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Atherogenicity of amino acids in the lipid-laden macrophage model system *in vitro* and in atherosclerotic mice: a key role for triglyceride metabolism☆

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Abstract

Atherosclerosis-related research has focused mainly on the effects of lipids on macrophage foam cell formation and atherogenesis, whereas the role of amino acids (AAs) was understudied. The current study aimed to identify anti- or pro-atherogenic AA in the macrophage model system and to elucidate the underlying metabolic and molecular mechanisms. J774A.1 cultured macrophages were treated with increasing concentrations of each 1 of the 20 AAs. Macrophage atherogenicity was assessed in terms of cellular toxicity, generation of reactive oxygen species (ROS) and cellular cholesterol or triglyceride content. At nontoxic concentrations (up to 1 mM), modest effects on ROS generation or cholesterol content were noted, but six specific AAs significantly affected macrophage triglyceride content. Glycine, cysteine, alanine and leucine significantly decreased macrophage triglyceride content (by 24%–38%), through attenuated uptake of triglyceride-rich very low-density lipoprotein (VLDL) by macrophages. In contrast, glutamate and glutamine caused a marked triglyceride accumulation in macrophages (by 107% and 129%, respectively), *via* a diacylglycerol acyltransferase-1 (DGAT1)-dependent increase in triglyceride biosynthesis rate with a concurrent maturation of the sterol regulatory element-binding protein-1 (SREBP1). Supplementation of apolipoprotein E-deficient ($apoE^{-/-}$) mice with glycine for 40 days significantly decreased the triglyceride levels in serum and in peritoneal macrophages (MPMs) isolated from the mice (by 19%). In contrast, glutamine supplementation significantly increased MPM ROS generation and the accumulation of cholesterol and that of triglycerides (by 48%), *via* enhanced uptake of LDL and VLDL. Altogether, the present findings reveal some novel roles for specific AA in macrophage atherogenicity, mainly through modulation of cellular triglyceride metabolism.

Keywords: Amino acids; Atherosclerosis; Glutamine; Glycine; Macrophage foam cells; Triglyceride metabolism

1. Introduction

Atherosclerosis, a chronic inflammation of the arteries, is the underlying cause of cardiovascular diseases (CVDs), the major cause of death in most developed countries [1]. In the early stages of atherosclerosis development, blood monocytes migrate from the circulation into the aortic intima, where they differentiate into macrophages and accumulate lipids in a process known as macrophage foam cell formation, which is considered as the hallmark feature of early atherogenesis [2]. Macrophage lipid accumulation and foam cell formation are determined by the balance between three main metabolic mechanisms: (1) the uptake of lipoproteins by macrophages, (2) the rate of lipid biosynthesis within the macrophages, and (3) the clearance of lipids from the macrophages [2,3]. The oxidative status of macrophages is known to affect the above metabolic mechanisms and hence the atherogenicity of macrophages. Whereas anti-oxidative conditions can attenuate foam cell formation, pro-oxidative conditions can enhance it [3].

The atherosclerotic lesions and the macrophages isolated from those lesions contain not only cholesterol but also a substantial amount of triglycerides [4–6]. Accumulation of triglycerides in macrophages results from enhanced uptake of triglyceride-rich very low-density lipoprotein (VLDL) by the cells or from enhanced rate of triglyceride biosynthesis [6–10]. The uptake of triglyceride-rich lipoproteins by macrophages could be facilitated by receptor-mediated pathways that include the LDL receptor (LDLR) and members of the scavenger receptor family such as CD36 or the scavenger receptor B1 (SR-B1) [6–8]. As to the regulation of

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macrophage triglyceride biosynthesis, the key regulator of cellular biosynthesis of fatty acids and triglycerides is the sterol regulatory element-binding protein-1 (SREBP1) transcription factor. SREBP1 is synthesized as a membrane-bound precursor and is activated through proteolytic cleavage. Cleaved and mature SREBP1 translocates into the nucleus where it activates the transcription of genes required for triglyceride biosynthesis [11]. In addition, the key enzyme involved in triglyceride biosynthesis in macrophages is diacylglycerol acyltransferase-1 (DGAT1) which catalyzes the final step in triglyceride biosynthesis by attaching a long-chain fatty acyl-CoA to diacylglycerol [12].

