Leucine supplementation attenuates macrophage foam-cell formation: studies in humans, mice, and cultured macrophages

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ABSTRACT

Whereas atherogenicity of dietary lipids has been largely studied, relatively little is known about the possible contribution of dietary amino acids to macrophage foam-cell formation, a hallmark of early atherogenesis. Recently, we showed that leucine has anti-atherogenic properties in the macrophage model system. In the current study, an in-depth investigation of the role of leucine in macrophage lipid metabolism was conducted by supplementing humans, mice, or cultured macrophages with leucine. Macrophage incubation with serum obtained from healthy adults supplemented with leucine (5 g/d, 3 weeks) significantly decreased cellular cholesterol mass by inhibiting the rate of cholesterol biosynthesis and increasing cholesterol efflux from macrophages. Similarly, leucine supplementation to C57BL/6 mice (8 weeks) resulted in decreased cholesterol content in their harvested peritoneal macrophages (MPM) in relation with reduced cholesterol biosynthesis rate. Studies in J774A.1 murine macrophages revealed that leucine dose-dependently decreased cellular cholesterol and triglyceride mass. Macrophages treated with leucine (0.2 mM) showed attenuated uptake of very low-density lipoproteins (VLDL) and triglyceride biosynthesis rate, with a concurrent down-regulation of diacylglycerol acyltransferase-1 (DGAT1), a key enzyme catalyzing triglyceride biosynthesis in macrophages. Similar effects were observed when macrophages were treated with α-ketoisocaproate (KIC), a key leucine metabolite. Finally, both *in vivo* and *in vitro* leucine supplementation significantly improved macrophage mitochondrial respiration and ATP production. The above studies, conducted in human, mice, and cultured macrophages, highlight a protective role for leucine attenuating macrophage foam-cell formation by mechanisms related to the metabolism of cholesterol, triglycerides, and energy production.

Keywords*: Leucine, α-ketoisocaproate, macrophages, lipid metabolism, foam-cell formation*

Abbreviations: *MPM, mouse peritoneal macrophages; VLDL, very low-density lipoproteins; DGAT1, diacylglycerol acyltransferase-1; KIC, α-ketoisocaproate; CVD, cardiovascular diseases; BCAA, branched-chain amino acids; HFD, high-fat diet; MI, myocardial infarction; apoE$^{-/-}$, apolipoprotein E deficient; LDLR$^{-/-}$, low-density lipoprotein receptor deficient; I/R, ischemia/reperfusion; HMB, β-hydroxy-β-methylbutyrate; IVA, isovaleric acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate buffered saline; DCFH-DA, 2',7'-dichlorofluorescin-diacetate; FITC, fluorescein-isothiocyanate; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; FBS, fetal bovine serum; BSA, bovine serum albumin; LDH, lactate dehydrogenase; FCCP, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; WHR, waist-to-hip ratio; ATCC, American Type Culture Collection; ROS, reactive oxygen species; AggLDL, aggregated LDL; LSC, liquid scintillation counting; TLC, thin-layer chromatography; OCR, oxygen consumption rate; HDL, high-density lipoproteins; BCAT, branched-chain aminotransferase; BCKDH, branched-chain α-keto acid dehydrogenase.*

1. INTRODUCTION

Atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries (1), is the pathogenic mediator of most cardiovascular diseases (CVD), a leading cause of morbidity and mortality worldwide (2). A crucial early step in atherosclerosis development is the infiltration of monocytes from the circulation into the arterial wall (3), where they differentiate into macrophages. Intimal macrophages take up native and modified lipoproteins, accumulate lipids, and are transformed into foam-cells, a hallmark feature of early atherogenesis (1, 4). Although a considerable body of evidence supports the link between dietary fat and atherogenesis, relatively little is known about the contribution of other key dietary components, such as amino acids, to the process of macrophage foam-cell formation and the onset of atherosclerosis (5). Controversial findings have been reported regarding the relationship between specific amino acids, atherosclerosis, and CVD risk. For instance, excess dietary methionine (6), and its metabolite, homocysteine (7-9), as well as glutamate and glutamine (10, 11), have been associated with increased risk of atherosclerosis, CVD and related cardio-metabolic disorders.

Branched-chain amino acids (BCAA), leucine, isoleucine, and valine, are essential amino acids that share a distinct catabolic pathway (12). High circulating BCAA were found in subjects with dyslipidemia, obesity, and cardio-metabolic diseases (10, 13-17). Despite these associations, BCAA intakes were shown to be cardio-protective by improving parameters of inflammation, blood pressure, and insulin resistance, independent of metabolic conditions (18), and were associated with lower prevalence of obesity and decreased risk of diabetes development in men and women (19-21). Moreover, dietary BCAA were shown to possess cardio-protective effects in a heart-failure rat model (22), and to improve hepatic steatosis by modulating lipid synthetic

pathways in obese mice (23). Particularly, increasing evidence indicate that leucine has unique properties in metabolic regulation, beyond its known role in de novo protein synthesis (24, 25). The beneficial effects of leucine in lipid and glucose metabolism have been studied in animals and in humans (26). Dietary leucine attenuated obesity, hyperglycemia, and hypercholesterolemia (27), and ameliorated adipose tissue inflammation, hepatic lipid deposition, and insulin resistance in high-fat diet (HFD) fed mice (28). Recently, leucine intake was associated with improved measures of arterial stiffness and atherosclerosis in women (29). In C57BL/6 mice subjected to experimental myocardial infarction (MI), a high-leucine diet attenuated fibrosis and apoptosis, and improved cardiac structure, function, and post-MI survival (30). Moreover, leucine supplementation improved plasma lipid profile in apoE$^{-/-}$ mice protecting from atherosclerotic progression (31), and enhanced the hypolipidemic and athero-protective effects of nicotinic acid administration in low-density lipoprotein receptor deficient (LDLR$^{-/-}$) mice (32).

Due to its unique role in metabolism, there is a growing interest to study the metabolic effects of leucine catabolism-derived metabolites (**Figure 1**). For instance, the cardio-protective role of KIC, the first metabolite from leucine catabolism, was recently demonstrated by attenuated ischemia-reperfusion (I/R) injury in isolated mouse hearts (33). However, little is known about the effects of leucine and its metabolites in macrophage-foam cell formation or atherogenesis. Recently, we have screened for anti- or pro-atherogenic amino acids using a macrophage model system, and identified leucine as a potent amino acid which dose-dependently decreased macrophage cholesterol and triglyceride content *in vitro* (34). Nevertheless, whether leucine is able to inhibit macrophage foam-cell formation *in vivo*, and its lipid-lowering mechanisms, remain unclear. The current investigation aims to elucidate the role of leucine in macrophage lipid metabolism and the concomitant foam-cell formation process by

supplementing humans, mice, and cultured macrophages with leucine. Moreover, the effects of key leucine catabolites (KIC, β-hydroxy-β-methylbutyrate, HMB, or isovaleric acid, IVA) on lipid metabolism were assessed in the macrophage model system.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

L-leucine (L8912), IVA (129542), Dulbecco's modified Eagle's medium (DMEM, D5796), phosphate buffered saline (PBS, D8537), triglyceride determination kit (containing the T2449 triglyceride reagent and the F6428 free glycerol reagent), 2′,7′-dichlorofluorescin-diacetate (DCFH-DA, D6883), fluorescein-isothiocyanate (FITC, F7250), dimethyl sulfoxide (DMSO, D8418), dimethylformamide (DMF, 227056), glucose solution (G8769, 2.5 mM), and phosphatase inhibitor cocktail (P5726), were purchased from Sigma-Aldrich (St. Louis, MO, USA). KIC (Alfa Aesar H60076) and HMB (Alfa Aesar 42722) were purchased from Tzamal D-Chem Laboratories Ltd. Cholesterol measurement kit (11491458) and protease inhibitor cocktail tablets (cOmplete 11231400) were obtained from Roche Diagnostics (Mannheim, Germany). Leucine-deficient DMEM, heat inactivated fetal bovine serum (FBS), bovine serum albumin solution (BSA, 10%), sodium pyruvate solution (100mM), L-glutamine solution (200 mM), and penicillin-streptomycin-nystatin solution, were purchased from Biological Industries (Beit Haemek, Israel). [9,10-^{3}H(N)]-oleic acid (NET289001MC), [^{3}H]-acetic acid sodium salt (NET003005MC), and [1,2-^{3}H(N)]-cholesterol (NET139001MC), were purchased from Perkin Elmer (Waltham, MA, USA). Lactate dehydrogenase (LDH) determination kit and silica gel plates (60F254) were purchased from Merck (Darmstadt, Germany). Exclusion chromatography PD-10 columns (17-0851-01) were obtained from GE healthcare (Buckinghamshire, UK). Bradford reagent was purchased from Bio-Rad (Hercules, CA,

USA). RNA purification kit (MasterPure TM) was obtained from Epicentre Biotechnologies (Madison, WI, USA). cDNA preparation kit and Absolute Blue qPCR ROX mix were purchased from Thermo Scientific (Epsom, UK). Seahorse XF medium (102353-100), and Seahorse XF Cell Mito Stress Test Kit (103015-100) including oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and a mix of rotenone/antimycin A, were from Seahorse Bioscience (Agilent Technologies distributor, Petach Tikva, Israel). L-leucine approved for human consumption was obtained from Fagron Inc (St. Paul, MN, USA).

