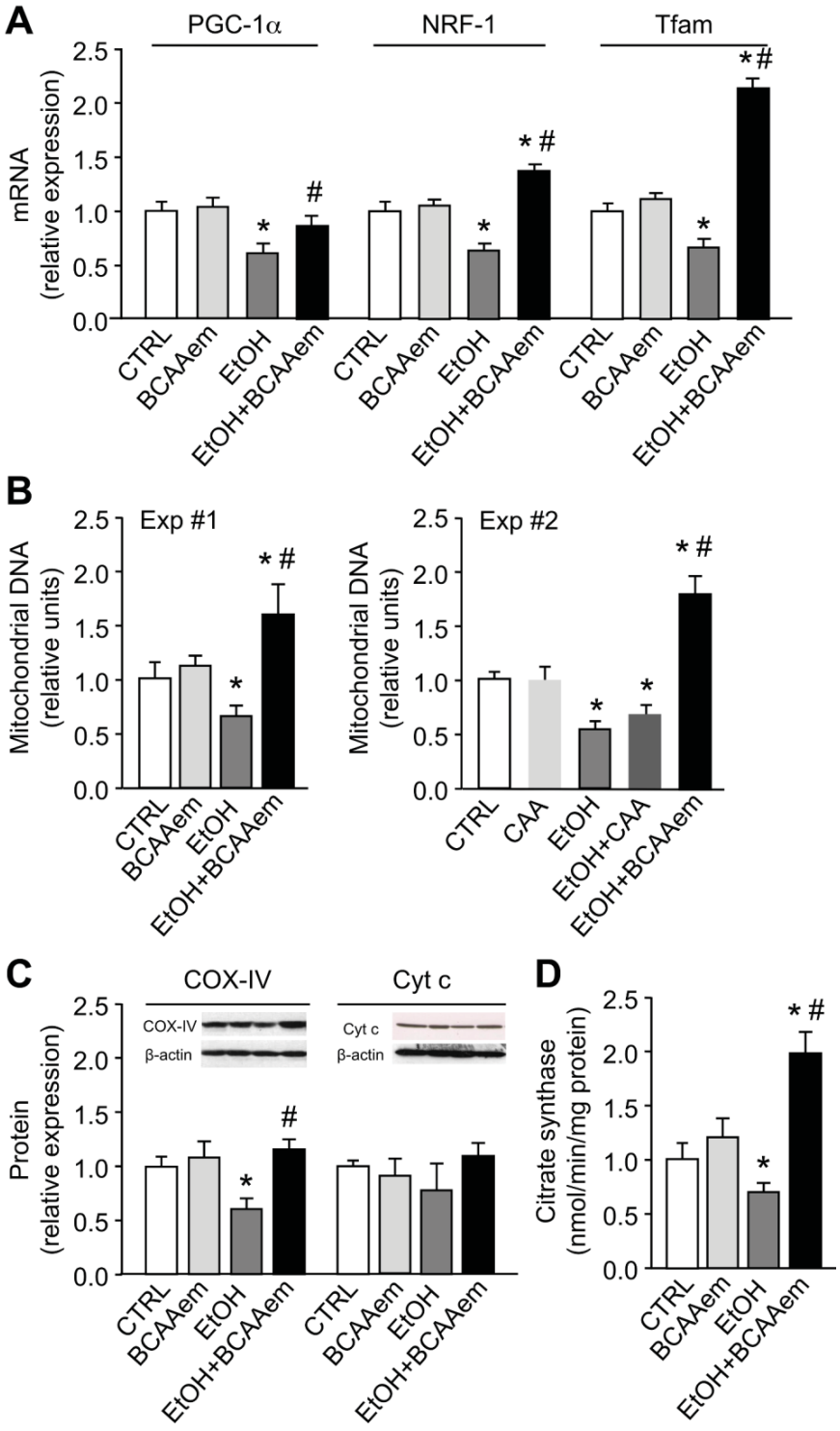

Figure 4

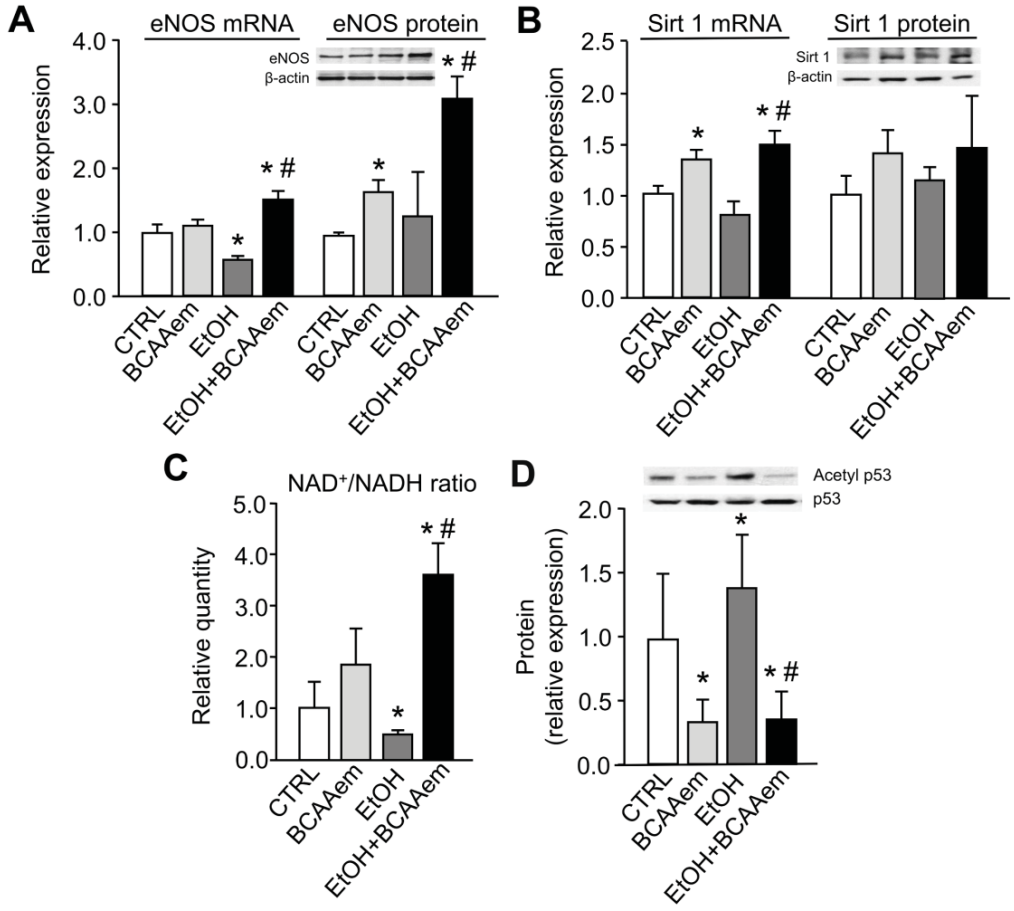

Figure 5

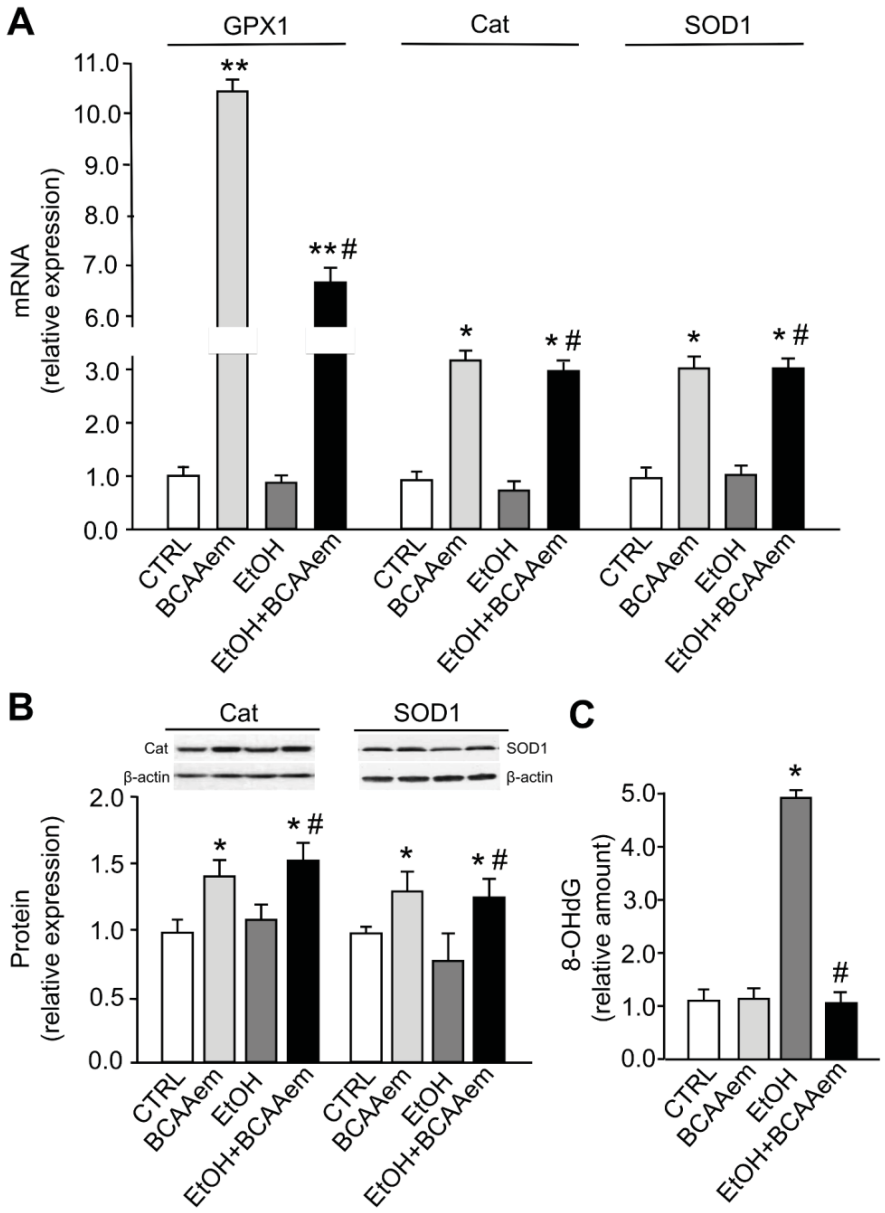

Figure 6

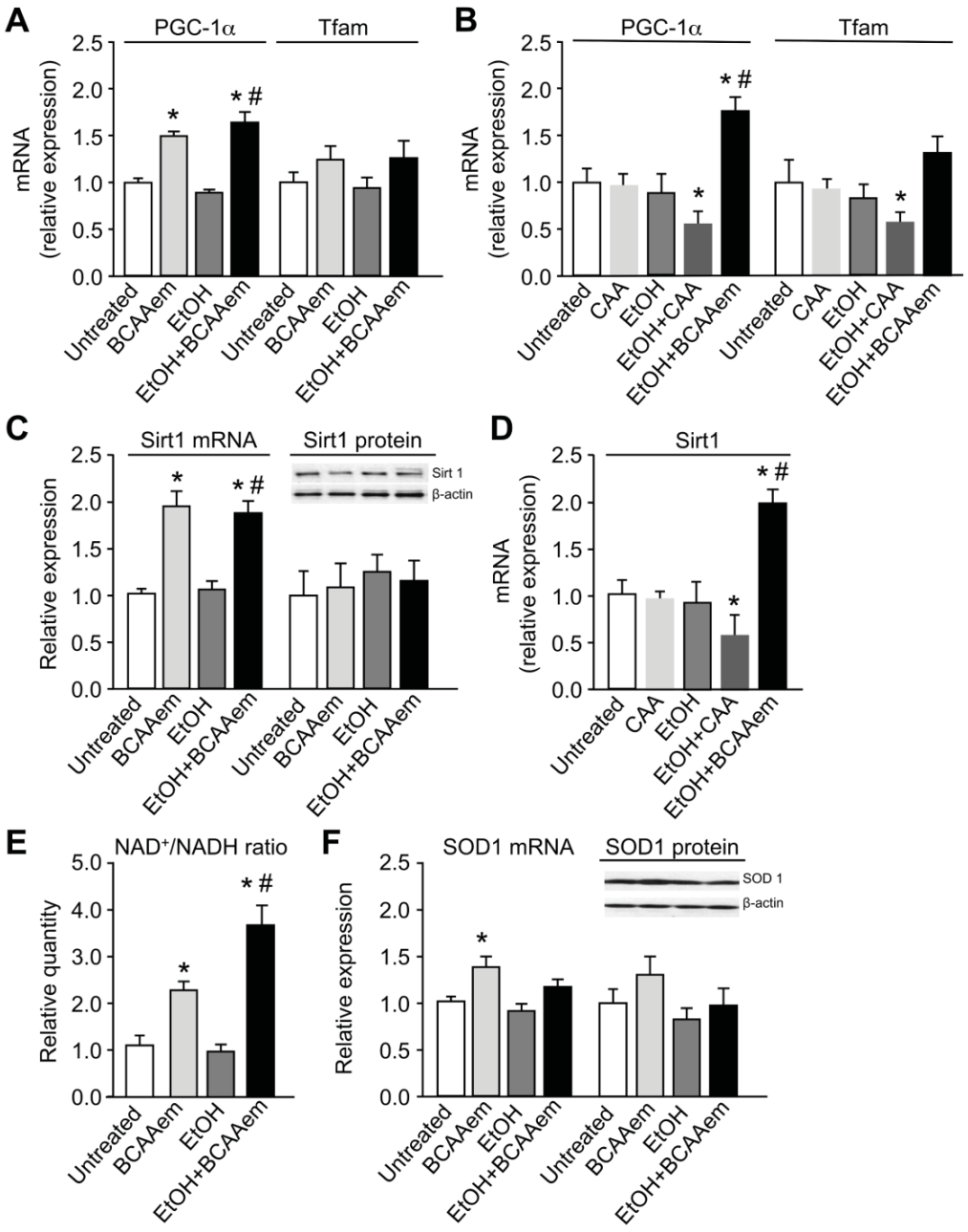

Figure 7

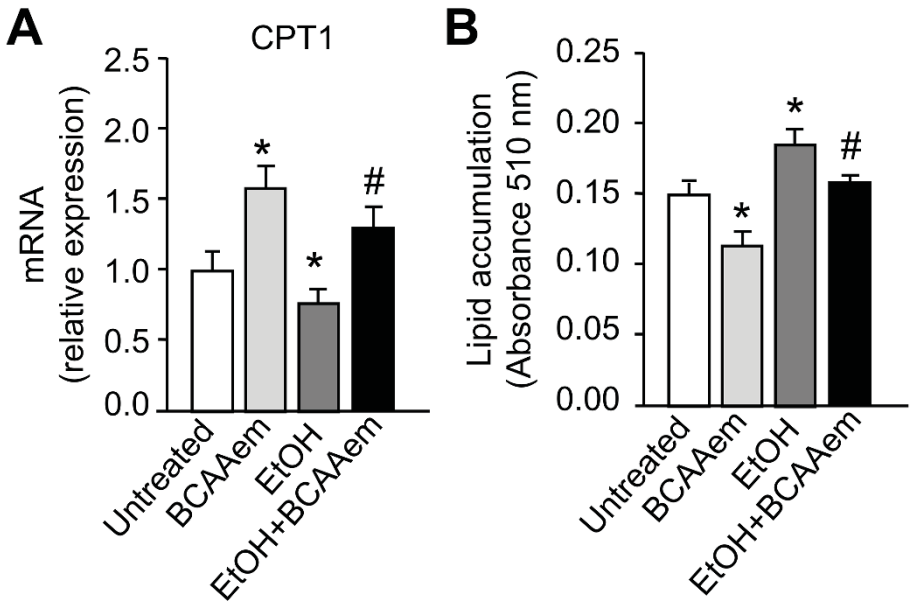

Figure 8

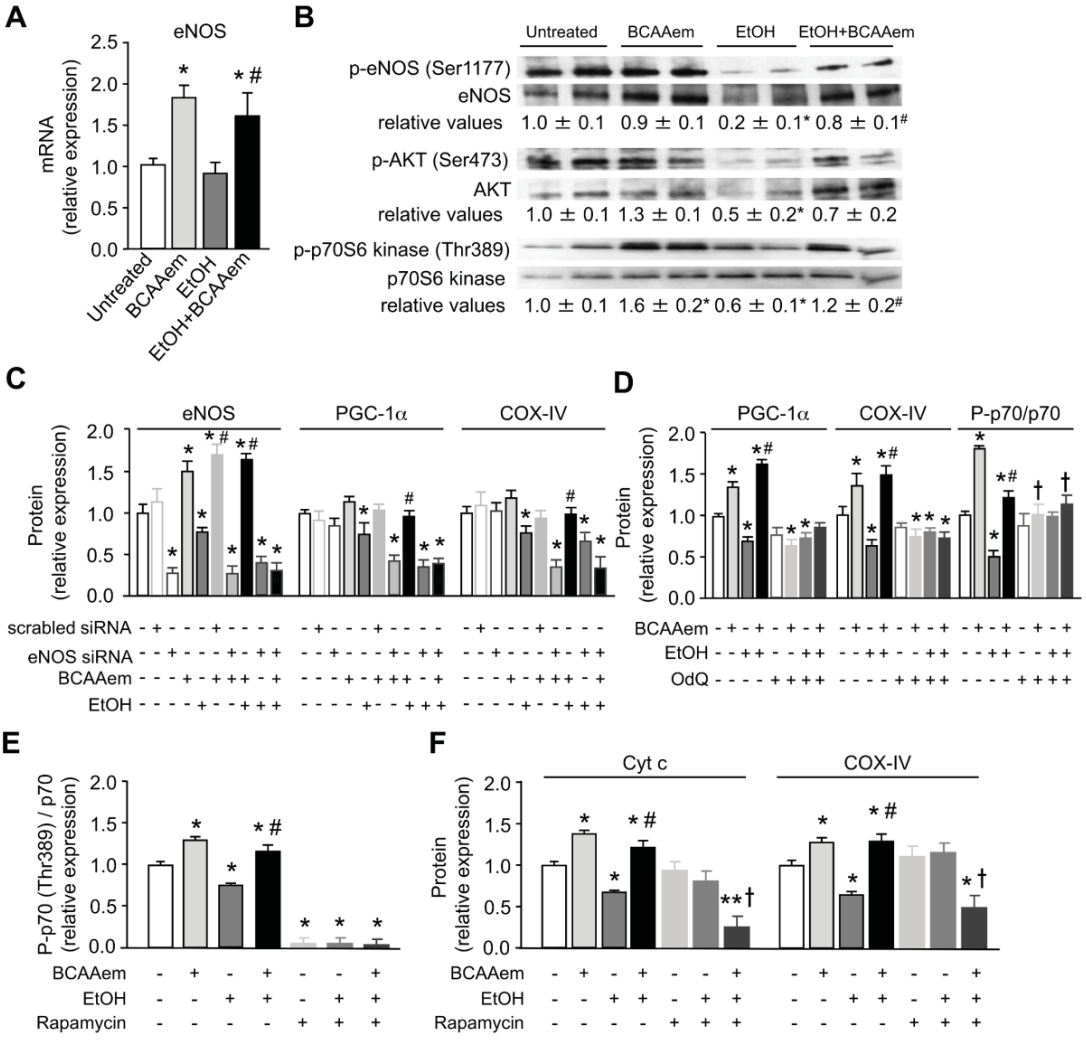

Table 1. *Composition of diets used in the present work*

Diet ingredients	Composition, grams/L					
	CTRL	EtOH	CAA	BCAAem	EtOH+ CAA	EtOH+ BCAAem
Casein	41.4	41.4	41.4	41.4	41.4	41.4
