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RESEARCH ARTICLE | *Nutrient Sensing, Nutrition, and Metabolism*

A specific amino acid formula prevents alcoholic liver disease in rodents

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Tedesco L, Corsetti G, Ruocco C, Ragni M, Rossi F, Carruba MO, Valerio A, Nisoli E. A specific amino acid formula prevents alcoholic liver disease in rodents. *Am J Physiol Gastrointest Liver Physiol* 314: G566–G582, 2018. First published January 25, 2018; doi:10.1152/ajpgi.00231.2017.—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, although 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. Nicotinamide adenine dinucleotide levels and sirtuin 1 (Sirt1) expression, as well as endothelial nitric oxide (eNOS) and mammalian/mechanistic target of rapamycin (mTOR) signaling pathways, were downregulated 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 antireactive oxygen species genes. The amino acid-protective effects likely reflect activation of sirtuin 1-endothelial nitric oxide synthase-mammalian target of rapamycin pathway able to regulate the cellular energy balance of hepatocytes exposed to chronic, alcoholic damage.

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 (ADH), catalase (Cat), NADPH-oxidase, xanthine oxidase, and the microsomal alcohol oxidizing system, lead to acetaldehyde accumulation in liver. Acetaldehyde can be oxidized to acetate, mainly through aldehyde dehydrogenases (ALDH), which are mitochondrial, nicotinamide dinucleotide (NAD^+)-dependent enzymes, so 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 posttranslational 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 1α (PGC-1α) (56, 80). Moreover, Lieber and coworkers (49) have demonstrated that Sirt1 and PGC-1α, acting as a complex, can physiologically regulate the activity of each other in liver of alcohol-consuming animals.

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 with reduced activities of all of the respiratory complexes (except complex II) (22), impaired mitochondrial protein synthesis (17), mitochondrial DNA (mtDNA) damage (12), and ribosomal defects (10), has been suggested to cause failing

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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 ALD 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 Health and complied with The National Animal Protection Guidelines. For *experiment 1*, a total of 26 male Wistar rats (3 mo 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 wk, corresponding to a final concentration of 6.2% (vol/vol)]; *3*) the BCAAem group (BCAAem, $n = 6$) was fed with a control liquid diet, in which EtOH was replaced by isocaloric maltose dextran and supplemented with BCAA mixture (10 g/l), that provided 1.5 $g \cdot kg^{-1} \cdot day^{-1}$ BCAAem; and *4*) the EtOH plus BCAAem group (EtOH + BCAAem, $n = 7$) was fed with a Lieber-DeCarli liquid diet containing EtOH and BCAAem ad libitum.

For *experiment 2*, a total of 30 male Wistar rats (3 mo old) were divided into five groups: *1*) the pair-fed group (Ctrl, $n = 6$); *2*) the EtOH group (EtOH, $n = 6$); *3*) the EtOH plus BCAAem group ($n = 6$) were fed as in *experiment 1*; *4*) the casein-amino acid group (CAA, $n = 6$) was 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 $g \cdot kg^{-1} \cdot day^{-1}$ CAA supplement; and *5*) the EtOH plus CAA group (EtOH + CAA, $n = 6$) was 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). The composition of each diet is described in Table 1. The amino acid concentrations of BCAAem used were previously found to be active in rodents and mimic the recommended daily dose for humans (23). Dietary treatments were prolonged for 8 wk, in a 12-h:12-h light/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 killed by guillotine. Left lobe of liver was fixed for microscopy analysis, whereas the rest of the organ was quickly frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Sample preparation. Liver ($n = 4$ animals/group) was weighted, homogenized in cold methanol:water (vol/vol, 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 min at 4°C. Two microliters of supernatant was directly loaded onto the ultra-performance liquid chromatography (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 solution was 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. Three mixtures of amino acids were next prepared on the basis of DP and CE values: *mix 1*,

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

	Composition, g/l					
Diet Ingredients	Ctrl	EtOH	CAA	BCAAem	EtOH + CAA	EtOH + BCAAem
Casein	41.4	41.4	41.4	41.4	41.4	41.4
L-Cysteine	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	—	—	0.90	3.05	0.90	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	—

The different diets are isocaloric. EtOH, ethanol; BCAAem, branched-chain amino acid-enriched mixture; CAA, casein-amino acid. *Ethanol is expressed as percentage (vol/vol).

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 time-of-flight mass spectrometry and Product Ion mode, according to the *m/z* values reported in Table 2. The source parameters were as follows: gas 1, 33 psi; gas 2, 58 psi; curtain gas, 25 psi, temperature 500°C, and IonSpray Voltage Floating 5,500 V.

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 (Cernusco sul Naviglio; Agilent Technologies Italia, Milan, Italy). The chromatographic column was Acquity HSS (T3 C18 2.1 × 100 mm, 1.7 μm; Waters, Milford, MA), whereas the mobile phase was *1)* 1 mM tridecafluoroheptanoic acid (TDFHA) in water, *2*) 1 mM TDFHA in acetonitrile. A gradient from the latter 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, Framingham, MA). 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 hematoxylin and eosin (H and E).

Transmission electron microscopy analysis. Liver was removed, fixed with 2.5% glutaraldehyde in cacodylate buffer (pH 7.4, 0.2M), and postfixed for 1 h with 1% OsO_4 in the same buffer. The samples were processed with standard procedures for embedding in Araldite (Sigma) and polymerized at 60°C for 72 h. Thick sections (~1 μm) were stained with Epoxy Tissue Stain (no. 14950; Electron Microscopy Sciences, Hatfield, PA). 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 ×5,000 using electron microscope film (Kodak electron film 4489, 6.5 × 9.0 cm; Rochester, NY). Five different section levels of each sample were examined. The total area examined was ~140,000.00 μm^2 in each group. We have previously shown that cytochrome *c* oxidase and peroxisome staining were markedly reduced, whereas 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, Corning, NY). Six hours 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 2,000,000 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 for the different assays.

Moreover, HepG2 cells were seeded in six-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 six-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. They were then 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 Bio-Rad Model 680 microplate reader (Segrate, Italy) (66).

