Basic nutritional investigation

Conjugated linoleic acid increases intracellular ROS synthesis and oxygenation of arachidonic acid in macrophages

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Abstract

Objective: Conjugated linoleic acids (CLAs) have potential antiatherosclerotic properties: they may inhibit atherosclerotic processes by reducing the intensity of inflammatory processes. However, in vivo studies have shown that the application of trans-10, cis-12 CLA in obese men increased their oxidative stress.

The objective of this study was to determine whether CLA can lead to an increase in oxidative stress and to isoprostane synthesis in macrophages.

Methods: Monocytes from peripheral blood and human monocytic leukemia cells were used in this study. Monocytes were differentiated to macrophages, and were incubated with 30 µM cis-9, trans-11 CLA and trans-10, cis-12 CLA or linoleic acid for 2 days. In some experiments the inhibitors of peroxisome proliferator-activated receptor-α (PPAR-α) or respiratory chain were added. After incubation, synthesis of reactive oxygen species (ROS), total cellular concentration of adenosine triphosphate, concentration of 8-epi-prostaglandin F2α, activity of cytoplasmic phospholipase A2 (cPLA2), activity of mitochondria, and expression of mRNA of PPAR-α were measured.

Results: In cells cultured with CLAs intercellular ROS synthesis increased. In this condition the mitochondrial energy potential was high, and the inhibitors of the respiratory chain and PPAR-α reduced ROS concentration. At the same time, the cPLA2 activity was abolished. In contrast, 8-iPF2αIII synthesis increased in CLA cells.

Conclusion: Cultivation of cells with CLA leads to an increased ROS synthesis, partly by PPAR-α mechanism. An increase in ROS concentration and inhibition of cPLA2 activity can stimulate oxygenation of arachidonic acid and contribute to an increase in 8-epi-PF2αIII level and in the apoptosis process in macrophages. © 2008 Elsevier Inc. All rights reserved.

Keywords: Macrophages; Conjugated linoleic acid; Reactive oxygen species; Isoprostanes

Introduction

Conjugated linoleic acid dienes (CLAs) are fatty acids found in food [1,2]. Their most common isomers are cis-9, trans-11 C18:2 isomers and trans-10, cis-12 C18:2 isomers [1]. In animals, CLAs have antiatherosclerotic properties; they are responsible for a decrease in the concentration of atherogenic lipoproteins of plasma and for normalization of plasma glucose levels [3–5]. CLAs also inhibit atherosclerotic processes in the body, e.g., by decreasing the intensity of inflammatory processes in cells [6]. The antiatherosclerotic function of CLA, well documented in animals [7], might be useful in the prevention of athero-
sclerosis. However, the application of trans-10 CLA, cis-12 isomer of CLA in the diet of obese men was shown to increase their oxidative stress (measured by the concentration of isoprostane 8-epi-prostaglandin F2α [8-epi-PGF2α,III] in urine) and increased C-reactive proteins (by 110%) in the body [8]. Trans-10, cis-12 CLA isomer turned out to be the activator of lipid peroxidation and oxidative stress and contributed to an increase in inflammatory marker concentration [8].

Studies on laboratory animals have proved that the metabolic action of CLA in the cell is based on stimulation of receptors activated by peroxisome proliferators [9,10]. As demonstrated, CLAs belong to the most potent agonists of such receptors [3]. Peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of nuclear receptors [11–13]. They are transcription factors that function as regulators of gene transcription related to lipid metabolism [14,15]. Ligands of PPARs are mainly fatty acids and their eicosanoid derivatives [11–13]. The level of expression of individual PPARs differs substantially for different tissue types [11–13]. Three main types of PPAR coded by separate PPAR genes have been identified: α, β, and γ. Two of these receptors, PPAR-α and PPAR-γ, are particularly active in macrophages [16]. The PPAR-α receptor undergoes the most intense expression in tissues characterized by intense catabolism of fatty acids [15]. Its main function is the regulation of genes engaged in mitochondrial and peroxisomal β-oxidation of fatty acids [14,15]. PPAR-α is required for the induction of genes involved in β-oxidation [15]. Both receptor types are activated by different agonists; PPAR-α is activated by polyunsaturated fatty acids (including CLA) [15]. CLA is also a natural ligand and activator of PPAR-α [3]. CLA isomers appear to be among the most avid fatty acid ligands of PPAR-α [3].