2.2. Clinical trial

A randomized interventional study was conducted in accordance with the principles of the Declaration of Helsinki (ID: 0566-16-RMB CTIL), and registered in the US National Library of Medicine (http://www.clinicaltrials.gov ID: NCT03180775).

2.2.1. Study population

Subjects were recruited at the Rambam Health Care Campus, Haifa, Israel. Volunteers were screened for eligibility after signing an informed consent document for all the study-related procedures. The inclusion criteria were healthy male subjects, in the age range between 18-50 years old. The exclusion criteria were any renal, pulmonary, or endocrinal condition, diabetes, cancer, or morbid obesity (BMI >40 kg/m^2), smokers, or consumption of more than two alcoholic drinks per day. Subjects who were using any kind of amino acid supplements were also excluded from the study.

2.2.2. Leucine administration

Each volunteer was asked to fast overnight before the beginning of the study. The day of the first visit (baseline, 0 h), subjects were provided with a solution of leucine (5 g dissolved in 250 mL of fresh water) which was consumed in a single dose. Afterwards, daily administration of leucine was carried out for 3 weeks. Leucine was provided in

packages containing 5 g of the soluble powder, which the participants dissolved in fresh water and consumed each morning throughout the study. Leucine dose (5 g/d) was set up based on the average leucine daily intake reported in adults (5.3±0.1 g/d) (35).

2.2.3. Anthropometric and laboratory measures

During the period between blood collections, the participants were allowed to continue with their usual activities and dietary habits, and were advised to avoid any other dietary supplements. Fasting blood samples were drawn from the patients on the first visit at baseline, after 1 h of the first leucine dose (1 h), and at end of the study (21 d). From each participant, serum was obtained by centrifugation (1000 g, 10 min) and stocked at -20 °C for further analyses. Biochemical parameters of the serum including the levels of leucine, glucose, lipid profile, liver enzyme, and renal function, were assessed at the Laboratory of Clinical Biochemistry of the Rambam Health Care Campus, Haifa, Israel, at baseline and at the end of the dietary intervention (21 d). Each participant was subjected to the following anthropometric measurements: body weight, height, waist and hip circumferences. Next, body composition was assessed using a segmental body composition monitor (Tanita®, Tokyo, Japan) (36), and the waist-to-hip ratio (WHR) was calculated by dividing waist circumference by hip circumference measures.

2.2.4. *Ex vivo* studies in human serum-supplemented J774A.1 macrophages

J774A.1 murine macrophage-like cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and maintained in a humidified incubator (37 °C, 5% CO_2) in DMEM (1% penicillin-streptomycin-nystatin solution, 1% L-glutamine, 1% sodium pyruvate) supplemented with 5% heat-inactivated FBS. For the *ex vivo* experiments, cultured J774A.1 macrophages were incubated overnight in serum-free DMEM (1% penicillin/streptomycin/nystatin solution, 1% L-glutamine, 1% sodium pyruvate), supplemented with 0.2% BSA and 2.5% serum obtained from the leucine-supplemented participants at baseline, 1 h, or at 21 d. Serum concentration was chosen

based on previous studies evaluating the effects of human serum addition on lipid metabolism in different cell types, such as MPM and Fu5AH hepatoma cells (37, 38). The human serum-mediated effects on macrophage lipids content and lipids metabolism were assessed as described in the following sections (**2.5.** Macrophage lipid contents and metabolism).

2.3. Mice study

Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the NIH, USA. The protocol for the study was approved by the Committee for Supervision of Animal Experiments of the Technion – Israel Institute of Technology (Approval number: IL045-04-16). Male C57BL/6, 8-weeks old mice (provided by Envigo, Jerusalem, Israel) were bred and housed in pathogen-free conditions at the Animal Care Facility of the Rappaport Faculty of Medicine, Technion.

2.3.1. Leucine administration to mice

Mice were randomly divided in 2 experimental groups (n=8): 1) Control group, which received standard chow and water with no supplementation, and 2) Leucine supplemented group, which received standard chow and leucine (1%, w/v) via their drinking water for 8 weeks. Leucine dose was set to double its amount provided by daily chow (average consumption). This dose was based on previous reports demonstrating the metabolic benefits of leucine without affecting food intake or weight gain in mice (30). Data on the macronutrient and the amino acid composition of the standard chow as provided by the manufacturer are detailed in the supporting information (Table S1). The food intake and body weight of the mice were monitored every two and seven days, respectively, during the course of the 8-week study.

2.3.2. Serum analyses

Blood was collected from the retro-orbital plexus of the mice under isoflurane anesthesia (via inhalation) after overnight fasting. The serum was separated from the clotted blood by centrifugation (1000 g, 10 min). Serum cholesterol and triglycerides were measured using the commercially available kits detailed in the Materials section. Serum leucine levels, determined by HPLC (34), were assessed at the Laboratory of Clinical Biochemistry of the Rambam Health Care Campus, Haifa, Israel.

2.3.3. Aortic and liver lipid analyses

Aortas and livers were rapidly removed from the euthanized mice and kept at -80°C. Aortas (including the ascending aorta, the aortic arch, and the descending thoracic aorta) were cleared of adhering fat and connective tissue before further analyses. Aorta or liver samples were homogenized in PBS (containing protease inhibitor and phosphatase inhibitor cocktails) using a Polytron Homogenizer (Kinematica AG, Littau, Switzerland) at 60W for 1 min. Aorta or liver homogenates were then centrifuged (5000 g, 20 min) and the supernatants were analyzed for protein levels by the Lowry assay (39). To assess the aortic or liver lipid composition, lipids were extracted from the homogenates with hexane:isopropanol (3:2, v:v), and the hexane phase was evaporated under nitrogen. The amounts of aortic or liver cholesterol or triglycerides were determined spectrophotometrically using the commercially available kits described in the Materials section. Data were normalized to protein levels.

2.3.4. Mouse peritoneal macrophages (MPM) isolation and culture

Three days after intraperitoneal injection of 3 mL of thioglycollate (40 g/L) into each mouse, MPM were harvested from the peritoneal fluids of the euthanized mice. MPM were then plated and incubated in a humidified incubator (37°C, 5% CO_2) in DMEM (1% penicillin-streptomycin-nystatin solution, 1% L-glutamine, 1% sodium pyruvate) supplemented with 5% heat-inactivated FBS, for further lipid content and metabolism

studies, as described in the following sections (**2.5.** Macrophage lipid contents and metabolism).

2.4. J774A.1 macrophage study

For experiments with leucine or its metabolites, cultured J774A.1 macrophages (ATCC, Rockville, MD, USA) were incubated overnight (20 h) in leucine-deficient DMEM (1% penicillin-streptomycin-nystatin solution, 1% L-glutamine, 1% sodium pyruvate) supplemented with 5% heat-inactivated FBS and increasing concentrations (0.02, 0.05, 0.1, 0.2, 1 and 2 mM) of leucine, KIC, HMB, or IVA. The range of concentrations was determined based on the physiological or supra-physiological plasma levels of leucine or its metabolites, KIC or HMB (IVA plasma levels in adults are not described), and on previous reports (14, 34, 40-43).

2.4.1. J774A.1 macrophage toxicity and proliferation

The effect of increasing levels of leucine or its metabolites on the toxicity of J774A.1 macrophages were assessed by measuring the release of LDH from the cells into the culture medium (34, 44). The remaining cells in the plates were dissolved in 0.1 M NaOH, for cellular protein quantification by the Lowry assay (39). Results were expressed as LDH units/mg cell protein. J774A.1 macrophages counting was performed after cell treatment with leucine (0.2 mM), in an automated cell counter (Countess II, Thermo Fisher, Israel).

2.4.2. J774A.1 macrophage oxidative stress – DCFH-DA assay

Intracellular generation of reactive oxygen species (ROS) in macrophages was evaluated in J774A.1 macrophages by the DCFH-DA probe as previously described (45). Cells were incubated with DCFH-DA (10 µM, in PBS) in the dark for 40 min at 37°C. Next, the cells were washed with PBS (2x), and the adherent cells were detached by gentle scraping, resuspended in PBS, and transferred to special tubes. Measurement

of cellular fluorescence was made by flow cytometry and performed using a BD LSRFortessa (BD Biosciences, San Jose, CA, USA).

2.5. Macrophage lipid contents and metabolism

2.5.1. Cellular cholesterol or triglyceride quantification

Cellular lipids were extracted twice with a mixture of hexane:isopropanol (3:2, v:v), and the hexane phase was evaporated under nitrogen. Cellular cholesterol or triglyceride contents were determined spectrophotometrically using the commercially available kits described in the Materials section. The remaining cells in the plates were dissolved in 0.1 M NaOH for cellular protein determination by the Lowry assay (39). Cholesterol or triglycerides mass values were normalized to cellular protein levels. In some experiments, J774A.1 macrophages were loaded overnight (20 h) with isolated VLDL (triglycerides-rich lipoprotein, 50 µg/mL) or with aggregated LDL (AggLDL, cholesterol-rich lipoproteins, 50 µg/mL). AggLDL were prepared by vortexing the isolated LDL during 90 s, resulting in a turbid solution. Then, cells were washed with PBS (1x) and incubated for 20 h with leucine (0.2 mM) in leucine-deficient DMEM. Next, macrophage lipids were extracted, and cellular cholesterol or triglycerides contents were determined as described above.