Atherosclerosis-related research has focused mainly on the effects of the different endogenous or exogenous lipids such as cholesterol, triglycerides and fatty acids on macrophage foam cell formation [13]. Unlike lipids, the contribution of amino acids (AAs) to atherogenesis and to the CVD risk was understudied. However, a growing body of evidence suggests an important role for certain AA in the pathogenesis of CVD and atherosclerosis development. For instance, association studies have demonstrated that the plasma levels of branched-chain AAs (BCAAs; valine, leucine and isoleucine) significantly and independently correlate with dyslipidemia and coronary artery diseases (CADs) [14–17]. Glutamate and glutamine were also found to be associated with CAD risk [17], and glutamine was identified as a biomarker associated with increased risk for both plaque and high intima–media thickness (IMT) [18]. In addition, methionine was shown to accelerate the development of atherosclerosis in animal models *via* impaired anti-oxidant activity, increased lipid peroxidation, lower ability of high-density lipoprotein (HDL) to stimulate cholesterol efflux from macrophages and through enhanced macrophage foam cell formation [19–22]. Nevertheless, the effects of the different AA on macrophage oxidative status or their lipid metabolism and the consequence macrophage foam cell formation remain unknown.

To address the gap of knowledge regarding the role of the different AA in macrophage atherogenicity, the aims of the current study were as follows: (1) to screen for anti- or pro-atherogenic AA in the J774A.1 macrophage model system, (2) to elucidate the underlying metabolic and molecular mechanisms by which specific AAs affect macrophage lipid metabolism, and (3) to study the *in vivo* effects of specific AAs that significantly affected macrophage atherogenicity *in vitro* by using the atherosclerotic apoE$^{-/-}$ mice model.

2. Materials and methods

2.1. Materials

All AAs (L-alanine A7469/A7627, L-arginine A8094/A5131, L-asparagine A4159/A8824, L-aspartate A7219/A9256, L-cysteine C7352, L-glutamate G8415/G1251, L-glutamine G8540/G3126, L-glycine G5417/G7126, L-Histidine H6034/H8125, L-isoleucine I7403/I2877, L-leucine L8912/L800, L-lysine L8662/L5626, L-methionine M5308/M6039, L-phenylalanine P5482/P2126, L-proline P5607, L-serine S4311/S4500, L-threonine T8441/T8625, L-tryptophan T8941/T0254, L-tyrosine W373605/T8909, L-valine V0513/V0500, L-citruline C7629, L-taurine T0625, DL-ornithine O2250, argininosuccinic acid A5707, β-alanine A9920, α-amino adipic acid A7275, L-allo-isoleucine I8754), the triglyceride determination kit (containing the T2449 triglyceride reagent and the F6428 free glycerol reagent), 2′,7′-dichlorofluorescin-diacetate (DCFH-DA), fluorescein-isothiocyanate (FITC), the diacylglycerol acyltransferase-1 (DGAT1) inhibitor–oleanolic acid, dihydrocumarin (DHC), dimethyl sulfoxide (DMSO) dimethyl formamide and homocysteic acid (H-9633) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Homocitrulline (F2995) was obtained from Bachem (Bubendorf, Switzerland). Cholesterol measurement kit (CHOL, 11491458) and protease inhibitor cocktail tablets (cOmplete 11231400) were obtained from Roche Diagnostics (Mannheim, Germany). Dulbecco's modified Eagle medium (DMEM), DMEM without AAs, phosphate-buffered saline (PBS), fetal bovine serum (FBS), bovine serum albumin (BSA), penicillin, streptomycin and enzyme-linked chemiluminescence (ECL) solution were all purchased from Biological Industries (Beit Haemek, Israel). [^{3}H]-oleic acid was purchased from PerkinElmer (Waltham, MA, USA). Lactate dehydrogenase (LDH) determination kit, methanol in high-liquid performance chromatography (HPLC) grade and silica gel plates (60F254) were purchased from Merck (Darmstadt, Germany). *O*-phthalaldehyde (OPA) was obtained from TCI (Tokyo, Japan). 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). Rodent chow was purchased from Altromin (Lage, Germany). Primary antibodies against LDLR (sc-11826, goat polyclonal antibody), CD36 (sc-9154, rabbit polyclonal antibody), SR-B1 (sc-67099, rabbit polyclonal antibody), SREBP1 (sc-13551 mouse monoclonal antibody) and DGAT1 (sc-32861, rabbit polyclonal antibody) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Primary antibody against actin (MAB1501, mouse monoclonal antibody) was purchased from Millipore (Temecula, CA, USA). Horseradish peroxidase-conjugated secondary antibodies (AffiniPure Donkey AntiMouse, Goat AntiRabbit or Rabbit AntiGoat polyclonal antibodies) were obtained from Jackson Immuno-Research (West Grove, PA, USA).