Quantitative RT-PCR analysis. Quantitative RT-PCR reactions were performed as described (73) and run with the iQ SybrGreenI SuperMix (Bio-Rad) on an iCycler iQ Real-Time PCR detection system (Bio-Rad). Briefly, RNA was isolated from tissue using the

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

	Precursor (*m/z*)	Product (*m/z*)	DP	CE	Weighted Regression
Mix 1					
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					
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					
Glutamic acid	148.061	102.056	80	18	$1/x^2$
Lysine	147.113	84.093	80	18	$1/x$
Arginine	175.120	116.072	80	18	$1/x$
Tryptophan	205.098	188.070	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*), declustering potential (DP) (V), collision energy (CE) (V), and weighted regression are indicated for each amino acid.

RNeasy Tissue Mini Kit (Qiagen, Milan, Italy). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). 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 with that of TATA-box-binding protein, referred to as ΔCT. The gene-relative levels were expressed as $2^{-(\Delta\Delta CT)}$, in which ΔΔCT equals ΔCT of EtOH-, 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, IL) as described by the manufacturer, in the presence of protease and phosphatase inhibitor cocktail (Sigma). Protein content was measured by the bicinchoninic acid protein assay (BCA; Pierce, Euroclone, Milan, Italy), and 50 μg of proteins was run on SDS-PAGE under reducing conditions. The separated proteins were then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). Proteins of interest were revealed with specific antibodies: anti-cytochrome *c* (Cyt *c*) oxidase subunit IV (COX 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-p70S6 kinase), anti-p70 S6 kinase, anti-Sirt1, anti-β-actin (all from Cell Signaling, Euroclone, Milan, Italy), anti-eNOS (Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 (from GeneSpin, Milan, Italy), anti- superoxide dismutase 1 [Cu-Zn] (SOD1; from Santa Cruz Biotechnology), anti-catalase (from Santa Cruz Biotechnology), at 1:1,000 dilution each.

The immunostaining was detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin for 1 h at room temperature. After the visualization of p-eNOS, acetyl p53, p-Akt, and p-p70 S6 kinase, filters were stripped with the Restore Western Blot Stripping Buffer (Euroclone) and further used for the visualization of total eNOS, total p53, total Akt, or total p70 S6 kinase. The amount of protein was measured using SuperSignal Substrate (Pierce, Euroclone) and quantified by densitometry with ImageJ (NIH, Bethesda, MD) software image analyzer.

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′		
Catalase			
Sense	5′-CATCGGCACATGAATGGC-3′	281	60
Antisense	5′-ACCTTGGTCAGGTCAAATGG-3′		
SOD1			
Sense	5′-TGAAGAGAGGCATGTTGGAG-3′	164	58
Antisense	5′-CCACCTTTGCCCAAGTCATC-3′		
β-Globin			
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′-GGAGAGGAGACAGACACCATCCA-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.

Mitochondrial DNA measurement. For mtDNA analysis, total DNA was extracted with QIAamp DNA extraction kit (Qiagen). The content of mtDNA was calculated using real-time quantitative PCR by measuring the threshold cycle ratio (ΔCT) of a mitochondrial-encoded gene NADH dehydrogenase subunit 1 (ND1) vs. a nuclear-encoded gene (β-globin) in the liver of EtOH-, BCAAem-, or CAA mixture-treated and control (Ctrl) rats, as described (27).

Citrate synthase activity measurement. The citrate synthase activity was measured spectrophotometrically at 412 nm at 30°C in liver tissue extracts (51). Liver samples were added to a buffer containing 0.10 mM 5,5-dithio-bis-(2-nitrobenzoic) acid, 0.50 mM oxaloacetate, 50.00 μM EDTA, 0.31 mM acetyl CoA, 5.00 mM triethanolamine hydrochloride, and 0.10 M Tris·HCl, pH 8.1. Citrate synthase activity was expressed as nanomoles of citrate produced per minute per milligram of protein. The data were normalized to total protein content, determined as reported above.

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). The samples were then 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. The NAD^+/NADH ratio was then 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 (JaICA, Hamamatsu, Japan) 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). 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-Keul's test, or Student's *t*-test. Data were presented as the means ± 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 wk resulted in a significant increase of the liver weight, together with fat accumulation, compared with 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 attributable 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 not in a statistically different manner (Fig. 1*B*), whereas 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).

