Influence of alpha-linolenic acid supplementation on murine macrophages RAW 264.7 activated with lipopolisaccharide

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Abstract

Chronic inflammation is characterized by excessive production of cytokines and eicosanoids and is associated with unsufficient resolution. Supplementation with n-3 fatty acids may result in a lower incidence of many inflammatory diseases.

The aim of this study was to determine the effect of α – linolenic acid (ALA) on the fatty acids profile of cell membranes and on the pro-inflammatory proteins cyclooxygenase – 2 (COX-2), prostaglandin E2 synthase (cPGES) and prostaglandin F2α receptor (FP) expression in murine RAW 264.7 macrophages, activated with lipopolysaccharide (LPS).

It has been shown that COX-2, cPGES as well as FP receptor expression was highest in cells activated by LPS. In macrophages supplemented with ALA and activated with LPS a pro-inflammatory protein levels were significantly reduced, suggesting anti-inflammatory activity of α-linolenic acid. There were also statistically significant changes in the fatty acid profile after incubation of the RAW 264.7 cells for 48 hours with ALA.

A deficiency or excess of specific fatty acids affect the cellular membrane fluidity, can also cause changes in cell morphology. Therefore it is appropriate to carry out further research on the ALA properties.

Keywords: α – linolenic acid, macrophage RAW 264.7, lipopolysaccharide, COX-2, cPGES, FP receptor.

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Abbreviations:

ALA, α-linolenic acid, COX-2, prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2); cPGES; cytosolic prostaglandine E2 synthase; FA, fatty acids, FP, prostaglandin F receptor; MUFA, monounsaturated fatty acids; SFA saturated fatty acids; UNSFA, unsaturated fatty acids.

Introduction

Polyunsaturated fatty acids (PUFAs) play an important role in the proper functioning of the organism. PUFAs include fatty acids (FA) of n-3 and n-6 family, differing with double bond position. In animal tissues, the reactions of fatty acids desaturation and elongation occur to a limited extent. In addition, these processes require the input of certain external polyunsaturated FA of vegetable origin, which synthesis in human organism is not possible [1,2].

Essential Fatty Acids (EFA) include linoleic acid (18:2 n-6) and **α-linolenic acid (18:3 n-3)**, which produce respectively, γ-linolenic acid (18:3 n-6), dihomo-γ-linolenic acid (20:3 n-6), arachidonic acid (AA, 20:4 n-6) and n-3 acids, i.e., eicosapentaenoic (EPA, 20:5 n-3) and docosahexaenoic acids (DHA, 22:6 n-3) [3].

PUFA of n-6 family are predominant in the typical Western diet, and therefore a general deficiency of n-3 acids is noted, which can cause an excessive release of pro-inflammatory metabolites, mainly arachidonic acid derivatives [4-9]. Too high a ratio of n-6 to n-3 acids provided with the diet is a risk factor for many diseases, including those of an inflammation nature. Reports of recent years clearly indicate that n-3 FA and their derivatives are essential for proper growth and development, act in an immunomodulating, anti-inflammatory, anti-arteriosclerosis, anticancer manner and are resolvents of inflammation [1,2,4-10].

Macrophages are involves in physiological processes such as the regulation of innate and acquired immunity, but they also play the role

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Tab. 1. Fatty acids [%] profile in RAW 264.7 cell membranes after ALA (2.5, 5 and 10 μmol) supplementation for 48h and LPS activation. Means ±SD, n=6.