Inflammatory reactions underlie the pathogenesis of the atherosclerotic process [17,18] and oxygen free radicals formed during inflammatory reactions contribute to aggravation of atherosclerotic lesions [19]. This is because free radicals synthesized in quantities exceeding the efficiency of antioxidative systems (such as the oxygenated/reduced glutathione system, antioxidant enzymes) lead to damage of biomolecules important for correct functioning of the cell [19]. Reactive oxygen species (ROSs) are toxic products formed as a result of a decrease of molecular oxygen in the cell [17,20]. In monocytes/macrophages, ROS sources are the mitochondrial electron transport chain, cyclo-oxygenases, lipo-oxygenases, cytochrome P-450 and nicotinamide adenine dinucleotide phosphate oxidase [17,20]. ROSs participate in the regulation of numerous cellular functions, e.g., cellular differentiation, intracellular signaling [21], gene expression regulation, and (through activation of caspases or Fas receptors) in apoptotic stimulation [22]. Macrophages are also a source of free radicals other than ROSs, e.g., reactive nitrogen species [23,24]. In granulocytes ROSs and reactive nitrogen species have a dual function: they function as signaling molecules and serve as modulators of protein and lipid kinases and phosphatases, receptors, ion channels, and transcription factors. They regulate expression of key cytokines and chemokines that further modulate the inflammatory response. During an inflammatory response, ROSs and reactive nitrogen species modulate phagocytosis, secretion, gene expression, and apoptosis [25].

Isoprostanes are mostly formed during non-enzymatic peroxidation of arachidonic acid (AA), bound to lipid membrane phospholipids, and catalyzed by ROSs [26]. In fact, isoprostanes are chemically stable prostaglandin isomers [19,26–28]. In monocytes, cells participating in the inflammatory process, isoprostanes (e.g., 8-epi-PGF2α,III) are synthesized predominantly by non-enzymatic free radical peroxidation of AA in membranes [28].

Macrophages play a key role in the inflammatory process. Thus far reports have indicated an anti-inflammatory function of CLAs in macrophages [10,29]. This report shows for the first time that CLAs are the substances that may increase the synthesis of ROSs and isoprostane 8-epi-PGF2α,III in macrophages. This mechanism may be based on CLA-induced inhibition of cytoplasmic phospholipase A2 (PLA2) [30], which potentially retains AA in membrane phospholipids and increases its susceptibility to ROSs.

The objective of the study was to determine whether two isomers of CLA predominant in food (cis-9, trans-11 C18:2 and trans-10, cis-12 C18:2), when administered to macrophages, had an effect on ROS synthesis, mitochondrial activity and isoprostane 8-epi-PGF2α,III production.

The cis-9, trans-11 CLA isomer was selected for testing because it is the CLA isomer predominant in the human diet, constituting >80% of all CLAs present in milk and milk-derived products [23]. The second isomer, trans-10, cis-12, is present in milk in trace amounts but was selected on the basis of its high metabolic activity in the cell [31–33].

Materials and methods

Reagents and sources

RPMI medium, glutamine, and antibiotics (penicillin and streptomycin) were from Sigma-Aldrich (Poznań, Poland). THP-1 was obtained from American Type Culture Collection (Rockville, MD, USA). Fetal bovine serum was from Gibco (Gibco, Paisley, UK). Phosphate buffered saline (PBS) and Lymphozyten separation medium were from PAP Laboratories (Vienna, Austria). CD14+ MicroBeads MS separation columns, nylon filters (30 µm), and MACS separator system were from Miltenyi Biotec (Auburn, CA, USA). CLAs were from Nu-Chek Prep (Elysian, MN, USA; 98% purity). The fatty acids for cell culture were dissolved in fatty acid–free albumin from Sigma-Aldrich. All reagents and
solvents for extraction of 8-epi-PGF\textsubscript{2\alpha}III were from Sigma-Aldrich. C\textsubscript{18} reverse-phase columns (JT Baker, Phillipsburg, NJ, USA) and the solid-phase extraction Vacuum Manifold system used for 8-epi-PGF\textsubscript{2\alpha}III extraction were from Supelco (Poznań, Poland). The 8-iPGF\textsubscript{2\alpha} immunoassay kits were from Oxis (Portland, OR, USA). Deionized water was from Sigma-Aldrich. The Bradford-based protein extraction measured kit was from Sigma-Aldrich. N,N,N',N'-tetramethyl-p-phenylenediamine and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich. An R-phycocerythrin-conjugated mouse anti-human monoclonal antibody set was obtained from BD Biosciences Pharmingen (USA).