2.5.2. Human serum-mediated cholesterol efflux

J774A.1 macrophages were pre-incubated with [^{3}H]-labeled cholesterol (2.5 µCi/ml) for 1 h at 37°C, followed by cell wash with PBS (x2) and further incubation for 3 h at 37°C, in the absence or presence of 2.5% serum obtained from the participants of the clinical trial, at baseline, 1 h, or at 21 d. At the end of the incubation period, the efflux media were collected and the [^{3}H]-labels in 500 µL of each sample were determined by liquid scintillation counting (LSC) (β-counter, Packard Tri Carb 2100TR, PerkinElmer, Waltham, MA, USA). The remaining cells in each well were washed with PBS (x2),

solubilized overnight in 0.1 M NaOH, and the amount of [^{3}H]-labels in 500 µL of each lysate was determined LSC. Total cellular [^{3}H]-cholesterol was calculated as the sum of the radioactivity in the efflux medium plus the radioactivity in the lysates. Human serum-mediated cholesterol efflux corresponded to the % of total [^{3}H]-cholesterol released into the medium (46). Results were expressed as % serum-mediated cholesterol efflux vs baseline.

2.5.3. Cholesterol biosynthesis rate

Macrophage cholesterol biosynthesis rate was assessed after a 4 h-incubation period at 37°C with [^{3}H]-acetic acid (3.3 µCi/mL) in serum-free DMEM supplemented with 0.2% BSA (47). At the end of the incubation time, cells were washed with PBS (x2), and lipids were extracted with hexane:isopropanol (3:2, v:v). After hexane evaporation, lipids were dissolved in $CHCl_3$ and separated by thin-layer chromatography (TLC) on silica gel plates, and developed in hexane:ether:acetic acid (130:30:1.5, v:v:v). Unesterified cholesterol spots were visualized by iodine vapor using an appropriate standard for identification, and [^{3}H]-labels were counted by LSC. The remaining cells in the plate were dissolved overnight in 0.1 M NaOH for cellular protein determination by the Lowry assay (39). [^{3}H]-labels counts were normalized to cellular protein levels, and expressed as cpm/mg cell protein.

2.5.4. Triglycerides biosynthesis rate

Macrophages were washed with PBS (1x) and the rate of triglycerides biosynthesis was assessed after a 4 h-incubation period at 37°C with [^{3}H]-oleic acid (3.3 µCi/mL) in serum-free DMEM supplemented with 0.2% BSA (34, 47, 48). At the end of the incubation time, cells were washed with PBS (x2), and lipids were extracted. After evaporation of the hexane phase, lipids were dissolved in $CHCl_3$ and separated by TLC on silica gel plates, and developed in hexane:ether:acetic acid (130:30:1.5, v:v:v). Triglycerides spots were visualized by iodine vapor using an appropriate standard for

identification, and [^{3}H]-labels were counted by LSC. The remaining cells in the plate were dissolved overnight in 0.1 M NaOH for cellular protein quantification by the Lowry assay (39). [^{3}H]-labels counts were normalized to cellular protein levels, and expressed as cpm/mg cell protein.

2.5.5. Triglyceride degradation rate

Following the triglyceride biosynthesis rate procedure, cells were washed with PBS (x2) and incubated for additional 4 h in serum-free DMEM supplemented with 0.2% BSA. At the end of the second incubation period, cells were washed with PBS (x2) and lipids were extracted. [^{3}H]-labels were analyzed as described above. The remaining cells in the plate were dissolved in 0.1 M NaOH for cellular protein determination by the Lowry assay (39). [^{3}H]-labels counts were normalized to cellular protein levels, and expressed as cpm/mg cell protein.

2.5.6. Isolation of lipoproteins

VLDL and LDL were isolated from plasma of healthy humans by discontinuous density gradient ultracentrifugation as previously described (47, 49). VLDL fraction was separated at d= 1.006 g/mL, and LDL fraction at d= 1.063. Protein concentration was determined by the Bradford protein assay.

2.5.7. Lipoprotein labeling and VLDL uptake analysis

Isolated VLDL (0.8 mg/mL) were labelled with FITC (0.2 mg/mL DMF) as previously described (34, 50). Macrophages were incubated for 4 h at 37°C with FITC-conjugated VLDL (24 µg/mL) in serum-free DMEM supplemented with 0.2% BSA. Next, cells were washed (x2) and resuspended in PBS to be transferred to special tubes. The uptake of FITC-conjugated VLDL by the macrophages was determined by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA) (34, 51).

2.6. Assessment of mitochondrial function in macrophages

Cellular oxygen consumption rate (OCR) was measured in J774A.1 macrophages and in MPM using a Seahorse Bioscience XF96 analyzer (Seahorse Bioscience, Billerica, MA) following the recommended procedure and conditions of the manufacturer. The key parameters of mitochondrial function, baseline macrophage respiration, mitochondrial ATP production, and maximal mitochondrial respiration, were estimated after oxygen consumption rate (OCR) measurements. This approach requires the use of mitochondrial inhibitors: oligomycin (blocks ATP synthase), FCCP (uncoupling agent, makes the inner mitochondrial membrane permeable for protons and disrupts mitochondrial potential), and rotenone and antimycin A (inhibit complexes I and III, respectively, to shut down mitochondrial respiration) (52, 53). Macrophages were seeded in XF96 cell culture microplates (V3-PS Seahorse Bioscience) and incubated at 37°C, 5% CO_2 for 20 h. The day prior to the assay, a sensor cartridge was hydrated overnight with Seahorse XF Calibrant (200 µL/well) in a non-CO_2 incubator, at 37°C. The day of the experiment, the assay medium was prepared by supplementing Seahorse XF Base Medium with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose, and the pH was adjusted to 7.4 with 0.1 M NaOH, followed by sterilization by filtration (0.22 µm), and kept at 37°C until use. The assay was initiated by carefully washing the cells (3x) with the assay medium, without disturbing the cell monolayer from each well, and 1 h-incubation with 180 µL of the same assay medium at 37°C in a CO_2-free incubator, to allow media temperature and pH to reach equilibrium before the first-rate measurement. For OCR measurement, stock solutions of oligomycin, FCCP, and a mix of rotenone and antimycin A were prepared in assay medium and loaded into the appropriate ports of the previously hydrated sensor cartridge as follows: Port A: Oligomycin 1 µM, 20 µL; Port B: FCCP 0.5 µM, 22 µL; Port C: Rotenone/antimycin A 100 µM, 25 µL. The corresponding calibration and equilibration times were followed by 3-4 cycles of mixing, waiting, and measurement (4, 0, and 3 min, respectively) after each injection. Once the program

finished, protein concentrations of the samples in each well were determined by the Lowry assay (39). Mitochondrial OCR results were used to calculate mitochondrial basal respiration, ATP production, and maximal mitochondrial respiration, and were reported in pmol/min/mg cell protein.

2.7. Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from J774A.1 macrophages with MasterPureTM RNA purification kit. cDNA was generated from 1 µg of total RNA with ThermoScientific cDNA kit. Using Absolute Blue qPCR ROX mix, products of the reverse transcription were subjected to qPCR using TaqMan gene expression assays with a Rotor-Gene 6000 amplification detection system. mRNA data were normalized to GAPDH used as the housekeeping gene. The primers used for mouse DGAT1, CD36, and GAPDH were designed by Primer Design (South Hampton, UK): DGAT1 sense primer, TGGTGGAATGCTGAGTCTG; DGAT1 antisense primer, GGTCAAAAATACTCCTGTCCTG; CD36 sense primer, CAGCAGCAAAATCAAGGTTAAA; CD36 antisense primer, TCGGGGTCCTGAGTTATATTT.

2.8. Statistical analysis

Results are presented as mean±SEM of at least three independent observations. Statistical analyses were performed using SPSS 17.0 software (SPSS Inc. IBM, Chicago, IL, USA). One-way ANOVA followed by Bonferroni post-hoc tests were used to compare the means between more than two experimental groups (i.e. different compounds concentrations vs. control). The Independent-Samples T test was used to compare the means between two experimental groups (i.e. specific compound vs. control). $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. Anti-atherogenic properties of human serum harvested following leucine supplementation to healthy volunteers

The effects of leucine on serum atherogenicity were studied in healthy male adults supplemented with leucine (5 g/d). Seven of the eight participants recruited completed the instructions on leucine supplementation throughout the whole period (3 weeks), and were included in the study. None of the participants reported any adverse effects related to the dietary intervention. Anthropometric and biochemical characteristics of the subjects at baseline and at 21 d are shown in Table 1. The enrolled subjects were men in the age range of 21-38 years, non-smokers. All participants were non-obese adults, with a mean BMI of 24.5±0.8 (range: 21.6-28.3), and no significant differences were observed in body weight, body fat (%) or WHR, throughout the study. Whereas serum glucose, lipid profile, and other biochemical parameters did not significantly change, leucine serum levels were significantly higher at the end of the intervention and increased by 36%, from 159.4±10.3 μM at baseline, up to 217.3±23.6 at 21 d ($p<0.05$).