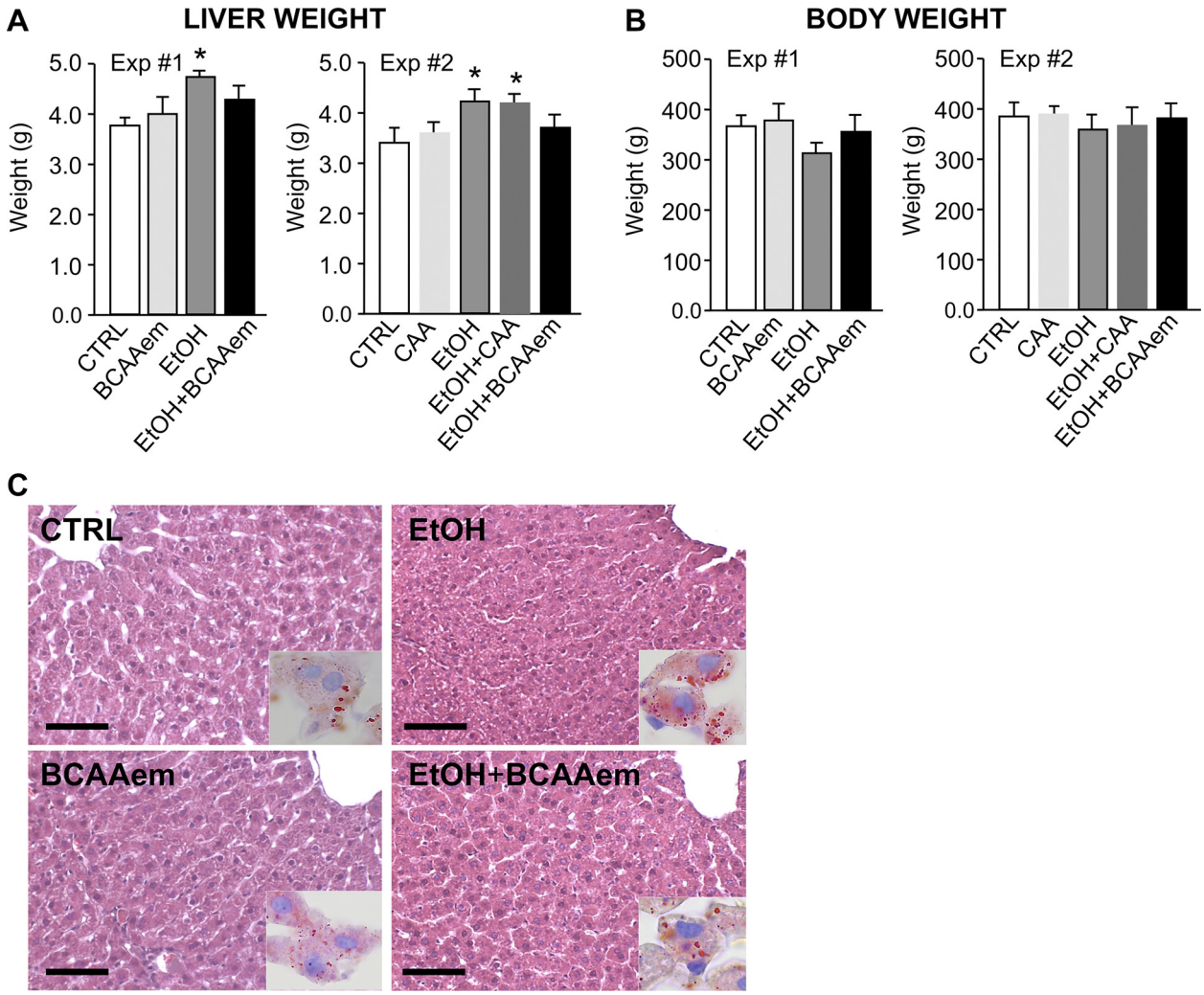

Fig. 1. Branched-chain amino acid-enriched mixture (BCAAem) supplementation normalizes liver and body weight of ethanol (EtOH)-consuming rats. Liver (*A*) and body weight (*B*) of pair-fed rats (pair-fed Ctrl, $n = 6$), consuming a control liquid diet in which EtOH was replaced by isocaloric maltose dextran, and rats fed with a Lieber-DeCarli liquid diet containing EtOH or BCAAem/casein-amino acid-enriched mixture (CAAem) or EtOH plus BCAAem/CAAem ($n = 6$–7) are shown. Data in *A* and *B* represent means ± 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 2 animals per group. Representative staining images from at least 3 independent experiments are reported.

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	4717.7 ± 850.0
Histidine	$527.9 \cdot 10^3 \pm 80.3 \cdot 10^3$	$606.8 \cdot 10^3 \pm 10.8 \cdot 10^3$	$461.1 \cdot 10^3 \pm 44.6 \cdot 10^3$	$406.0 \cdot 10^3 \pm 19.3 \cdot 10^3$
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	55.50 ± 2.41	52.20 ± 2.00	52.90 ± 2.50	51.50 ± 2.20
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	514.50 ± 63.30	382.60 ± 19.70	311.70 ± 71.89*	274.60 ± 39.70*
Valine	429.9 ± 46.7	455.3 ± 17.7	291.9 ± 61.1	290.8 ± 48.9

Values are means ± SD (pmol/mg of tissue), $n = 4$ animals/group. EtOH, ethanol; BCAAem, branched-chain amino acid-enriched mixture. *$P < 0.05$ shows difference vs. Ctrl group; #$P < 0.05$ shows difference vs. EtOH group.

Free amino acid levels were next measured in liver tissue. As reported in Table 5, arginine, leucine, and tryptophan concentrations were reduced by EtOH consumption. Although 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, although 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, whereas the A*mit*/A*cyt* ratio was lower in the EtOH-consuming group compared with 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

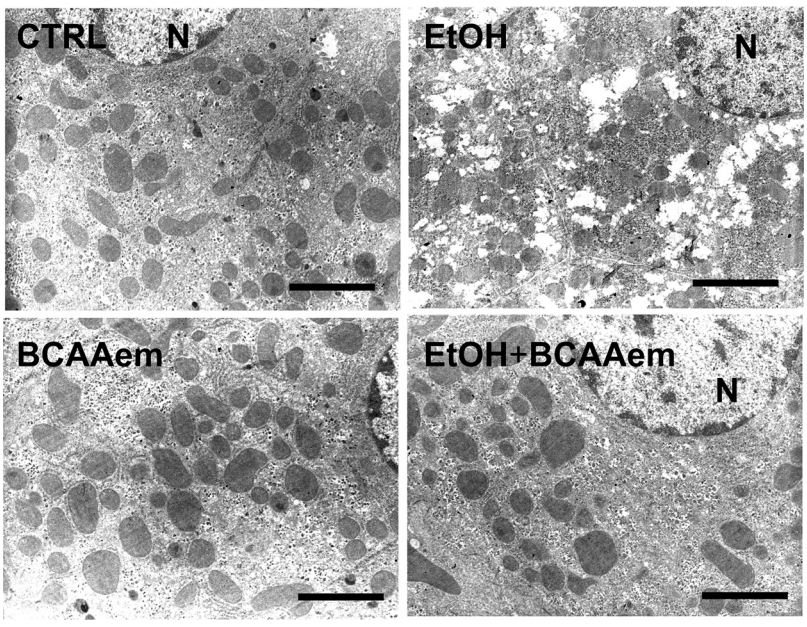

Fig. 2. Electron microscopy analysis shows that branched-chain amino acid-enriched mixture (BCAAem) supplementation ameliorated the liver mitochondrial damage induced by alcohol. The ethanol (EtOH)-consuming rats had fewer mitochondria than pair-fed animals, whereas the BCAAem supplementation renormalized their density. N, nucleus; scale bar = 0.1 μm (magnification ×5,200). Analyses were performed in 2 animals per group. Representative images from at least 3 independent experiments are reported.