M-PER mammalian protein extraction reagent was from Pierce (Rockford, IL, USA). An annexin V/fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from BD Pharmingen. Potassium cyanide was from POCH (Poland), and antimycin A, rotenone, and GW 6471 (inhibitor of PPAR-\(\alpha\)) were from Sigma-Aldrich.

MitoTracker green FM was from Molecular Probes (Europe BV, Leiden, The Netherlands).

JC-1 fluorochrome (5,5',6,6'-tetrachloro-1',13,3'-tetraethylenbenzimidazol-carbocyanine iodide) was from Molecular Probes, Europe BV.

**Experimental conditions**

Monocytes (CD14\(^+\)) were isolated from blood from healthy donors. Blood sampling was performed in accordance with the principles outlined in the Declaration of Helsinki [34]. First, peripheral blood mononuclear cells were isolated from anticoagulated blood using Lymphozyn separation medium [35]. Then, cells were passed through a 30-μm nylon filter to remove clumps and magnetically labeled with CD14\(^+\) MicroBeads. After incubation (15 min, 6°C) cells were separated in a column that was placed in the magnetic field of a MACS separator. The magnetically labeled CD14\(^+\) cells were retained in the column, and the unlabeled cells passed through. In the next step, after removal of the column from the magnetic field, the magnetically retained CD14\(^+\) cells were eluted using a separation buffer (PBS, pH 7.2, supplemented with 0.5% bovine serum albumin [BSA] and 2 mM ethylenediaminetetra-acetic acid). CD14\(^+\) cells were seeded with RPMI medium with 10% autologous serum. Cells were cultured at 37°C in a 5% CO\textsubscript{2} humid atmosphere in RPMI medium containing 2 mM glutamine, antibiotics, and 10% autologous human serum and then cultured for 7 d [17].

THP-1 cells were differentiated into macrophages by administering phorbol myristate acetate [36], and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum without fatty acid, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37°C in 5% CO\textsubscript{2}. The THP-1 monocytes were treated with 100 nM phorbol myristate acetate for 24 h, and then the adherent macrophages were washed three times with PBS and incubated with fatty acids for 48 h at 37°C. Fatty acids were added as a 4-mM stock solution dissolved in 1 mM of fatty acid-free BSA [18]. Isomers of CLA (cis-9, trans-11, and trans-10, cis-12) were used at final concentrations of 30 μM.

Incubation time and fatty acid concentration were selected on the basis of results obtained in preliminary experiments. Cells with or without fatty acids (incubated with BSA) were incubated for 2 d. The cells were harvested by trypsinization and a pellet was obtained by centrifugation (250g for 5 min). The cell count was determined with a Bright Line Hemacytometer (Sigma-Aldrich). Cell viability was examined using a trypan blue dye exclusion method. Protein concentration was measured by the Bradford method [37].

Differentiation of monocytes to macrophages was estimated by flow cytometry with an anti-CD68 antibody. After 7 d of incubation with fatty acids, the percentage of CD68 cells was assessed by flow cytometry (FACScan) using CellQuest software [38]. CD68 cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, and incubated with fluorescein-conjugated monoclonal FITC CD68 antibody for 30 min. Mouse immunoglobulin G2\(\alpha\) was used as an isotype control antibody (Becton Dickinson, Oxford, United Kingdom).

Macrophages cultured without BSA and fatty acids were estimated in this study as a negative control.

**Intracellular ROS formation**

After incubation CLA cells were preloaded (30 min at 37°C) with 5 μM DCFH-DA. DCFH-DA becomes fluorescent on oxidation to DCF by hydrogen peroxide within the cell [17,39–41]. Cells were harvested and the number of cells exhibiting increased fluorescence of oxidized DCF was measured by flow cytometry (FACScan, Becton Dickinson) as previously described [17]. To determine whether the respiratory chain was the main source of ROSs in this experiment, 1-h incubation of macrophages was carried out (after 2 d of cultivation with CLA).