Fatty acid	control	LPS	ALA 2.5	ALA 2.5 +LPS	ALA 5	ALA 5 +LPS	ALA 10	ALA10 +LPS
C12:0	4.3 ±1	5.5 ±1.3	5.3 ±1	2.0 ±0.6	3.3±0.6	3.3±0.2	2.3±0.2	1.2±1.0
C14:0	9.8 ±3	8.5 ±2.1	4.1 ±2	5.6 ±0.1	4.4±1.1	2.6±2.8	3.7±0.9	12.2±1.3
C16:0	26.5 ±1	14.0 ±3.2	16.6 ±5.2	18.9±1.4	9.7±2.8	19.8±1.3	6.2±1.0	9.9±0.9
C18:0	21.5 ±4	14.3 ±0.9	21.5 ±2.1	20.7 ±2.8	19.1±0.5	15.7±1.8	17.8±2.2	15.0±2.1
C16:1	1.0 ±0.2	2.5 ±1.0	11.2 ±3.1	10.5 ±3.6	9.6±3.8	11.5±1.1	8.5±3.0	8.1±3.3
C18:1	21.7 ±1	24.3 ±0,6	20.3 ±2.1	18.6±0.8	18.6±2.4	22.9±1.9	20.4±1.8	22.9±4.1
C18:2n-6	15.1 ±6	12.6 ±0,5	4.0 ±0.5	3.2±2.2	5.0±1.1	2.8±2.4	3.6±1.7	3.8±1.9
C18:3n-6	0.0	0.2 ±0.0	2.0 ±0.6	0.2±0.0	2.0±0.6	2.1±0.0	0.0	1.1±0.0
C20:3n-6	0.0	0.3 ±0.0	0.3 ±0.0	1.7±0.3	1.9±0.5	1.3±0.5	0.2±0.0	1.6±0.2
C20:4n-6	1.5±0.5	17.5 ±5.3	1.0±0.0	15.1±5.9	1.0±0.1	6.2±4.3	2.0±0.9	5.4±2.1
C18:3n-3	0.6 ±0.1	0.3 ±0.0	10.2 ±5.2	3.0±2.1	17.2±6.8	8.6±1.2	28.7±5.5	7.7±1.0
C20:3n-3	0.0	0.0	2.5 ±1.0	0.0	3.6±2.1	0.0	1.8±0.3	3.4±0.0
C20:4n-3	0.0	0.0	0.0	0.0	1.1±0.6	0.8±0.0	2.0±2.8	6.3±1.1
C20:5n-3	0.0	0.0	2.0±0.4	0.5±0.0	3.0±0.3	1.4±0.1	2.8±0.2	0.8±0.2
C22:6n-3	0.0	0.0	0.0	0.0	0.5±0.0	1.0±0.5	0.0	0.6±0.1

in pathological conditions, including inflammation. These cells are characterized by remarkable plasticity, they also exhibit pleiotropic effects. The phenotype of macrophages is dependent on signals received from the environment and cells polarization (M1–pro-inflammatory, M2–anti-inflammatory) and may be modified for example by supplementation with fatty acids [11,12]. Activation of macrophages results in, inter alia, synthesis of eicosanoids and other lipid mediators.

The aim of this study was to evaluate the anti-inflammatory or pro-resolving impact of ALA on the inflammatory state-related proteins and membrane fatty acids in the RAW 264.7 cells activated with LPS.

Materials And Methods

Cultured cells

Mus musculus murine macrophages RAW 264.7 (TIB-71, ATCC, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % Fetal Bovine Serum and 1 % antibiotic solution (100 IU/mL penicillin, 0.1 μg/mL streptomycin) (ATTC). Cells were maintained at 37 °C in humidified atmosphere of 5 % CO_2 in air and were finally seeded into a 6-well plate (Sarstedt, Germany) at a density of 5 x 10^5 cells/well in 2 mL of medium. At every step of the procedure, cell morphology was investigated by an inverted light microscope (Olympus, Japan). Cell viability during culturing was assessed with a Trypan Blue Exclusion Test. RAW 264.7 were supplemented with 2.5, 5 and 10 μmol of ALA (Sigma-Aldrich, USA) for 24h and 48h and after incubation activated with 10 ng/mL of lipopolysaccharide (LPS, from *E. coli*, Sigma-Aldrich). ALA was dissolved in ethanol. Control cultures received the same concentration of ethanol (the final content did not exceed 0.12 % v/v) as experimental cells. After incubation, cells were collected.

ApoTox-Glo Triplex Assay

After cells treatment, the ApoTox-Glo Assay was used to assess cell viability, the potential cytotoxicity of ALA, and cell apoptosis, was performed as per manufacturer instuctions (Promega, USA) as described previously [5,6].