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 attributable to the EtOH consumption. As expected, the hepatic mRNA levels of PGC-1α, nuclear respiratory factor-1 (NRF-1), and mitochondrial DNA transcription factor A (Tfam) were all lowered by EtOH consumption compared with 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 35.0 ± 1.5% compared with pair-fed rats, whereas its consumption with BCAAem increased mtDNA amount by 55.0 ± 2.6% and 138.0 ± 4.0% compared with pair-fed and EtOH group, respectively (Fig. 3*B*). In accord with liver weight results (Fig. 1*A*), CAA diet was unable to affect mtDNA amount either when supplemented with EtOH or alone (Fig. 3*B*). Although protein levels of both COX IV and Cyt *c*, as well as citrate synthase activity, were reduced in liver of EtOH-consuming rats compared with 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 the hepatic liver mitochondrial damage induced by EtOH consumption.

Because eNOS-dependent NO production was found to promote mitochondrial biogenesis in different cells, including hepatocytes (30, 57), we analyzed eNOS mRNA and protein levels in livers of both pair-fed controls and EtOH-consuming rats, supplemented with or without BCAAem. A reduction by 38.0 ± 2.1% of eNOS mRNA level was evident in the EtOH group compared with the pair-fed group, whereas 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 with 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 further investigated 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 an ~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 occurred 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 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), Cat, and SOD [Cu-Zn], also known as SOD1 mRNA levels were reduced in EtOH group compared with 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, whereas the BCAAem supplementation

Table 6. *Mitochondrial morphometric analysis in liver tissue*

	N*mit*/100 μm^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 ± 4.10*	0.08 ± 0.01*
EtOH + BCAAem	0.71 ± 0.11	26.12 ± 4.40*†	0.16 ± 0.03
ANOVA	$F = 8.70$; $P = 0.0001$	$F = 65.95$; $P = 0.0001$	$F = 13.85$; $P = 0.0001$

Values are means ± SD. 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 are shown. BCAAem, branched-chain amino acid-enriched mixture; EtOH, ethanol. *$P < 0.05$ vs. Ctrl; †$P < 0.05$ vs. EtOH-fed animals.

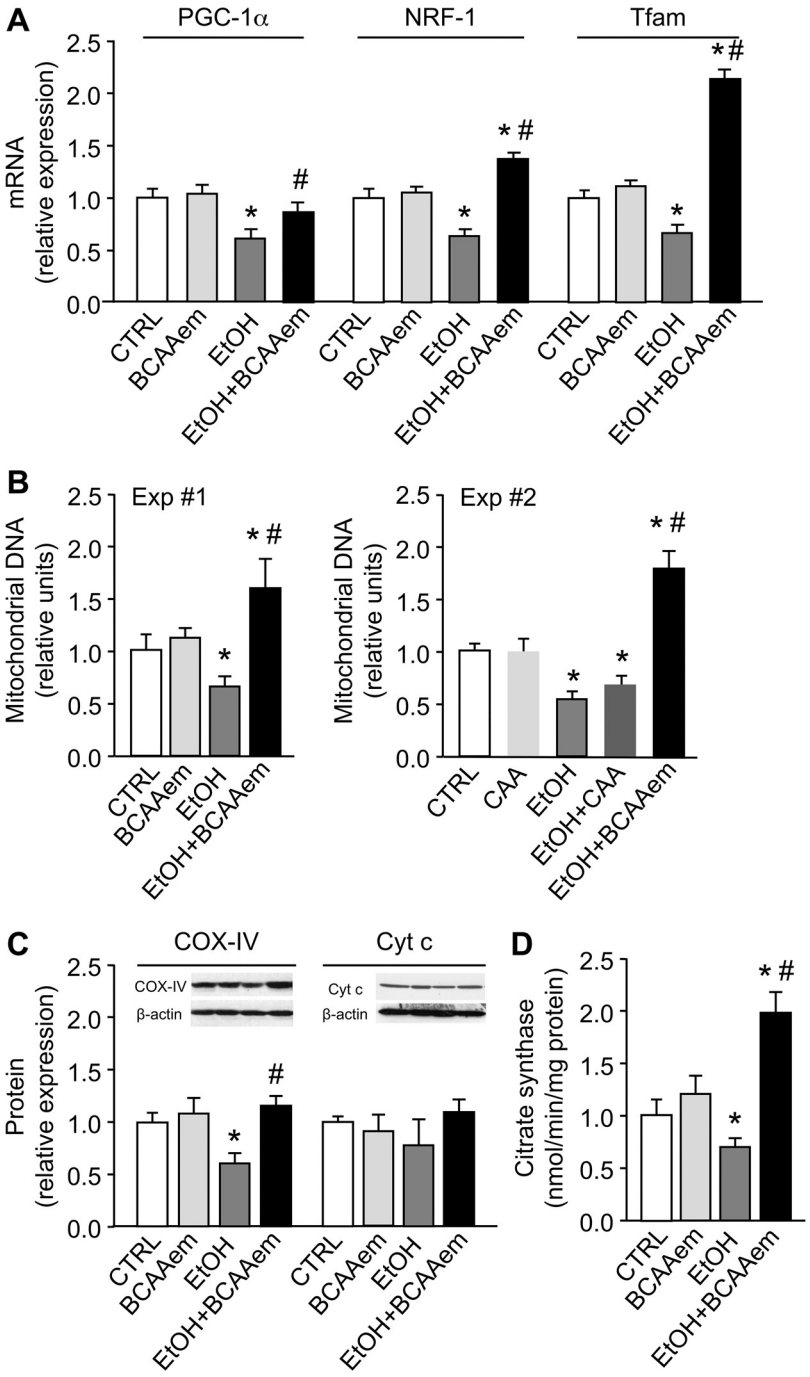

Fig. 3. Mitochondrial biogenesis and function markers are decreased in liver of ethanol (EtOH)-consuming rats, whereas supplementation with branched-chain amino acid-enriched mixture (BCAAem), unlike casein-amino acid (CAA) diet, restores them to control levels. *A*: peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1-α), nuclear respiratory factor-1 (NRF-1), and mitochondrial DNA (mtDNA) transcription factor A (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 compared with those of the Ctrl rats taken as 1.0. *C*: cytochrome *c* (Cyt *c*) oxidase subunit IV (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 means ± SD, $^*P < 0.05$ vs. Ctrl; $\#P < 0.05$ vs. EtOH-consuming rats.