For some cells a KCN (1 mM) mixture of antimycin A (5 μg/mL; a mitochondrial complex III inhibitor) and rotenone (10 μM; a mitochondrial complex I inhibitor) was added for 24 h before ROS measurements.

In other experiments the PPAR-\(\alpha\) inhibitor GW 6471 was added (50 μM) to cells with fatty acids or BSA 48 h before ROS measurements.

**Fluorescent microscopy:** imaging of mitochondria by Mito Tracker Green FM and fluorometric determination of membrane potential by JC-1

MitoTracker Green FM is frequently used as a fluorescent probe in mitochondrial imaging [42].

To stain the mitochondria, macrophages cultivated for 48 h with fatty acids were incubated in a medium containing...
MitoTracker (0.2 μg of fluorochrome in 1 mL of RPMI medium) for 25 min at 37°C in an incubator. After incubation, the preparations were examined under a fluorescent microscope (Axioskop Zeiss, Germany) using a 09 filter (filter set 09-487909-000; Carl Zeiss, Germany).

Mitochondrial membrane potential was estimated with JC-1. The macrophages cultivated with fatty acids were incubated with lipophilic cationic fluorochrome JC-1. The medium containing JC-1 with the final concentration of 10 μg/mL was prepared from the initial solution containing 1 mg of JC-1/1 mL of dimethyl sulfoxide. The cells were incubated with fluorochrome in an incubator for 25 min and then examined under a fluorescent microscope.

JC-1 exhibits potential dependent accumulation in mitochondria and displays two colors of fluorescence: green and red/orange/yellow [43]. A Δψ above 80–100 mV causes an orange fluorescence emission (emission maximum at 590 nm) due to reversible formation of JC-1 aggregates in polarized mitochondria. The green fluorescence is characteristic for a monomeric form of the dye that accumulates only in depolarized mitochondria with low mitochondrial membrane potential (ΔΨ <80–100 mV).

High-performance liquid chromatographic separation of adenosine triphosphate (and other purines) in macrophages cultured with fatty acids

After incubation with BSA or fatty acids, the cells were washed with PBS buffer (Sigma-Aldrich) at +4°C and centrifuged (500g) for 10 min at +4°C. The centrifuged cells were suspended in 200 μL of PBS and deproteinized with 200 μL of 1.3 mol/L of HClO4. The samples were centrifuged (14 000g) for 10 min at +4°C. Two hundred microliters of supernatant was neutralized with 20 μL of 1 M K2HPO4 (Fluka, Poznań, Poland) to achieve a pH range of 6.0–7.0. The samples were centrifuged again (under the same conditions) and the supernatant was frozen at -70°C until further analysis. The concentrations of purines (adenosine-5’-triphosphate [ATP], adenosine-5’-diphosphate [ADP], adenosine-5’-monophosphate [AMP], and adenosine) were determined in the prepared samples.

For high-performance liquid chromatographic analysis, a Hewlett-Packard chromatographic system (HP 1100, Austria) was used. The analytical column (100 × 4.6 mm) was packed with 3 μm of Hypersil BDS-C18 (Aqilent Technologies, Wilmington, DE, USA). The following buffers were used: buffer A contained 150 mM KH2PO4 and 150 mM KCl adjusted to pH 6.0 with K2HPO4; buffer B was a 15% KCl adjusted to pH 6.0 –7.0. The samples were centrifuged again (under the same conditions) and the supernatant was frozen at 70°C until further analysis. The analytical column (100 × 4.6 mm) was packed with 3 μm of Hypersil BDS-C18 (Aqilent Technologies, Wilmington, DE, USA). The following buffers were used: buffer A contained 150 mM KH2PO4 and 150 mM KCl adjusted to pH 6.0 with K2HPO4; buffer B was a 15% solution of acetonitrile in buffer A. Peaks were detected by absorption measurements at 254-nm wavelength.