Next, serum samples that were obtained from the participants at baseline, 1 h and 21 d (n=7 per each time point) were used to study the effects of leucine supplementation on macrophage atherogenicity *ex vivo*. J774A.1 macrophages were incubated for 20 h in serum-free DMEM supplemented with 2.5% human serum samples. Cholesterol content was significantly reduced in cells incubated with 21 d-serum compared to baseline serum (by 15%, $p<0.05$, **Figure 2A**). To explain the observed decrease in macrophage cholesterol content, the effects of the human serum on cholesterol metabolism in J774A.1 macrophages were studied next by determining the rate of cholesterol biosynthesis and cholesterol efflux. Accordingly, J774A.1

macrophages incubated with 21 d-serum showed a significant reduction in cholesterol biosynthesis rate (by 11%, $p<0.05$, **Figure 2B**) when compared with cells incubated with baseline-serum. Moreover, leucine supplementation for 21 d resulted in a significant increase of the serum ability to induce cholesterol efflux from macrophages (10%, $p<0.01$, **Figure 2C**).

3.2. Lipid-lowering effects in MPM and liver from leucine-supplemented mice

Similar to the human study, leucine supplementation had no significant effects on the mice body weight, or serum lipid profile (Table 2). However, leucine significantly decreased the cholesterol content in MPM by 30% ($p<0.05$, **Figure 3A**) in relation with a significant decrease in cholesterol biosynthesis rate compared to the control group (by 59%, $p<0.05$, **Figure S1A**). However, no significant difference was found in the MPM triglyceride content between both groups (**Figure 3B**). The lipid-lowering effects of leucine supplementation were observed in other tissues as well. In livers isolated from leucine-supplemented mice a significant decrease in both cholesterol and triglyceride content was found (13%, $p<0.05$, and 45%, $p<0.01$, respectively, **Figure 3C and D**). Finally, while no significant effects were observed in the aortic cholesterol content, a trend toward decreased triglyceride content was found in aortas isolated from leucine-supplemented mice, in comparison with the control group (by 51%, $p=0.052$, **Figure S1B**).

3.3. Lipid-lowering effects of leucine in J774A.1 macrophages

To gain more insight into the metabolic mechanisms by which leucine attenuates lipid accumulation in macrophages, J774A.1 murine macrophages were incubated with increasing concentrations of leucine with or without lipid loading (VLDL for triglyceride loading, and AggLDL for cholesterol loading). In accordance with the *in vivo* data,

leucine supplementation induced a dose-dependent decrease in macrophage cholesterol mass by up to 30% at 0.2-1.0 mM ($p<0.001$, **Figure 4A**). Accordingly, leucine (0.2 mM) inhibited the accumulation of cholesterol in macrophages loaded with AggLDL. As shown in **Figure 4B**, an overnight incubation with 50 μg/mL of AggLDL caused a significant increase in cholesterol mass in control macrophages (2.2-fold, $p<0.001$), which was significantly attenuated in the leucine-treated cells (by 34%, $p<0.001$).

Leucine treatment resulted in a significant and dose-dependent decrease in macrophage triglyceride content, from 23% already at 0.02 mM ($p<0.05$), up to 56% at the higher concentration tested (2 mM, $p<0.001$, **Figure 4C**). In accordance, leucine-supplementation significantly attenuated the accumulation of triglycerides in VLDL-loaded macrophages. Whereas an overnight incubation with VLDL (50 μg/mL) caused a significant increase in cellular triglyceride mass (66%, $p<0.001$), the triglyceride content in VLDL-loaded macrophages was significantly decreased after leucine supplementation (0.2 mM) to values even lower than those observed in non-loaded cells (by 62%, $p<0.001$, vs VLDL-loaded macrophages; and by 37%, $p<0.05$, vs non-loaded macrophages, **Figure 4D**).

3.4. The effects of leucine on triglyceride metabolism in cultured J774A.1 macrophages

As leucine supplementation to J774A.1 macrophages was found to have a marked effect of decreasing macrophage triglyceride accumulation, metabolism of triglycerides was assessed next in J774A.1 macrophages treated with non-toxic levels of leucine (0.2 mM, **Figure S2**). Macrophage triglyceride content is determined by a balance between the rate of cellular triglyceride biosynthesis, the rate of their hydrolysis, and the uptake of triglyceride-rich lipoproteins by macrophages (e.g. VLDL) (54, 55). The effect of leucine on macrophage triglyceride biosynthesis rate was determined by measuring the

incorporation of [^{3}H]-oleic acid into cellular triglycerides. As shown in **Figure 5A**, J774A.1 macrophages treated with leucine showed a marked decrement in triglyceride biosynthesis rate by 77% ($p<0.001$), compared to control cells. Accordingly, DGAT1, a key enzyme in the triglyceride biosynthetic pathway in macrophages (56), was significantly down-regulated (by 30%, $p<0.05$, **Figure 5B**). Additionally, degradation of triglycerides in leucine-treated macrophages was studied after incubating cells loaded with [^{3}H]-oleic acid in free-serum medium. Leucine addition to cultured macrophages resulted in a non-significant increase of the triglyceride degradation rate compared to control cells (43% vs 38%, $p>0.05$, respectively). Using FITC-labeled VLDL, leucine treatment was found to decrease the macrophage uptake of VLDL by 20% ($p<0.01$, **Figure 5C**) with a non-significant trend towards decreased mRNA levels of its transporter, CD36 (**Figure 5D**).

3.5. KIC, the first leucine metabolite, decreases macrophage lipid content

According to the catabolic pathway that characterizes BCAA, leucine is first converted to KIC (**Figure 1**), which is subsequently decarboxylated and converted to the corresponding acyl-CoA derivative (12), or alternatively, oxidized to HMB (57). To determine if the observed protective effects of leucine against lipid accumulation can also be exerted by its metabolites, J774A.1 macrophages were supplemented with increasing concentrations of KIC, HMB, or IVA (0-2 mM), and their effects on lipid contents were assessed. At the examined concentrations, these compounds showed no cytotoxic effects on J774A.1 macrophages (**Figure S3**). A significant and dose-dependent decrease in cellular triglyceride and cholesterol mass were observed after treating the cells with KIC. Similar to leucine supplementation, KIC treatment (0.2 mM) caused a significant reduction in macrophage cholesterol mass (26%, $p<0.01$, **Figure 6A**) and triglyceride mass (50%, $p<0.001$, **Figure 6B**), related to a marked inhibition of

triglyceride biosynthesis rate compared to the control cells (73%, p<0.001, **Figure S4**). In contrast, upon adding increasing concentrations of HMB or IVA (0-2 mM), no significant effects on macrophage cholesterol or triglyceride content were noted (**Figure 6C-F**).

3.6. *In vitro* and *in vivo* leucine supplementation improves macrophage mitochondrial function

Given the importance of mitochondria in maintaining normal vascular cell function, there is growing evidence showing that mitochondrial dysfunction may be correlated with atherogenesis and CVD risk (58). To evaluate how macrophage mitochondrial energy status is affected by *in vitro* and *in vivo* leucine supplementation, macrophage OCR was measured with an extracellular flux analyzer (Seahorse Bioscience XF-96, **Figure 7A**). OCR measurements were made in J774A.1 macrophage incubated with leucine (0.2 mM), and in MPM harvested from the leucine-supplemented mice. In J774A.1 macrophages, leucine significantly increased mitochondrial basal respiration (by 54%, p<0.001), ATP production (by 39%, p<0.001), and maximal respiration (by 25%, p<0.01) compared to the control cells (**Figure 7B-D**). Accordingly, in MPM from the leucine-supplemented mice, a significant increase in the above three parameters was observed (by 28, 31, and 36%, p<0.05, **Figure 7E-G**).

4. DISCUSSION

Macrophage foam-cell formation is a hallmark feature of early atherogenesis (1, 4). Recently, we have studied the anti- or pro-atherogenic effects of all amino acids using a macrophage model system, and identified leucine as a potent amino acid able to decrease macrophage lipid content *in vitro* (34). In the current investigation we studied,

for the first time, both the *in vivo* and *in vitro* roles of leucine in macrophage foam-cell formation by supplementing humans, mice and cultured macrophages with leucine. The key findings of the present study are: 1) Leucine decreases macrophage lipid accumulation as determined *ex vivo* (humans), *in vivo* (mice), and *in vitro* (J774A.1 macrophages); 2) the metabolic mechanisms by which leucine decreases macrophage lipid content include inhibition of cellular cholesterol or triglyceride biosynthesis rate, enhancement of cholesterol efflux from macrophages, and attenuation of VLDL uptake; 3) leucine improves macrophage mitochondrial respiration *in vitro* and *in vivo* as shown by OCR measurements; 4) unlike other examined leucine metabolites (HMB and IVA), KIC, the first leucine metabolite, is able to mimic the lipid-lowering effects of leucine *in vitro* by decreasing macrophage cholesterol and triglyceride contents.