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, although the amount of 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. 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. We first 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*).

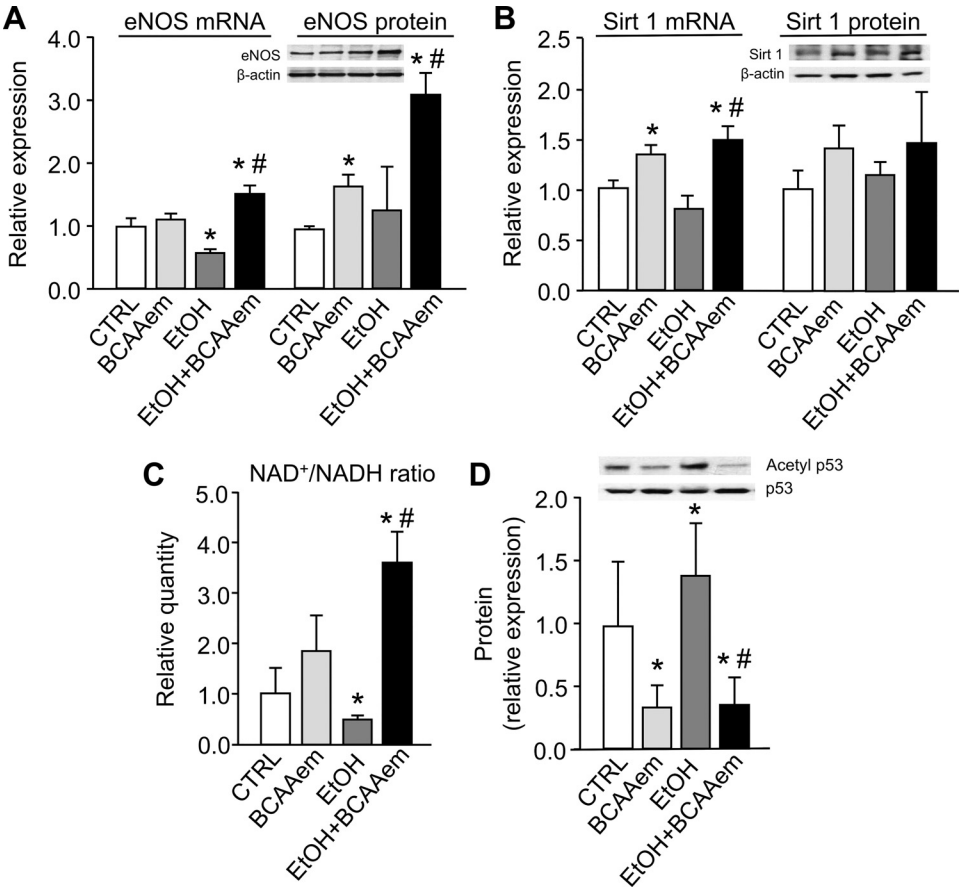

Fig. 4. Endothelial nitric oxide synthase (eNOS) and sirtuin 1 (Sirt1) expression in liver, in addition to nicotinamide dinucleotide (NAD^+)/NADH ratio and Sirt1 activity, are reduced by ethanol (EtOH) consumption, whereas branched-chain amino acid-enriched mixture (BCAAem) supplementation renormalizes them to control levels. eNOS (*A*) and Sirt1 (*B*) 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 an 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 were detected by densitometric analysis relative to the total p53. Values of Ctrl were taken as 1.0 ($n = 5$ experiments). All data represent means ± SD, $*P < 0.05$ vs. Ctrl; $\#P < 0.05$ vs. EtOH-consuming rats.

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 with untreated HepG2 cells (data not shown). Accordingly, the NAD^+/NADH ratio was reduced by only 10% in alcohol-treated compared with 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 with 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, whereas both their protein and mRNA levels increased by ~30% compared with 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. Because 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 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 compared with untreated HepG2 (Fig. 7*A*). On the contrary, BCAAem upregulated 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 with 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 ex-