The adenylate energy charge (AEC) was evaluated according to the formula by Atkinson and Walton [44]:

$$\text{AEC} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

Measurements of non-enzymatic oxidation 8-epi-PGF2α,III

After 7 d of incubation with fatty acids, cells and supernatant were collected and frozen at −80°C until 8-epi-PGF2α,III isolation. Lipid fractions were extracted from cells with a Folch mixture [45] (chloroform:methanol 2:1, v/v) with butylated hydroxytoluene (0.05%) as an antioxidant, the mixture was intensively vortex-mixed, and 0.043% MgCl2 was added. The mixture was vortex-mixed again and centrifuged (3824 for 10 min). Downer phase was taken and evaporated under N2. The residue after evaporation was saponified and hydrolyzed with 15% KOH and methanol (1:1, v/v). The mixture was vortex-mixed again and incubated at 37°C for 30 min. Thereafter, 1 mM of HCl was added and the mixture was applied under a separation column (Bakerbond spe, RP-18, J.T. Baker) [44]. The 8-epi-PGF2α,III was eluted from C18 Sep Pak by a mixture of ethyl acetate:methanol (1:1, v/v) and samples were evaporated under N2, resuspended in dilution buffer, and assayed as described by the Oxis protocol.

Measurement of PLA2 activity

After incubation, cells (macrophages from blood) with CLA (5 μM final concentration) ionophore (A 23187) added to the cells were incubated for 1 h at 37°C with gentle agitation [46]. After incubation, the cells and the medium were used for extraction of total lipids using a Folch mixture (2:1, v/v) of chloroform:methanol containing 0.01% (w/v) butylated hydroxytoluene as an antioxidant, as described previously in detail [30]. Measurement of PLA2 activity in macrophages in THP-1 was described in detail previously [30].

Complement DNA synthesis and quantitative real-time polymerase chain reaction

Total RNA was extracted from cell cultures using a Qiagen kit (Gibco). The quality of RNA was confirmed by denaturation gel electrophoresis and analysis on an Agilent 2100 Bioanalyser (Agilent Technologies, Santa Clara, CA, USA).

To confirm regulation of the PPARs, a quantitative expression analysis was performed by real-time polymerase chain reaction (PCR) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene, as described in detail previously [46]. Subsequently, cDNA was subjected to real-time PCR in a reaction mixture containing QuantiTect SYBR Green PCR (Qiagen, Valencia, CA, USA) mix and primers. The following primer sequences were used in this study: PPAR-α forward primer: 5′-GGACGTGCTTCCTGCTTCAT-3′; reverse primer: 5′-CACCATCGCGACCAGATG-3′; GAPDH forward primer: 5′-GCCAGCGGAGCCACATC-3′; reverse primer: 5′-GCCAATACGACCCAAA-3′. All real-time PCR reactions were performed on a DNA Engine Option II
(MJ Research, GMI Inc., Ramsey, MN, USA). The thermal profile included initial denaturation for 15 min at 95°C followed by 40 amplification cycles of denaturation for 30 s at 72°C. After PCR amplification, melting curve analysis was performed with a temperature profile slope of 1°C/s from 35°C to 95°C. A negative control without the cDNA template was run with every assay to ensure overall specificity. The expression rates were calculated as detailed previously [47,48].

**Western blot**

Antibodies against PPAR-α and peroxidase/anti-mouse immunoglobulin G1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cell monolayers were washed in PBS and lysed with lysis buffer (M-PER mammalian protein extraction reagent, apoprotein 10 mg/mL, pepstatin A 1 mg/mL, sodium orthovanadate 1 M, sodium fluoride 1 M, sodium pyrophosphate 100 mM, leupeptin 10 mg/mL, ethylene-diaminetetra-acetic acid 500 mM, and phenyl methyl sulfonyl fluoride 10 mg/mL). Total protein was separated in 10% sodium dodecylsulfate/polyacrylamide gel electrophoresis, and proteins from gels were blotted onto nitrocellulose membranes at 100 mA for 1 h at 4°C. Membranes were then incubated for 1 h with antibodies direct against PPAR-α (1:500) or with a monoclonal anti–β-actin (clone AC-74, Sigma-Aldrich). Bound antibody was detected by using the appropriate horseradish peroxidase–conjugated antibody. Signals were visualized by chemiluminescence (Amersham, Buckinghamshire, United Kingdom) [17].