L-Cystein	0.5	0.5	0.5	0.5	0.5	0.5
DL-Methionine	0.3	0.3	0.3	0.3	0.3	0.3
Corn Oil	8.5	8.5	8.5	8.5	8.5	8.5
Olive Oil	28.4	28.4	28.4	28.4	28.4	28.4
Safflower Oil	2.7	2.7	2.7	2.7	2.7	2.7
Maltose Dextrin	115.2	25.6	115.2	115.2	115.2	25.6
Cellulose	10	10	10	10	10	10
Mineral Mix	8.75	8.75	8.75	8.75	8.75	8.75
Vitamin Mix	2.5	2.5	2.5	2.5	2.5	2.5
Choline Bitartrate	0.53	0.53	0.53	0.53	0.53	0.53
Xanthan Gum	3	3	3	3	3	3
Ethanol	-	6.2*	-	-	6.2*	6.2*
Histidine	-	-	0.26	0.27	0.26	0.27
Isoleucine	-	-	0.43	1.56	0.43	1.56
Leucine	-	-	0.9	3.05	0.9	3.05
Lysine	-	-	0.75	1.32	0.75	1.32
Methionine+cysteine	-	-	0.36	0.54	0.36	0.54
Phenylalanine	-	-	0.48	0.16	0.48	0.16
Threonine	-	-	0.41	1.08	0.41	1.08
Tryptophan	-	-	0.12	0.02	0.12	0.02
Valine	-	-	0.53	1.96	0.53	1.96
Alanine	-	-	0.29	-	0.29	-
Arginine	-	-	0.34	-	0.34	-
Aspartic acid	-	-	0.69	-	0.69	-
Glutamic acid	-	-	2.17	-	2.17	-
Glycine	-	-	0.17	-	0.17	-
Proline	-	-	1.01	-	1.01	-