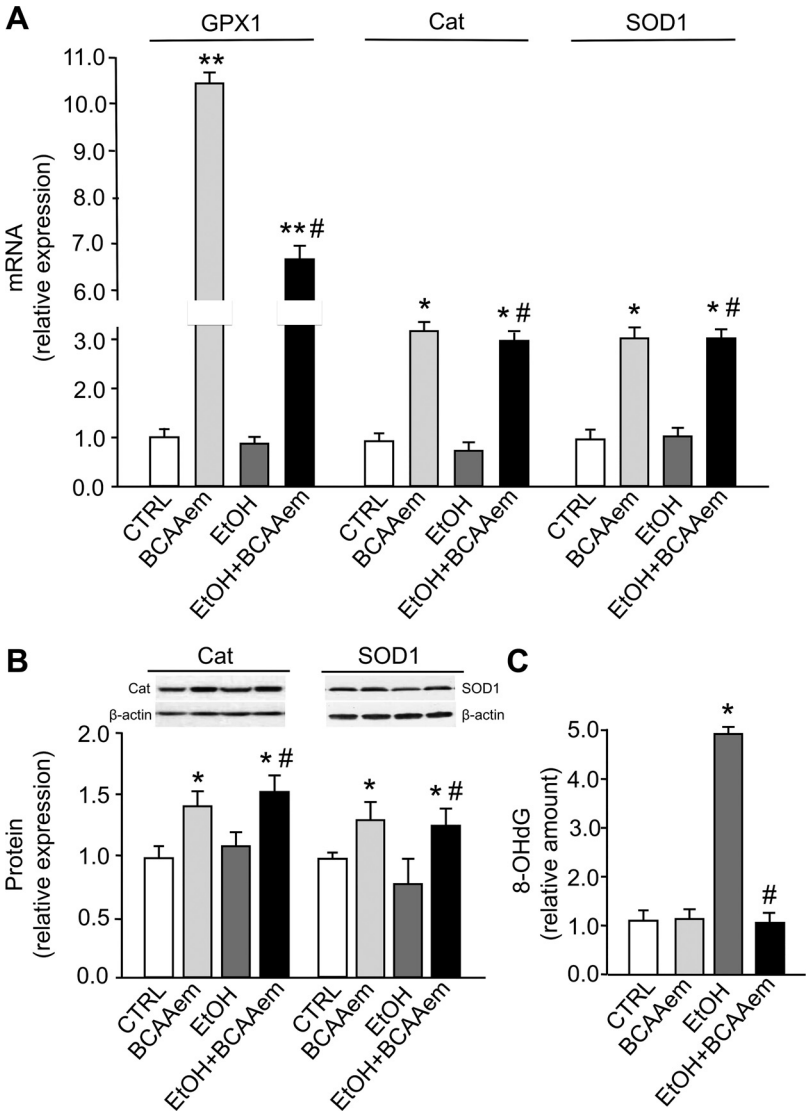

Fig. 5. Ethanol (EtOH) consumption induces oxidative damage in liver, whereas branched-chain amino acid-enriched mixture (BCAAem) supplementation coordinated an antioxidant effect. *A*: glutathione peroxidase 1 (GPX1), catalase (Cat), and superoxide dismutase 1 (SOD1) mRNA levels were analyzed by means 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-hydroxy-2'-deoxyguanosine (8-OhdG) production in liver of EtOH- and BCAAem-treated mice ($n = 3$ experiments). $*P < 0.05$ and $**P < 0.01$ vs. Ctrl rats; $\#P < 0.05$ vs. EtOH-consuming rats.

pression 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 with the untreated cells (Fig. 8*A*) However, 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 further strengthen the role of eNOS on mitochondrial protection by BCAAem supplementation, HepG2 cells were transfected with siRNA against eNOS or nontargeting siRNA as a negative control. We verified that eNOS siRNA reduced eNOS protein levels by 70% (Fig. 8*C*).

Although 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 (cGMP) (57). Thus we examined the effect of the selective guanylate cyclase inhibitor ODQ. Coincubation 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, 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 regulating, among other things, eNOS activity (53). On this basis, we investigated Akt function, measured as phosphorylation in Ser473, in untreated or ethanol-treated HepG2 cells, with or without BCAAem. We ob-

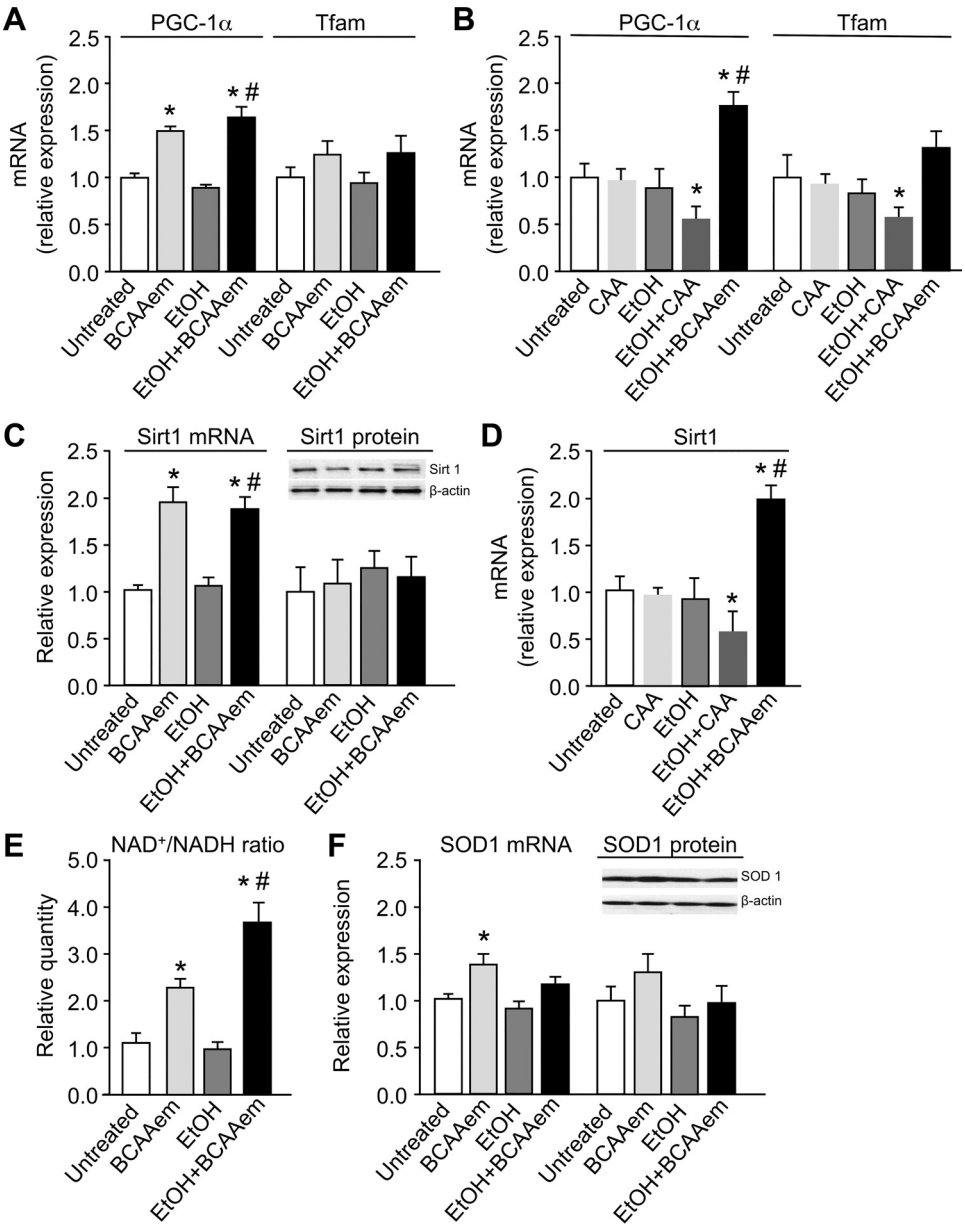

Fig. 6. Mitochondrial biogenesis markers, in addition to sirtuin 1 (Sirt1) expression and nicotinamide dinucleotide (NAD^+)/NADH ratio, are decreased in HepG2 cells exposed to ethanol (EtOH), whereas supplementation with branched-chain amino acid-enriched mixture (BCAAem) restores them to control levels. *A–D*: peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1-α), mitochondrial DNA transcription factor A (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^+/NADH ratio in HepG2 cells was measured by means of a NAD^+/NADH quantification kit. The values were normalized to protein content. *F*: superoxide dismutase 1 (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 means ± SD (n = 5 experiments). *$P < 0.05$ vs. untreated cells; #$P < 0.05$ vs. EtOH-treated cells.

served 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 with untreated cells (Fig. 8*B*).