Leucine is a key amino acid of the BCAA group (accounting for >20% of total dietary protein), which has received great attention due to its unique function in metabolic regulation, beyond its fundamental role as substrate for protein synthesis (59). Leucine has been shown to play an important cardio-metabolic role and to affect CVD (10, 16, 22, 30, 31). High dietary leucine intake has been associated with lower prevalence of overweight status, obesity, and regulation of glucose homeostasis in mice and humans (26). Since adverse effects of high intakes of leucine have not been clearly identified, to date, no upper limit or safe intake for this BCAA have been established. In our human clinical trial, a leucine dose of 5 g/d was set up based on the average leucine daily intake reported in adults (5.3±0.1 g/d) (35). As a result, this daily dose allowed a significant increase in leucine serum values at the end of the intervention (21 d), compared to baseline values. In contrast, leucine-supplementation did not affect the serum lipid profile, glucose concentration or other biochemical parameters that were quantified. Further studies involving longer dietary leucine interventions would be useful

to determine if changes in serum lipid profile can be observed in healthy subjects and also in individuals with increased CVD risk.

Anti- or pro-atherogenicity of human serum is determined by its ability to attenuate or induce lipid accumulation in cultured macrophages (60). In the current study, the effects of leucine on human serum atherogenicity were studied for the first time. Although leucine supplementation to healthy adults did not alter their serum lipid profile and only increased leucine levels, incubation of J774A.1 macrophages with serum obtained from participants 21 d after leucine supplementation, significantly decreased macrophage cholesterol content, paralleled with a decrease in macrophage cholesterol biosynthesis rate. Furthermore, the effect of leucine supplementation on serum-mediated cholesterol efflux from macrophages was evaluated. Cholesterol efflux is critical for maintaining lipid homeostasis in macrophages, thus its measurement constitutes a predictor of atherosclerotic burden (61). By incubating the cells with human serum obtained at 21 d of leucine supplementation, a significant increase in the serum-mediated cholesterol efflux from macrophages was observed. Considering that HDL, the key mediator of cholesterol efflux from macrophages (61), was not changed by leucine supplementation, these findings highlight an important role for leucine in macrophage cholesterol homeostasis, which is independent of the anti-atherogenic properties of HDL. However, we cannot discard a possible capacity of leucine to promote structural and functional changes in the HDL particles, which could improve their ability to remove cholesterol from macrophages (cholesterol efflux), as well as a possible effect of leucine on the capacity of the ATP-binding cassette transporters ABCA1/ABCG1 to facilitate cholesterol efflux from the macrophages to HDL. The findings of the current clinical-*ex vivo* studies demonstrate novel metabolic mechanisms by which leucine inhibits macrophage foam-cell formation and highlight the need to study the anti-atherogenic role of leucine in larger cohorts, including CVD patients.

In accordance with the human study, leucine supplementation to mice did not change their plasma lipid profile, but significantly reduced cholesterol content in macrophages isolated from the leucine-supplemented mice, in relation with decreased cholesterol biosynthesis rate. Moreover, similar to previous studies where leucine was found to decrease hepatic triglyceride accumulation in mice with fatty liver (62), we found a significant decrease in hepatic cholesterol and triglycerides in leucine-supplemented mice. Since accumulation of lipids in the liver (hepatic steatosis) represents a risk factor for the onset and progression of metabolic syndrome and atherosclerosis (63), the lipid-lowering effects currently observed in livers isolated from leucine-supplemented mice as well as in MPM indicate the cardio-metabolic benefits of this amino acid. Our findings are consistent with previous studies that demonstrated the cardio-metabolic protection by dietary leucine supplementation. For instance, doubling leucine intake in mice fed HFD, significantly decreased plasma total cholesterol and LDL levels, improved insulin resistance, glucose and cholesterol metabolism, and attenuated weight gain (27).

Although without statistical significance, we found that aortas isolated from leucine-supplemented mice showed a trend toward decreased triglyceride content ($p=0.052$). Recently, leucine was reported to improve the serum lipid profile by decreasing VLDL and LDL, and increasing HDL levels, which contributed to attenuate the progression of atherosclerotic plaques in apoE$^{-/-}$ mice. Moreover, the hepatic cholesterol content in those mice was significantly reduced by leucine supplementation (31). Overall, findings from the current investigation, and from the above mentioned reports, support a beneficial role of leucine in lipid metabolism and its ability to inhibit tissue lipid accumulation, which is a key feature of both fatty liver disease and atherosclerosis.

In accordance with our previous reports on the lipid-lowering effects of leucine in macrophages (34), the current investigation revealed that treating J774A.1 macrophages

with physiological (up to 0.2 mM, (43)) and supra-physiological (1 and 2 mM) concentrations of leucine resulted in a dose-dependent reduction of cellular lipid content. Moreover, both AggLDL-induced cholesterol accumulation and VLDL-induced triglyceride accumulation were significantly abolished by treating J774A.1 macrophages with physiological leucine levels. Interestingly, the *in vitro* studies demonstrated a marked effect of leucine on macrophage triglyceride metabolism. First, leucine significantly attenuated the macrophage uptake of VLDL, which are major endogenous carriers of triglyceride in the blood circulation, and are implicated in atherosclerosis development (64). Uptake of VLDL contributes to triglyceride accumulation and the concomitant foam-cell formation (65, 66). In addition to attenuating macrophage VLDL uptake, leucine caused a marked decrease in the rate of triglyceride biosynthesis associated with down-regulation of DGAT1, a key enzyme in triglyceride biosynthetic pathway in macrophages (56). However, unlike the prominent inhibition of triglyceride accumulation observed in leucine-treated J774A.1 macrophages, cellular triglyceride content remained unaffected after leucine supplementation *in vivo*. This discrepancy could be explained by a different effect exerted on triglyceride metabolism when leucine is directly added to the culture medium *in vitro*, while *in vivo*, leucine is found in a more complex matrix of serum components, which are very likely to influence the utilization of leucine by the cells, highlighting the different outcomes regarding lipid metabolism in macrophages after direct *in vitro* leucine exposure versus oral *in vivo* leucine administration.

Leucine catabolism comprises a first reversible transamination reaction, exclusive to the BCAA catabolic pathway, catalyzed by branched-chain aminotransferase (BCAT) to generate KIC, a key leucine metabolite (**Figure 1**). KIC is next decarboxylated and converted to its corresponding acyl-CoA derivative (isovaleryl-CoA) via branched-chain α-keto acid dehydrogenase (BCKDH), to finally generate

substrates for the tricarboxylic acids cycle (acetyl-CoA or acetoacetate) (12). KIC can follow an alternative pathway via cytosolic oxidation to generate HMB, which might be involved in the pathway of cholesterol biosynthesis (57). Similar to leucine and unlike the other examined leucine metabolites, we found that treating macrophages with KIC dose-dependently decreased both cholesterol and triglyceride contents in macrophages. Moreover, KIC and leucine markedly inhibited the rate of triglyceride biosynthesis in a similar manner. Given the reversibility of the BCAT transamination reaction, both leucine and KIC can be interconverted, depending on the metabolic needs of the cell (12). According to previous studies, the effects observed after KIC cell-supplementation are likely a result of its transamination back to leucine, as it was found in different cell models (40, 67).

Macrophage lipid accumulation also represents one of the mechanisms for mitochondrial dysfunction in atherosclerosis (2). Therefore, we assessed the effects of *in vitro* and *in vivo* leucine supplementation on macrophage mitochondrial energetic status. Leucine improved mitochondrial energetic status, represented by a significant increase in mitochondrial basal and maximal respiration, as well as mitochondrial ATP production, in both leucine-treated J774A.1 macrophages and in MPM from leucine-supplemented mice. Mitochondria are critical for cellular function and ATP generation, which is essential for metabolic processes, including lipid homeostasis. In macrophages, the inhibition of mitochondrial ATP production and function by pharmacologic (oligomycin) or genetic (Pgc-1α deletion) means, resulted in a significant reduction in cholesterol efflux capacity of the cells (68). Mitochondrial dysfunction has been mechanistically linked to the progression of atherosclerosis in the apoE$^{-/-}$ mice model, due in part to defects in oxidative phosphorylation (69). On the other hand, augmented mitochondrial respiration resulted in reduction of necrotic core areas in atherosclerotic plaques in apoE$^{-/-}$ mice, significantly increased cell proliferation and decreased the number of cells undergoing

apoptosis. Also, macrophages from these atherosclerotic mice with genetically augmented mitochondrial respiration, showed higher OCR after uncoupling with FCCP (respiratory reserve capacity), and increased abundance of both mitochondrial respiratory complexes I and III, compared with atherosclerotic controls (70). Based on these recent findings, the capacity of leucine to increase mitochondrial respiration in macrophages *in vitro* and *in vivo* reveals a new potential athero-protective feature of this amino acid, although the underlying molecular/biochemical mechanisms remain unclear.