Western blot

Cells lysate were prepared using M-PER mammalian protein extraction reagent (Thermo Scientific, USA) with protease inhibitor cocktail set III (Calbiochem, Merck, Germany). Protein concentrations were determined using the Bradford reaction. Aliquots (40μg) will be solubilised in a Laemmli buffer with 2% mercaptoethanol (BioRad) and subjected to 10% SDS-polyacrylamide gel electrophoresis as described below [4-6]. We used primary antibodies: anti-cyclooxygenase-2 (COX-2), anti-prostaglandin E2 synthase, anti-GAPDH diluted 1:1000 in Signal+

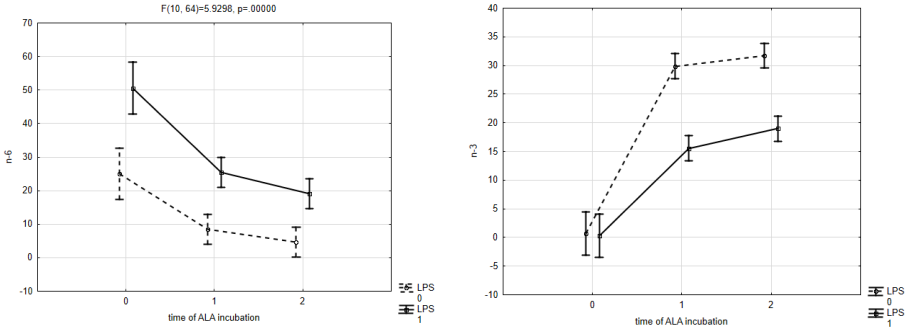

Fig. 1. n-3 and n-6 fatty acids [%] content in RAW 264.7 cells after LPS activation and according to the time of 10 μmol ALA incubation (1-after 24h, 2-after 48h).

for Western Blot (GeneTex) and anti-FP receptor (Cayman Chemical) diluted 1:200 in Signal+, and secondary antibody Easy Blot anti rabbit IgG (HRP) (GeneTex, 1:2000). Proteins were detected using the Western blotting detection kit Clarity Western ECL Luminol Substrate (Bio-Rad, USA). The integrated optical density of the bands were quantified using Chemi Doc Camera with Image Lab software (BioRad).

FA profile

Lipids from the RAW 264.7 cell membranes were extracted with chloroform–methanol solution (2:1 v/v). Fatty acid methyl esters (FAME) were synthesized using 10% BF_3 in methanol at 100°C. Heptadecanoic acid was used as an internal standard. FAME analyzes were performed using gas chromatography (GC)-Agilent 6890N with a J&W DB-23 capillary column (60 m, ID 0.25 mm, 0.25 μm) and FID detector as described earlier [5,6]. Retention times of FAME standards were used to identify fatty acids. Peak areas were measured using an integrator function (ChemStation). The results for fatty acid composition were expressed as relative % of total fatty acids.

Statistical analysis

All data are presented as means ±SD of at least six independent experiments. Comparisons between study groups were determined by two-way ANOVA followed by Scheffe's *post-hoc* test. Calculations were performed using Statistica 12 (StatSoft, USA) software, and statistical significance was established as $p \leq 0.05$.

Results

No cytotoxic effects were observed in the RAW 264.7 cells supplemented with 2.5, 5 and 10 μmol ALA for 24 and 48h and activated with LPS. Cell viability varied from 100% to 99% after incubation with LPS. There were no apoptotic cells after treatment with compounds.

Fatty acids profile in RAW 264.7 cell membranes differed significantly after ALA supplementation and after LPS activation. An increase of n-3 fatty acids was observed after incubation of cells with 2.5, 5 and 10 μmol of ALA for 48h (Table 1, Figure 1).

In macrophages membrane after LPS activation and after incubation with 5 and 10 μmol of ALA, the presence of EPA and DHA fatty acids was noticed. In the same samples a decrease of n-6 fatty acids in the cell membranes was observed (Table 1, Figure 1).

COX-2, cPGES and FP-receptor expression

Statistically the highest amount of COX-2 compared to control was observed in the RAW 264.7 cells incubated with LPS (p=0.01). Statistically the lowest expression of this protein was observed in the cells incubated with 10 uL ALA (p=0.00) (Fig. 2A). The highest expression of cPGES protein was observed in LPS treated cells (p=0.02). After ALA supplementation and LPS expression of this protein in RAW 264.7 cells was decreased with statistical significance (p=0.00) (Fig. 2B). In macrophages incubated with ALA and LPS the level of FP-receptor was statistically lower when compared to the LPS alone groups (p=0.01) (Fig. 2C).