Because 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 with 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 study, 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 demon-

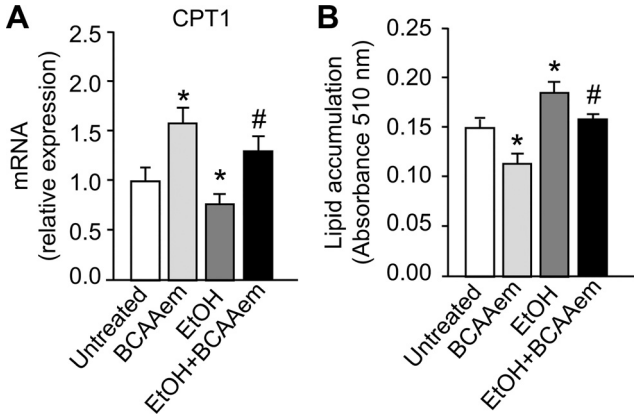

Fig. 7. Branched-chain amino acid-enriched mixture (BCAAem) reduces fat accumulation in HepG2 cells. *A*: carnitine palmitoyltransferase I (CPT1) mRNA levels were analyzed by means of quantitative RT-PCR. Relative expression values of the untreated cells were taken as 1.0. *B*: lipid droplets were measured by Oil Red O staining and then dissolved in isopropanol and quantified by reading the absorbance at 510-nm wavelength. All data represented means ± SD ($n = 5$ experiments). *$P < 0.05$ vs. untreated cells; #$P < 0.05$ vs. ethanol (EtOH)-treated cells.

strated 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, which 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 BCAAem. Accordingly, BCAAem was also able to upregulate CPT1 expression, the key enzyme in mitochondrial β-oxidation of fatty acids, whose expression was decreased in EtOH-fed animals.

Impaired fatty acid oxidation in alcohol-consuming rats, and its recovery by BCAAem supplementation, could therefore underlie the mechanism involved in the EtOH damage and in the protective effect of the amino acid supplement, respectively. Although the stimulatory effect of BCAAem supplementation on CPT1 expression might be linked to the ketogenic potential of leucine and lysine (59), our experimental conditions seem to exclude this hypothesis because ketogenesis is strictly linked to a shortage of carbohydrates, whereas HepG2 cells were maintained in 2 g/l glucose culture medium. Moreover, the increased expression of SOD1, GPX1, and Cat observed in BCAAem-fed rats also indicates a protective action of the amino acid supplement against the EtOH-induced oxidative stress (45), which was after all confirmed by the ability of BCAAem to reduce 8-OHdG amount in liver DNA of the alcoholic animals.

Hepatic ethanol metabolism occurs mainly via oxidation by means of ADH and ALDH. Both ADH and ALDH use NAD^+ as an enzymatic cofactor, which is reduced to NADH. Consequently, during ethanol oxidation, the NAD^+/NADH ratio is significantly decreased, altering the cellular redox state and triggering several adverse events (60). These include inhibition of tricarboxylic acid cycle and reduction of fatty acid oxidation, which may lead to hepatic steatosis (75). Moreover, changes of NAD^+/NADH ratio may also be linked to a decreased Sirt1 deacetylase activity, given that Sirt1 is a NAD^+-dependent enzyme whose activity improves mitochondrial 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 an increase of Sirt1 activity.

Reduction of Sirt1 and PGC-1α, which 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 a therapeutic supplement in patients with 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 BCAA whose breakdown products are acetyl-CoA and acetoacetate, is the most important ketogenic amino acid in humans (8). Great advances are presently in progress toward a molecular definition of leucine-dependent mTORC1 activation to coordinate eukaryotic cell growth and metabolism with environmental inputs, including nutrients and growth factors (79). In particular, research has established a central role for mTOR in regulating numerous essential cell processes, from protein synthesis to autophagy, and mTOR