In conclusion then, by studying the role of leucine in macrophage lipid metabolism and its mitochondrial energetic status *in vivo* and *in vitro*, the current study revealed a novel inhibitory effect of leucine in macrophage foam-cell formation, the hallmark of early atherogenesis. Thus, leucine ability to modify macrophage lipid metabolism with a simultaneous enhancement of mitochondrial respiration, may constitute a potential strategy to attenuate atherosclerosis development. Clearly, the overall favorable effects exerted by this unique amino acid involve multiple mechanisms, and further investigation, including leucine supplementation to humans for extended periods of time, and the use of atherosclerotic models, are warranted to fully establish the therapeutic approach of leucine supplementation.

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Table 1. Human anthropometric and biochemical characteristics at baseline visit and after leucine supplementation for 21 days.

	Baseline	After Supplementation (21 d)	*p* value
Anthropometric data			
Age (years)	27.6±2.0		
Height (meters)	1.8±0.0		
Body weight (kilograms)	76.2±3.1	76.3±3.06	0.979
BMI (height/weight^2)	24.5±0.8	24.5±0.78	0.977
Body fat (%)	17.3±1.3	17.9±1.34	0.767
WHR	0.87±0.0	0.87±0.02	0.874
Serum Biochemical data			
Leucine (µmol/L)	**159.4±10.33**	**217.3±23.62**	**0.044***
Glucose (mg/dl)	85.1±3.9	89.6±2.9	0.382
Triglycerides (mg/dl)	94.1±17.3	88.5±9.7	0.791
Cholesterol (mg/dl)	173.3±7.8	177.2±8.8	0.748
LDL-C (mg/dl)	109.0±6.9	111.5±7.7	0.812
HDL-C (mg/dl)	45.5±3.1	48.0±4.2	0.636
Total Protein (g/dl)	7.2±0.1	7.3±0.1	0.603
Albumin (g/dl)	4.6±0.1	4.7±0.1	0.740
BUN (mg/dl)	13.3±0.8	14.4±1.8	0.563
Creatinine (mg/dl)	0.8±0.0	0.8±0.0	0.817
Uric acid (mg/dl)	5.5±0.3	5.3±0.3	0.714

AST (U/L)	22.0±2.5	19.4±1.6	0.410
ALT (U/L)	20.1±2.8	18.1±2.5	0.605
GGT (U/L)	19.6±3.1	23.2±4.4	0.508
ALP (U/L)	59.3±4.3	63.3±3.9	0.508
LDH (U/L)	160.6±9.1	160.0±9.6	0.966

BMI, Body mass index; WHR, waist-to-hips ratio; LDL-C, low-density lipoprotein; HDL-C, high-density lipoproteins; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase. *p* values for comparison between baseline and supplementation outcomes. n= 7 healthy subjects. ***p<0.05 vs baseline**. Values are given as mean±SEM. Independent-Samples T test was used to compare the means between baseline and after supplementation values. p<0.05 was considered statistically significant.

Table 2. Body weight gain, consumption patterns, and serum biochemical parameters in C57BL/6 male mice treated with water (control), or with leucine-supplemented water (1%, w/v), for 8 weeks.

	Control	Leucine (1%, w/v)	*p* value
BW gain (g) [a]	5.6±0.4	5.9±0.6	0.706
Food consumption (mg/BW/d)	100.6±1.8	106.0±2.6	0.100
Leucine consumption (mg/BW/d) [b]	**1.4±0.0**	**3.3±0.1**	**0.000*** **
Serum Biochemical data			
Leucine (µmol/L)	**114.4±6.3**	**139.2±4.5**	**0.046***
Triglycerides (µmol/L)	270.3±6.5	255.8±6.2	0.110
Cholesterol (µmol/L)	198.1±4.7	187.6±4.5	0.110

[a] Calculated from the difference in body weight at the start and at the end of the study.

[b] Total leucine consumption was calculated from the amount of leucine reported in the chow diet plus the quantity of leucine consumed in the drinking water by the mice.

BW, body weight. n= 8 mice per group. ***p<0.05, ****p<0.001 vs Control group**. Data are given as mean±SEM. Independent-Samples T test was used to compare the means between values from control and leucine groups. p<0.05 was considered statistically significant.

FIGURE LEGENDS

Figure 1. Leucine catabolic pathway. Gray-dashed squares indicate the main enzymes of the pathway. Names in bold letters in the figure correspond to the studied compounds and their chemical structure. **α-KIC=** α-ketoisocaproate; **HMB=** β-hydroxy-β-methylbutyrate; **α-KG=** α-ketoglutarate; **IV-CoA=** Isovaleryl–Coenzyme A; **HMG-CoA=** Hydroxymethylglutaryl–Coenzyme A; **TCA** cycle**=** Tricarboxylic acids cycle. **BCAT=** Branched-chain aminotransferase; **BCKDH=** Branched-chain ketoacid dehydrogenase.

Figure 2. Atherogenicity of human serum following leucine supplementation in J774A.1 macrophages. Serum was harvested from blood collected from each participant at baseline (0 h), after 1 h of leucine consumption, and at the end of the study (21 d). J774A.1 macrophages were incubated for 20 h with 2.5% human serum, followed by analyses of: **(A)** Cellular cholesterol mass; **(B)** Cholesterol biosynthesis rate; and **(C)** Serum-mediated cholesterol efflux from macrophages**,** evaluated after 3 h of cell incubation with 2.5% human serum. Data are shown as % compared to baseline (mean±SEM). n= 7 healthy subjects. ***p<0.05, **p<0.01 vs. baseline**.

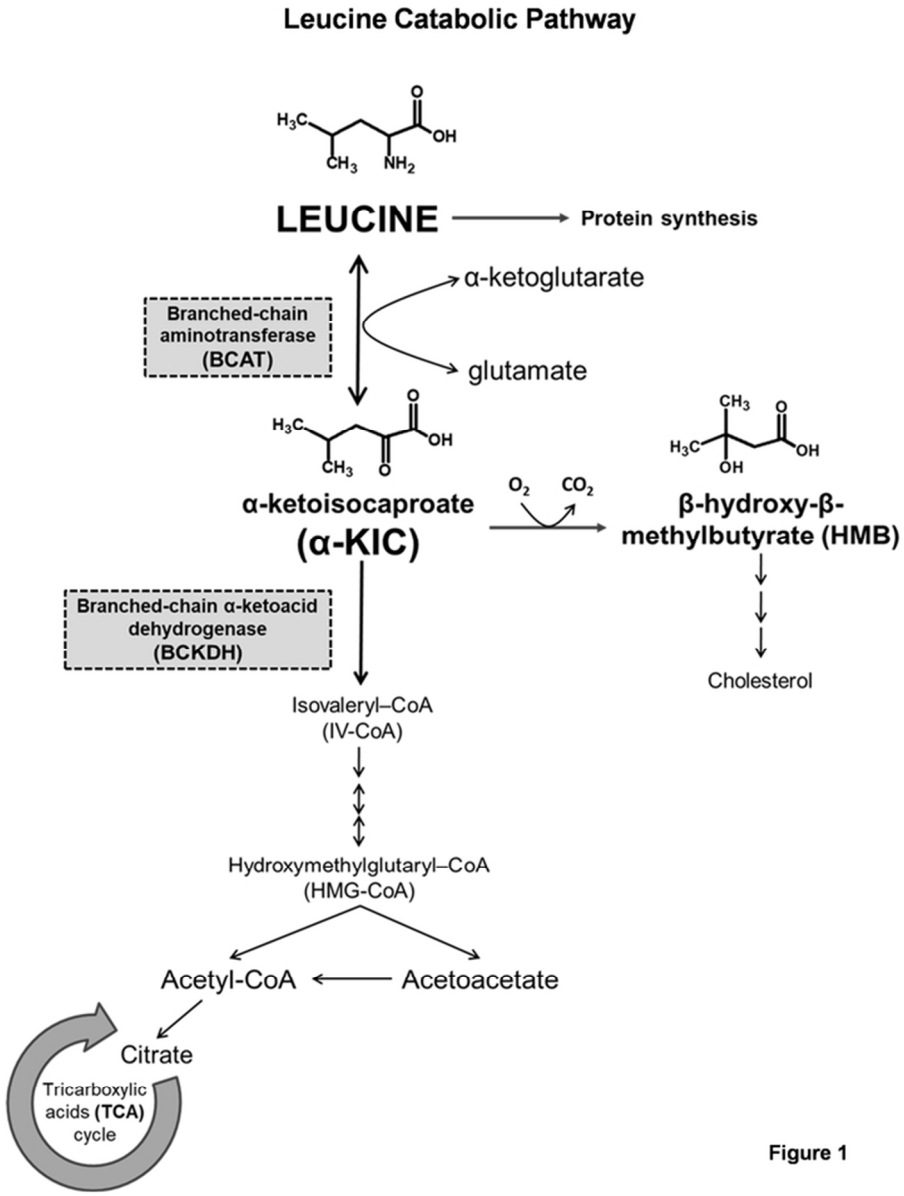
Figure 3. Lipid content in MPM and liver from leucine-supplemented mice. Mouse peritoneal macrophages (MPM) were harvested and livers were excised from C57BL/6 male mice treated with water (Control), or with leucine-supplemented water (1%, w/v), for 8 weeks as described under the Experimental Procedures section. Lipid content in MPM and in livers after lipid extraction and determination of: **(A, C)** Cholesterol mass; **(B, D)** Triglyceride mass. n= 8 mice per group. ***p<0.05, **p<0.01 vs. Control group**. Data are given as mean±SEM.