Dicussion

In the present study murine macrophages RAW 264.7 cells were incubated with α-linolenic acid for 48h. Then, cells were activated with LPS for 24h. Our major findings are as follows: 1. supplementation of RAW 264.7 cells with ALA or LPS

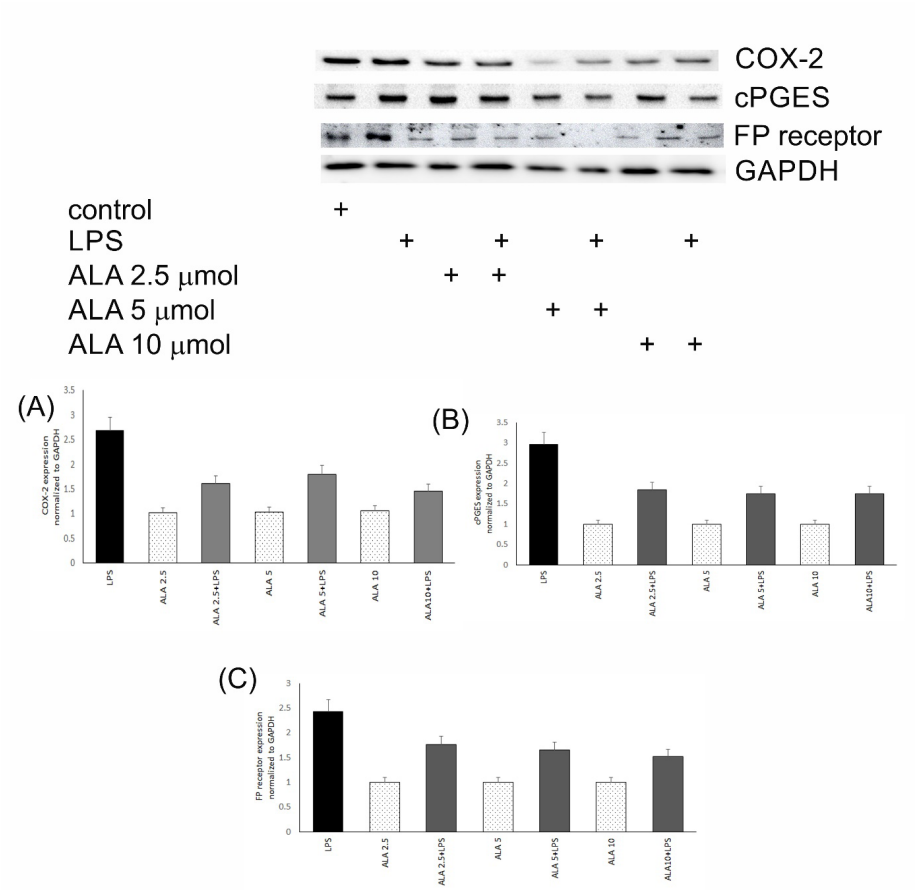

Fig. 2. Representative blots and relative expression of COX-2 (A), cPGES (B) and FP-receptor (C) in RAW 264.7 cells supplemented with ALA (2.5, 5 and 10 μmol) for 48h and activated with LPS.

had no effect on viability or apoptosis, 2. there were differences in the content of membrane fatty acids upon activation with LPS and upon ALA supplementation, 3. activation of cells with LPS resulted in increased expression of COX-2, cPGES and FP-receptor, which demonstrates the key role of these enzymes in the inflammatory process, 4. macrophages incubated with ALA and activated with LPS had a decrease in cyclooxygenase-2, prostaglandin E synthase and FP-receptor expression, compared to ALA+LPS cells, which demonstrates the anti-inflammatory properties of the compound.

In the study of Carotenuto et al. (2016) it was demonstrated that ALA reduces apoptosis and had protective effect on C2C12 myoblasts. This effect was associated with a decrease in caspase-3 activity and an increase of the Bcl-2/Bax ratio [13]. The anti-inflammatory potential of ALA attributed to COX – 2 inhibition was also assayed using the rat paw edema test [14]. The fatty acids profile in the phospholipids of cell membranes depends

on the diet and metabolic processes of the cell. A deficiency or excess of each FA may affect the membranes fluidity, may also induce changes in cell morphology [2,15]. This is extremely important because the fatty acids are involved in key biochemical processes, in cell signaling, inflammation, and regulation of gene expression. An unbalanced diet can lead to disorders of lipid metabolism, which is a risk factor for many diseases, including obesity, diabetes, inflammation or tumors. Changing the fatty acid composition of the membrane cells also affects their properties, function, cell signaling and also alteres phagocytic capacity of macrophages [10,16-18]. These effects appear to mediated at the membrane level suggesting the important roles that fatty acids play in membrane order, lipid raft structure and function, and membrane trafficking. Thus, the fatty acid composition of membrane cells influences their function and the cell membrane contents of n-6 and n-3 fatty acids are important [2,4-12].