Serine	-	-	0.57	-	0.57	-
Tyrosine	-	-	0.52	-	0.52	-

*Ethanol is expressed as % (vol/vol). The different diets are isocaloric.

Table 2. *Ion transitions, instrument settings and weighted regression for amino acid detection*

MIX_1

AA	Precursor (*m/z*)	Product (*m/z*)	DP	CE	Weighted regression
Serine	106,051	60,044	30	15	1/x
Asparagine	133,061	74,024	30	15	$1/x^2$
Tyrosine	182,082	165,053	30	15	$1/x^2$
Threonine	120,066	56,049	30	15	1/x

MIX_2

AA	Precursor (*m/z*)	Product (*m/z*)	DP	CE	Weighted regression
Glycine	76,040	30,033	40	15	1/x
Alanine	90,056	44,053	40	15	1/x
Leucine	132,103	86,096	40	15	1/x
Isoleucine	132,103	69,072	40	15	1/x
Valine	118,087	72,081	40	15	$1/x^2$
Proline	116,071	70,065	40	15	1/x
Histidine	156,077	109,830	40	15	1/x
Methionine	150,059	104,053	40	15	1/x
Aspartic acid	134,045	74,023	40	15	1/x
Glutamine	147,077	84,015	40	15	1/x
Phenylalanine	166,087	119,964	40	15	1/x

MIX_3

AA	Precursor (*m/z*)	Product (*m/z*)	DP	CE	Weighted regression
Glutamic acid	148,061	102,056	80	18	$1/x^2$
Lysine	147,113	84,093	80	18	1/x
Arginine	175,12	116,072	80	18	1/x
Tryptophan	205,098	188,07	80	18	1/x

Table reports the mass spectrometry parameters as determined by infusion of each amino acid. In each standard mixture, name, precursor (*m/z*), product (*m/z*), DP, declustering potential (V), CE, collision energy (V) and weighted regression are indicated for each amino acid.