Figure 4. Effects of leucine on J774A.1 macrophage lipids. Quantifications were performed after 20 h of cell incubation with leucine (0.2 mM), followed by cellular lipid extraction and determination of: **(A)** Cholesterol mass; **(B)** Cholesterol mass after an overnight cell loading with aggregated LDL (AggLDL, cholesterol-rich lipoproteins, 50 µg/mL); **(C)** Triglycerides mass; and, **(D)** Triglycerides mass after an overnight cell loading with VLDL (triglycerides-rich lipoprotein, 50 µg/mL). n= 6. ***p<0.05**, ****p<0.01, *****p<0.001 vs Control** (0, with no addition)**. ###p<0.001 vs cholesterol-rich or triglyceride-rich Control**, respectively. Data are given as mean±SEM.

Figure 5. Effects of leucine on triglyceride metabolism in cultured J774A.1 macrophages. Quantifications were performed after 20 h of cell incubation with leucine (0.2 mM) followed by **(A)** Triglyceride biosynthesis rate after 4 h of cell incubation with [^{3}H]-oleic acid; **(B)** qRT-PCR analysis of DGAT1 gene expression; **(C)** VLDL uptake, using FITC-VLDL (24 µg/mL); and, **(D)** qRT-PCR analysis of CD36 gene expression. n= 6. ***p<0.05**, ****p<0.01, *****p<0.001 vs Control** (with no addition). Data are given as mean±SEM.

Figure 6. Effects of leucine metabolites (KIC, HMB, and IVA) in J774A.1 macrophage lipid content. Quantifications were performed after 20 h of cell incubation with increasing concentrations of leucine metabolites (0-2 mM), followed by cellular lipid extraction and determination: **(A, C, E)** Cholesterol mass; and **(B, D, F)** Triglyceride mass. **KIC=** α-ketoisocaproate, **HMB=** β-hydroxy-β-methyl-butyric acid, and **IVA=** Isovaleric acid. n= 6. ***p<0.05**, ****p<0.01, *****p<0.001 vs Control** (with no addition). Data are given as mean±SEM.

Figure 7. Mitochondrial energetic status of J774A.1 macrophages and mouse peritoneal macrophages (MPM). Quantifications in cultured J774.A1 macrophages were performed after 20 h of cell incubation with leucine (0.2 mM). Mouse peritoneal macrophages (MPM) were harvested from C57BL/6 male mice treated with water (Control), or with leucine-supplemented water (1%, w/v), for up to 8 weeks as described under the Experimental Procedures section. All oxygen consumption rate (OCR) parameters were calculated after the injection of oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), or rotenone + antimycin A. Macrophage mitochondrial energetic status determined by: **(A)** OCR parameters; **(B, E)** Mitochondrial basal respiration; **(C, F)** Mitochondrial ATP production; and **(D, G)** Maximal mitochondrial respiration. n= 8 mice per group. ***p<0.05, **p<0.01, **p<0.001 vs Control group**. Data are given as mean±SEM.



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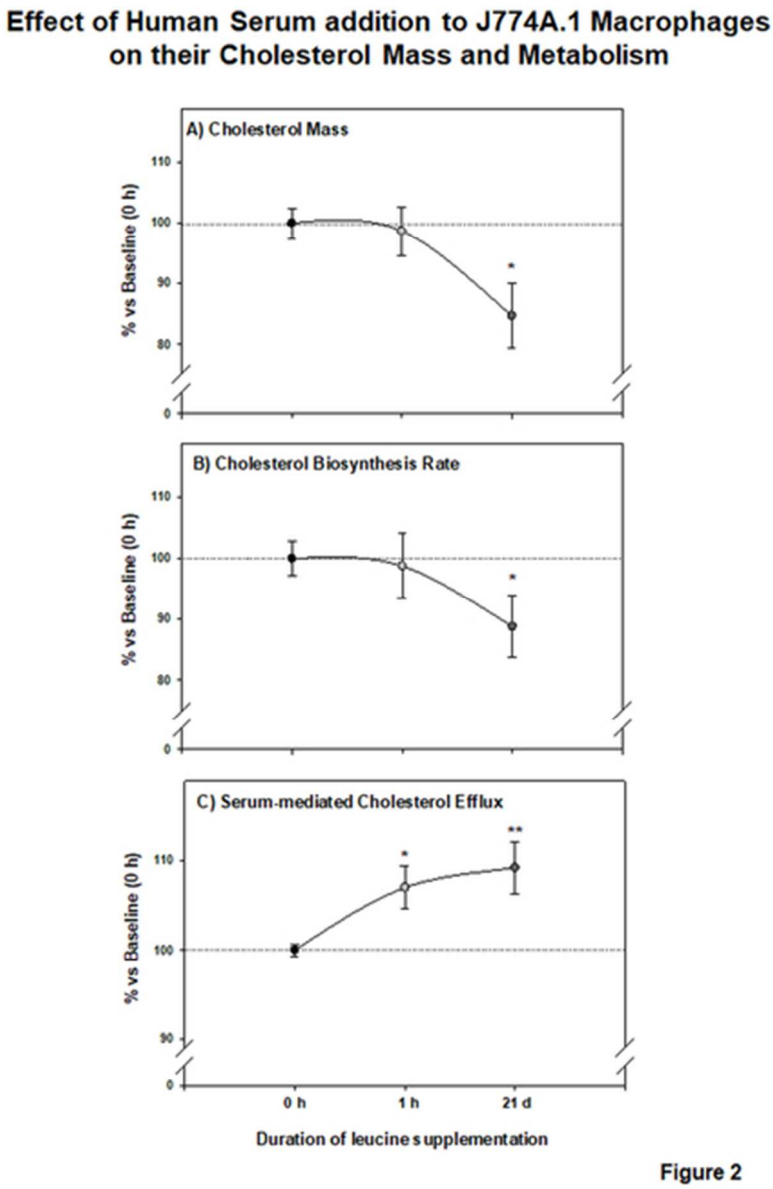

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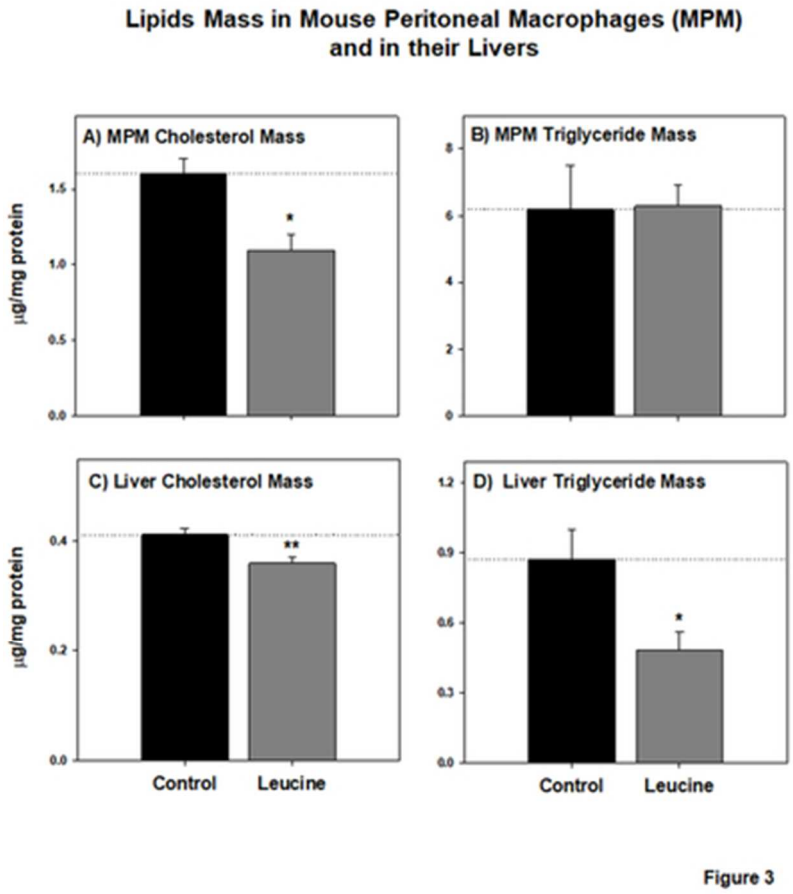