In the presented study we analyzed the effect of the ALA supplementation on the fatty acids profile in membranes of RAW 264.7 cells. There were significant changes in the fatty acid profile in all groups of macrophages, those LPS-activated as well as in those supplemented with ALA in relation to the control group. It was found that the LPS-activated RAW 264.7 cells displayed significantly increased n-6 fatty acids, with the exception of LPS-activated and ALA supplemented macrophages. Differences in the membrane fatty acid profiles of cells supplemented with ALA may result from the use of FA for biosynthesis of eicosanoids and from differential expression of genes involved in the synthesis, elongation, and desaturation of FA at the inflammatory conditions.

It was demonstrated in the studies a protective role of EPA and DHA in HepG2, A549, HUVEC and RAW 264.7 cells activated with LPS, BaP, or other polycyclic aromatic hydrocarbons [4-9]. The above fatty acids and their derivatives (maresins, protectin D1 and Resolvin D1) exhibited repression of COX-2 on mRNA and protein level, as well as repression of FP receptor [5]. They also significantly reduced the amount of 8-isoP-GF2α in lung epithelial cells A549 and vascular endothelial cells HUVEC [4-7]. Moreover, the observed beneficial effect of EPA and DHA supplementation on the expression of genes associated with regulation of inflammatory processes [6]. The results obtained for RAW 264.7 cells suggest the new, dependent on receptor CB2, mechanism related to the response to environmental stress in the macrophages activated by LPS and BaP after DHA supplementation [8].

The dietary intake of fatty acids affects production of eicosanoids, which are potent immune mediators being mainly synthesized from eicosapentaenoic and arachidonic acid. n-3 PUFAs competitively inhibit n-6 PUFA arachidonic acid metabolism, thus reducing generation of the pro-inflammatory eicosanoids, as well as the cytokines synthesis from an inflammatory cells [10,17,19]. The n-3 FA inhibit the activity of the pro-inflammatory transcription nuclear factor κB (NF-κB), which induces the expression of many pro-inflammatory genes that encode adhesion molecules, cytokines, chemokines, and other effectors of the innate immune response [2,10,11,12].

Our results suggested that ALA plays a role in enhancement of the anti-inflammatory and resolving defence and has a high pharmacological and therapeutic value.

Acknowledgements

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Resumo

Kronika inflamo estas karakterizita de troa produktado de citokinoj kaj ejkozanoidoj kaj estas asociita kun ilia nesufiĉa resolucio. Suplementado de n-3 grasaj acidoj povas rezultigi pli raran incidencon de multaj inflamaj malsanoj. La celo de ĉi tiu studo estis determini la efikon de α-linolenika acido (ALA) sur la profilo de acidaj grasoj de ĉelaj membranoj je la por-inflamaj proteinoj de ciklooksigenazo-2 (COX-2), sintezo de prostaglandino E2 (cPGES) kaj la esprimo de la receptoro de prostaglandino F2α (FP) en musaj RAW 264.7 makrofagoj, aktivigitaj per lipopolisaĥaridoj (LPS). Oni pruvis ke COX-2, cPGES, kaj ankaŭ la esprimo de FP receptoro estis la plej alta en la ĉeloj aktivigitaj per LPS. En makrofagoj, al kiuj ALA estis suplementita, kaj tiuj aktivigitaj per LPS oni konstatis signife reduktitan kvanton de por-inflamaj proteinoj, sugestante antiinflaman efikon de α-linolenika acido. Ekzistis observitaj statistike signifaj ŝanĝoj en la grasa acido profilo post inkubacio de la RAW 264.7 ĉeloj dum 48 horoj kun ALA. Nesufiĉeco aŭ troo de specifaj grasaj acidoj influas la membranan fluecon de la ĉeloj, povas ankaŭ kaŭzi ŝanĝojn en la ĉela morfologio. Tial indas plia esplorado de la ecoj de ALA.

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