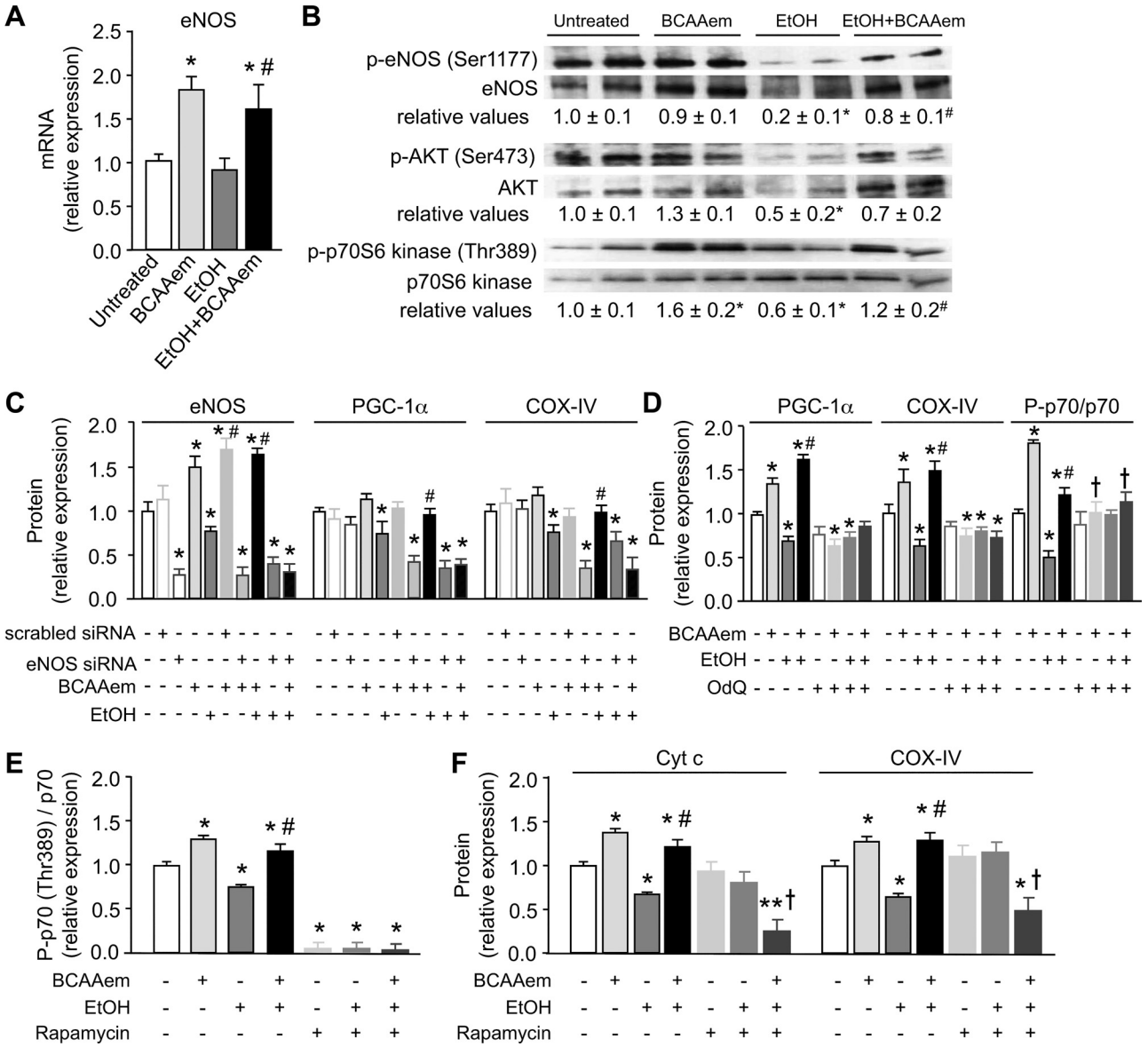

Fig. 8. Endothelial nitric oxide synthase (eNOS) and mammalian/mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling are involved in the protective effect of branched-chain amino acid-enriched mixture (BCAAem) supplementation in HepG2 cells. *A*: eNOS mRNA was analyzed by means of quantitative 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, peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), and cytochrome *c* oxidase subunit IV (COX-IV) protein levels were measured by immunoblot analysis in HepG2 cells transfected with either siRNA against eNOS or nontargeting siRNA and treated with ethanol (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 cells, treated with EtOH or BCAAem alone or with EtOH plus BCAAem coincubated with or without 5 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) for 2 days. Phosphorylation of p70S6 kinase (*E*) and cytochrome *c* (Cyt *c*) and COX-IV protein levels (*F*) were detected by immunoblot analysis in HepG2 cells with or without 100 nM rapamycin. Values of untreated or vehicle-treated cells were taken as 1.0. Representative immunoblots of 5 reproducible ones are shown. All data represent means ± SD ($n = 5$ experiments). *$P < 0.05$ and **$P < 0.01$ vs. untreated or vehicle-treated cells; #$P < 0.05$ vs. EtOH-treated cells; †$P < 0.01$ vs. BCAAem plus EtOH-treated cells.

signaling dysfunction is implicated in cancer and diabetes development, as well as in aging (70).

Leucine supplementation has beneficial effects in malnourished elderly people with skeletal muscle dysfunction (i.e., sarcopenia) and in other disorders. Moreover, leucine promotes mitochondrial biogenesis, in addition to increased protein synthesis (26). Malnutrition and its major component sarcopenia are known to be primarily responsible for the adverse clinical consequences in patients with liver disease (25). EtOH and its metabolites act on skeletal muscle, and the consequences of liver disease result in disturbed proteostasis (i.e., protein homeostasis) and sarcopenia. Leucine supplementation and mitochondrial biogenesis-promoting agents are presently in active evaluation to prevent and reverse sarcopenia in patients with ALD and cirrhosis (25).

Finally, tryptophan catabolism in mammals, whose first step is mediated by tryptophan dioxygenase, an enzyme mainly confined to the liver, is known to be implicated in the synthesis

of the nicotinamide ring of NAD^+ (8). A greater liver tryptophan availability would therefore lead to an increase in activity of hepatic tryptophan dioxygenase and thus NAD^+ synthesis. This is consistent with our present results on the BCAAem efficacy to normalize the reduced NAD^+/NADH ratio and Sirt1 activity in liver of EtOH-consuming rats. Plasma levels of tryptophan are reduced in patients with alcoholic liver cirrhosis (65), and clinical studies have confirmed that tryptophan protects liver from nonalcoholic fatty liver disease (NAFLD) and 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 healthily influence 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 the fact 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-wk consumption of a 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 high-fat-diet-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 coactivated. 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 here and elsewhere (36).

Finally, casein-amino acids, different from 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 beneficial 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 ALD 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 with mixed, not conclusive, results. Mixed results can be due to the complex mechanisms involved and the clinical differences between patients with acute or recurrent disorders. Moreover, the mode of administration, i.e., oral and parenteral administration, inclusion criteria, and additional patient and intervention characteristics, may influence the intervention benefits.

Our present in vivo and in vitro results may suggest that patients with diverse liver diseases need specific amino acid formulas. Moreover, mitochondrial dysfunction may be considered as a target of beneficial effect of BCAAem, strengthening the hypothesis that focusing on mitochondrial function to screen molecules and/or nutrients for liver disease therapy is a promising strategy. Similarly, the Sirt1-eNOS-Akt-mTORC1 signaling pathway may be considered a therapeutic target of liver diseases. This result suggests that NO donors and/or Sirt1 agonists, in addition to mTOR modulators, might prevent the EtOH-induced hepatic steatosis.

In summary, our findings therefore support the hypothesis that only some specific amino acid mixtures are able to protect liver against alcohol damage acting on mitochondria, possibly through a Sirt1-eNOS-Akt-mTORC1 cross-talk signaling pathway. Thus, if that were also true in humans, the dietary supplementation of the present or improved formula might represent a promising strategy for prevention and treatment of ALD.

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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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