Table 3. *Rat primers for quantitative RT-PCR*

Gene	Primer Sequences		PCR Product (bp)	T_a (°C)
Tfam	*Sense*	5'- CAGAGTTGTCATTGGGATTGGG -3'	140	60
	Antisense	5'- GCATTCAGTGGGCAGAAGTC -3'		
NRF1	*Sense*	5'- TATCCGAAAGAGACAGCAGACAC -3'	130	60
	Antisense	5'- CTTAAAGACAGGGTTGGGTTTGG -3'		
PGC1α	*Sense*	5'- CCACTACAGACACCGCACACATC -3'	141	60
	Antisense	5'- TCTCTGCGGTATTCGTCCCTCTT -3'		
eNOS	*Sense*	5'- CACAGGCATCACCAGGAAGAAG -3'	98	60
	Antisense	5'- CCTTCACACGCTTCGCCATC -3'		
TBP	*Sense*	5'- GCAGCCTCAGTACAGCAATC -3'	167	60
	Antisense	5'- TGGTGTGGCAGGAGTGATAG 3'		
ND1	*Sense*	5'- GGACCTAAGCCCAATAACGA -3'	348	58
	Antisense	5'- GCTTCATTGGCTACACCTTG -3'		
GPX1	*Sense*	5' – CAGGAGAATGGCAAGAATGAAGAG -3'	145	60
	Antisense	5' – ACTGGGTGCTGGCAAGGC -3'		
Cat	*Sense*	5'- CATCGGCACATGAATGGC -3'	281	60
	Antisense	5' – ACCTTGGTCAGGTCAAATGG -3'		
SOD1	*Sense*	5'- TGAAGAGAGGCATGTTGGAG -3'	164	58
	Antisense	5' – CCACCTTTGCCCAAGTCATC -3'		
βglobin	*Sense*	5'- CTTCTGGCTATGTTTCCCTT -3'	237	58
	Antisense	5'- GTTCTCAGGATCCACATG -3'		

T_a, temperature of annealing

Table 4. *Human primers for quantitative RT-PCR*

Gene	Primer Sequences		PCR Product (bp)	T_a (°C)
Tfam	*Sense*	5'- AGATTGGGGTCGGGTCAC -3'	184	60
	Antisense	5'- GACAACTTGCCAAGACAGATG -3'		
NRF1	*Sense*	5'- ACTCGTGTGGGACAGCAAGC -3'	200	60
	Antisense	5'- ATGGTGAGAGGCGGCAGTTC -3'		
PGC1α	*Sense*	5'- GACCCCAGAGTCACCAAATGAC -3'	132	60
	Antisense	5'- TTGGTTGGCTTTATGAGGAGGA -3'		
eNOS	*Sense*	5'- TGACCCTCACCGCTACAACATC -3'	103	60
	Antisense	5'- TGATTTCCACTGCTGCCTTGTCT -3'		
CPT1	*Sense*	5'- GGAGAGGAGACAGACACC ATCCA -3'	243	60
	Antisense	5'- CAAAATAGGCCTGACGACACCTG 3'		
ACOX1	*Sense*	5'- TGGTGAAGAAGATGAGGGAGT -3'	126	60
	Antisense	5'- AGCAAGGTGGGCAGGAAC -3'		
SOD1	*Sense*	5'- GAGACGGGGTGCTGGTTTGC -3'	82	60
	Antisense	5'- ACGCCGAGGTCCTGGTTCC -3'		
Sirt1	*Sense*	5'- GGGAGGCGGAGGCAGAGG -3'	154	60
	Antisense	5'- TCGTCGTCGTCGTCTTCGTC -3'		
TBP	*Sense*	5'- AGGCACCACAGCTCTTCCAC -3'	130	60
	Antisense	5'- CCCAGAACTCTCCGAAGCTG -3'		

T_a, temperature of annealing.