2.2. In vitro study: J774A.1 macrophages

2.2.1. J774A.1 macrophages and AA incubation studies

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 regular DMEM containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FBS. For AA experiments, DMEM without AA containing 1000 U/L penicillin, 100 mg/L streptomycin and 5% heat-inactivated FBS was supplemented with increasing concentrations (0.2, 1 or 5 mM) of each 1 of the 20 AAs, followed by sterilization by filtration (0.22 μm). The AA concentration in FBS was determined by HPLC (detailed below in Section 2.2.5). The AA profile of the FBS and the final AA concentrations in the medium are detailed in Supplementary Table 1. The range of concentrations (0.2, 1 or 5 mM) was determined to study both the physiological levels of AA [23] and also supra-physiological levels as previously used in various *in vitro* studies [24–26]. J774A.1 macrophages were treated with DMEM without AA, supplemented with increasing concentrations of each AA for 20 h. Control cells were treated with DMEM without AA. In some experiments, the cells were treated with specific AA in the absence or presence of the inhibitor of DGAT1, the triterpenoid oleanolic acid (75 μM), with equivalent ethanol level as vehicle control (0.1%) [9,10,27].

2.2.2. Macrophage toxicity — LDH release assay

The effects of increasing levels of each 1 of the 20 AAs on the toxicity of J774A.1 macrophages (1×10^6 cells) were assessed by measuring the release of LDH from the cells into the culture medium as previously described [9,10,28,29].

2.2.3. Macrophage oxidative stress — DCFH-DA assay

The effects of increasing levels of each 1 of the 20 AAs on intracellular generation of reactive oxygen species (ROS) in J774A.1 macrophages (1×10^6 cells) were determined with the DCFH-DA probe as previously described [9,10,28–30]. Briefly, following AA treatments, the cells were washed with PBS and incubated for 40 min with 10 μM of DCFH-DA at 37 °C in the dark. Then, the cells were washed with PBS, and the adherent cells were detached by gentle scraping. Measurements of cellular fluorescence were determined by flow cytometry and performed using BD LSRFortessa (BD Biosciences, San Jose, CA, USA).

2.2.4. Macrophage cholesterol or triglyceride mass

Following AA treatments, the lipids of J774A.1 macrophages (1×10^6 cells) were extracted with hexane/isopropanol (3:2, vol:vol), and the hexane phase was evaporated under nitrogen. The content of cellular cholesterol or triglycerides was determined as previously described [9,10,28–30], using the commercially available kits detailed in Section 2.1. The remaining cells in the plates were dissolved in 0.1 M NaOH, and an aliquot was taken for the measurement of cellular protein by the Lowry assay [31]. Cholesterol or triglyceride data were normalized to cellular protein levels.

2.2.5. Macrophage AA contents: HPLC analysis

Following AA treatments, J774A.1 macrophages (9×10^6 cells) were washed twice with cold PBS, and the adherent cells were detached by gentle scraping. The PBS was then discarded by centrifugation (3 min, 16,000g, 4 °C). Then, 100 μl of cold 5% trichloroacetic acid was added to the precipitates and centrifuged (10 min, 16,000g, 4 °C). The supernatant was transferred into glass tubes with 100 μl of 80% ether. The solution was vortexed and left for ether evaporation. The evaporation step was repeated three times. After the ether was completely evaporated, samples were stocked at 4 °C for further analysis. For sample extraction and derivatization, 200 μl of the samples were added to 1 ml of methanol supplemented with 1 mM homocysteic acid as internal standard and centrifuged (5 min, 13,000g, room temperature). The supernatants (800 μl) were transferred to clear HPLC vials for automatic pre-column derivatization with OPA reagent solution. Twenty microliters of 4% mercaptoethanol were mixed with 40 μl of 0.1 M borate buffer supplemented with 1% mercaptoethanol, 10 μl of extracted sample and 10 μl of methanol. The HPLC system used for analysis was equipped with a pump (PU-2080; Jasco, Tokyo, Japan), with a gradient unit (LG-980-02; Jasco) and a fluorescence detector (FP-1520, Jasco). Derivatization and sample injection were carried out with Waters autosampler (717 plus; Waters, Milford, MA, USA), using a reversed-phase column C-18 (150×4.6 mm, 3 μm, Supelcosil LC-18 dB, Supelco). The samples rack was maintained at 8 °C. Buffer A was 0.04 M phosphate buffer supplemented with 20% methanol, pH=5.5, and buffer B was 0.01 M phosphate buffer supplemented with 80% methanol. Gradient conditions were as follows: 100% of A from 0 to 5 min, 99% of A from 5 to 35 min, 97% of A from 35 to 36 min, 90% of A from 36 to 55 min, 80% of A from 55 to 70 min, 20% of A from 70 to 79 min, and 10% of A from 79 to 86 min. Flow rate was set at 0.9 ml/min from 0 to 8 min, 1 ml/min from 8 to