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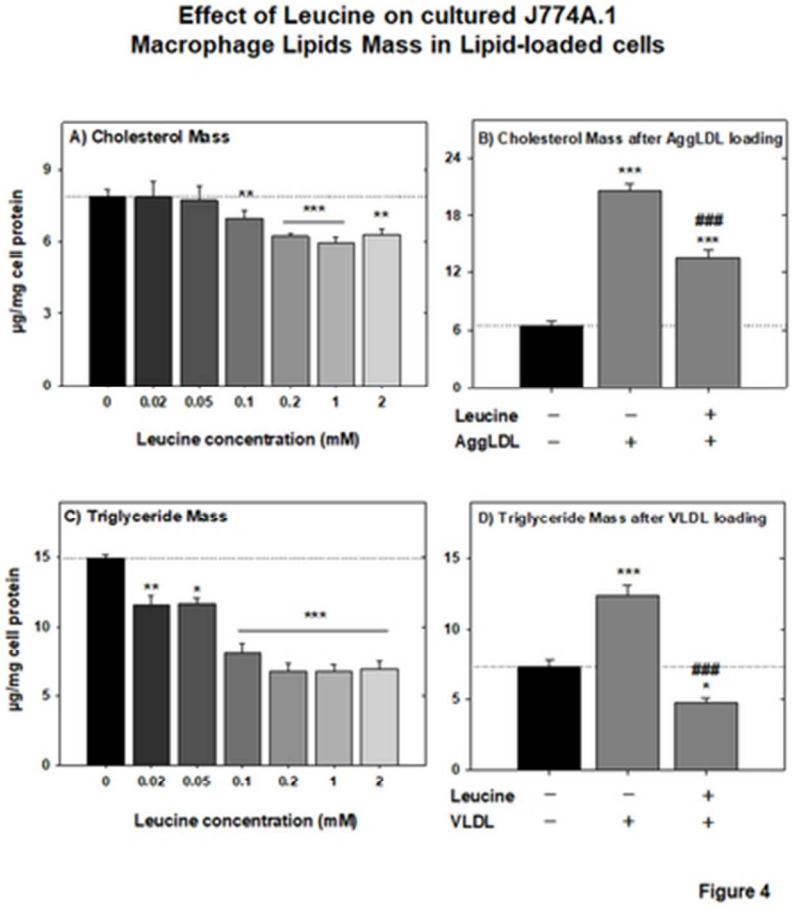

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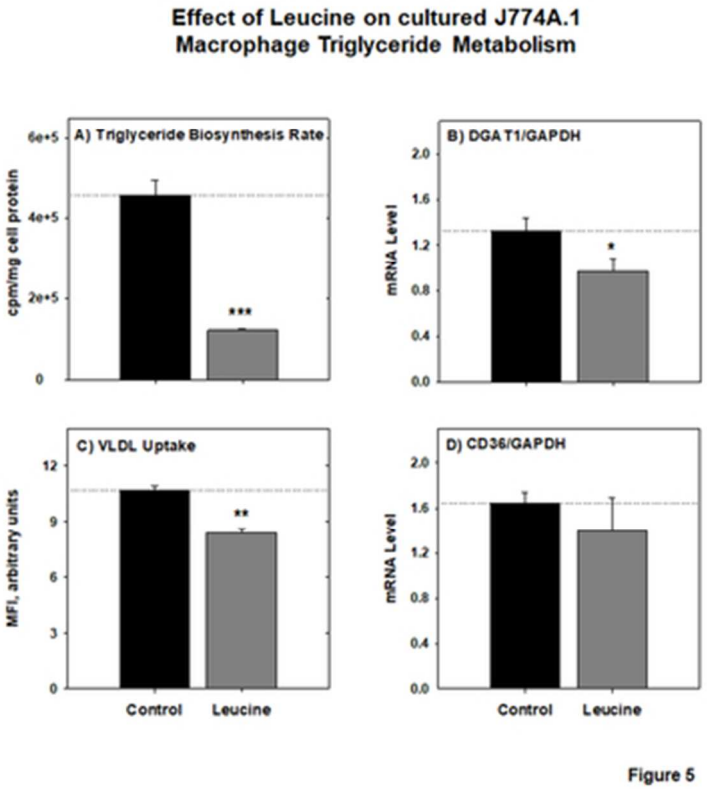

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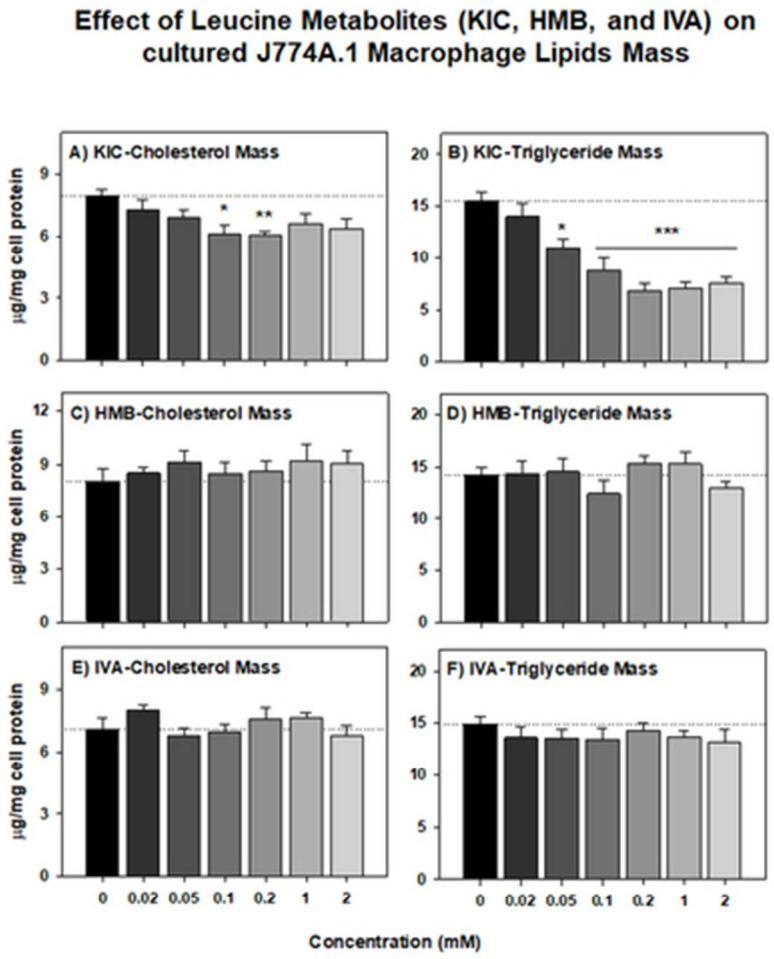

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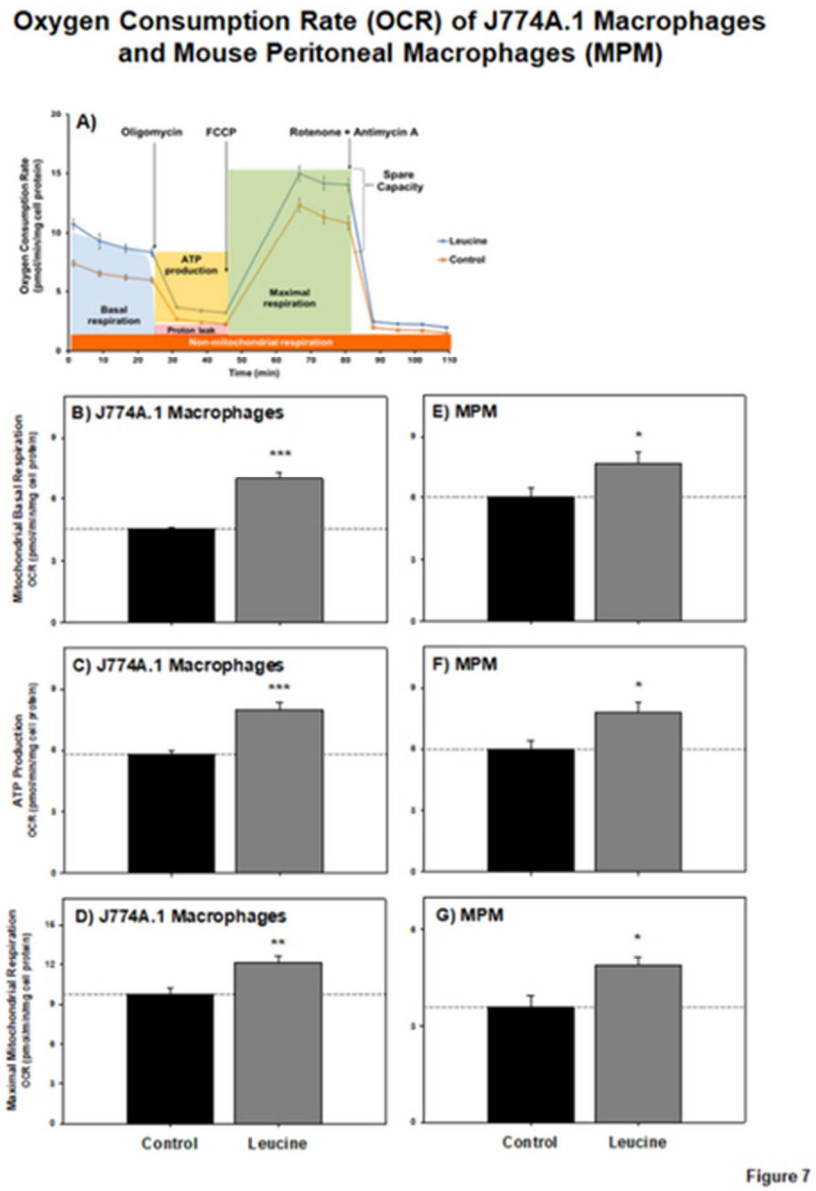

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