Table 5. *Amino acid concentrations measured using chromatography in liver*

	CTRL	BCAAem	EtOH	EtOH+BCAAem
Alanine	670.1 ± 65.7	832.0 ± 25.0	691.2 ± 128.0	679.0 ± 110.0
Arginine	6.6 ± 0.7	4.3 ± 0.8	2.46 ± 0.5*	4.3 ± 0.7#
Asparagine	8.9 ± 2.4	7.9 ± 2.7	8.1 ± 2.1	6.9 ± 2.8
Glycine	409.8 ± 78.2	479.8 ± 32.0	424.1 ± 52.0	440.7 ± 67.2
Glutamic acid	5232.0 ± 430.0	5394.0 ± 438.0	5970.5 ± 832.0	4717.70 ± 850
Histidine	$527.9 \cdot 10^3 \pm 80.3 \cdot 10^3$	$606.8 \cdot 10^3 \pm 10.8 \cdot 10^3$	$461.1 \cdot 10^3 \pm 44.6 \cdot 10^3$	$406.0 \times 10^3 \pm 19.3 \cdot 10^3$
Isoleucine	141.9 ± 29.7	144.4 ± 4.5	92.9 ± 16.6*	93.2 ± 17.9*
Leucine	195.0 ± 56.4	189.1 ± 81.9	110.2 ± 18.8 *	188.9 ± 92.4#
Lysine	72.2 ± 16.8	90.5 ± 19.7	62.3 ± 9.6	53.3 ± 6.4
Methionine	12.9 ± 5.5	17.9 ± 5.3	11.9 ± 3.9	17.8 ± 4.7
Phenylalanine	32.0 ± 13.2	36.4 ± 4.8	27.7 ± 10.2	28.8 ± 5.2
Proline	33.7 ± 5.2	33.5 ± 1.3	27.2 ± 8.7	24.6 ± 4.2
Serine	348.7 ± 33.9	362.4 ± 9.9	220. 7 ± 58.4*	215.4 ± 43.0*
Threonine	55.5 ± 2.41	52.2 ± 2.0	52.9 ± 2.5	51.5 ± 2.2
Tryptophan	$1252.8 \cdot 10^3 \pm 226.4 \cdot 10^3$	$1350.4 \cdot 10^3 \pm 74.54 \cdot 10^3$	$787.1 \cdot 10^3 \pm 112.7 \cdot 10^3$*	$1038.2 \cdot 10^3 \pm 169.9 \cdot 10^3$#
Tyrosine	514.5 ± 63.3	382.6 ± 19.7	311.7 ± 71.89 *	274.6 ± 39.7*
Valine	429.9 ± 46.7	455.3 ± 17.7	291.9 ± 61.1	290.8 ± 48.9

Values are reported as means ± SD (pmol/mg of tissue), n = 4 animals/group; *P value < 0.05 shows difference *vs.* CTRL group, $^{\#}P$ value < 0.05 shows difference *vs.* EtOH group.

Table 6. *Mitochondrial morphometric analysis in liver tissue*

	N*mit*/100μm^2	A*mit*	A*mit*/A*cyt*
CTRL	0.82 ± 0.14	12.32 ± 1.75	0.11 ± 0.02
BCAAem	0.88 ± 0.17	13.48 ± 1.86	0.13 ± 0.03
EtOH	**0.52** ± 0.13 *****	**16.89** ± 4.1 *****	**0.08** ± 0.01 *****
EtOH+BCAAem	0.71 ± 0.11	**26.12** ± 4.4 * †	0.16 ± 0.03
ANOVA	F = 8.70; P = 0.0001	F = 65.95; P = 0.0001	F = 13.85; P = 0.0001

Mitochondrial density (N*mit/100 μm*^{2}), mitochondrial area (A*mit*), and mitochondrial to cytoplasmic area (A*mit*/A*cyt*) ratio measured in liver tissue of the various experimental groups. Values are given as mean ± SD. * $P < 0.05$ vs CTRL, † $P < 0.05$ *vs*. EtOH-fed animals.