**Assay of apoptosis**

Cells were analyzed by flow cytometry by staining with propidium iodide using FACScan cytofluorometry. The measurement of apoptosis was carried out with a Becton-Dickinson annexin V/FITC (Oxford, UK) apoptosis detection kit according to the manufacturer’s instructions.

![Fig. 1. An intense green fluorescence in the cytoplasm after incubation with MitoTracker Green FM probe in cytoplasm of macrophages cultured with (A) the control bovine serum albumin, (B) trans-10, cis-12 conjugated linoleic acid diene isomer, (C) cis-9, trans-11 conjugated linoleic acid diene isomer, and (D) linoleic acid. THP-1 macrophages were cultured with fatty acids for 48 h as described in MATERIALS AND METHODS.](image)
Statistical analysis

All results are expressed as mean ± standard error. Because the distribution in most cases deviated from normal (Shapiro-Wilk test), non-parametric tests were used. For related samples, significance was first checked with Friedmann’s analysis of variance, and significant results were subjected to the Wilcoxon matched-pair test. Analysis was done with Statistica 6.1 (Statsoft, Poland). \( P < 0.05 \) was considered statistically significant.

Results

Effect of CLA on intracellular ROS synthesis in macrophages

Cells cultivated with CLA and LA exhibited bright green fluorescence, indicating the presence of active and intact mitochondria in the cytoplasm (Fig. 1A–D). After incubation of macrophages with the mitochondrial probe JC-1, yellow-orange fluorescence from the polarized mitochondria was visible, with a correct potential of the intracellular mitochondrial membrane (\( \Delta \Psi_m \) 80–100 mV; Fig. 2A–D).

The culture of macrophages with CLA and LA enhanced the synthesis of ROSs in macrophages \( (P \leq 0.02, \) macrophages from THP-1; \( P \leq 0.04, \) macrophages from blood; Table 1). In macrophages from THP-1, increased ROS synthesis was found in the environment of the \( \text{trans}-10, \text{cis}-12 \) CLA isomer \((+38\% \text{ versus BSA}, P \leq 0.01)\) and \( \text{cis}-9, \text{trans}-11 \) CLA \((+23\%, P \leq 0.01)\), with a trend toward increased ROS synthesis in cells cultivated with LA (Table 1). Similarly, in macrophages obtained from blood, increased ROS synthesis was noted in cells cultivated with both CLAs \((+20\% \text{ for } \text{trans}-10, \text{cis}-12 \text{ CLA}, P \leq 0.01; +14\% \text{ for } \text{cis}-9, \text{trans}-11, P \leq 0.05; \) Table 1).

To determine whether the respiratory chain is the main source of ROSs in macrophages cultivated with fatty acids, 1-h incubation of macrophages (cultivated earlier for 48 h with fatty acids) with 1 mmol/L of KCN (cytochrome oxidase inhibitor) was performed. In the presence of KCN, ROS synthesis (expressed as percentage of
Increased ROS synthesis (Table 2, Fig. 3A–D). Cultivated with LA and with GW 6471 tended to show 28% reduction of the DCF fluorescence; another decreased in the cells cultivated with both isomers of CLA. Yet only one isomer, trans-10, cis-12 CLA, decreased in the cells cultivated with both isomers of CLA.

Effect of fatty acids on ROS synthesis in macrophages cultured with fatty acids*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DCF fluorescence intensity</th>
<th>Percent of control</th>
<th>BSA</th>
<th>421 ± 103</th>
<th>100 ± 63</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trans-10, cis-12 CLA</td>
<td>589 ± 122†</td>
<td>+38</td>
<td>+20</td>
<td>120 ± 68§</td>
<td></td>
</tr>
<tr>
<td>Cis-9, trans-11 CLA</td>
<td>539 ± 116†</td>
<td>+23</td>
<td>+14</td>
<td>114 ± 47§</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>560 ± 262</td>
<td>+33</td>
<td>+10</td>
<td>110 ± 57</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>400 ± 261</td>
<td></td>
<td>101 ± 57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BSA, bovine serum albumin; CLA, conjugated linoleic acid diene; DCFH-DA, 2',7'-dichlorofluorescein diacetate; ROS, reactive oxygen species

* Macrophages were cultured with fatty acids for 48 h. Cells were the incubated with DCFH-DA (25 µmol/L) for 30 min and final fluorescence intensity was measured by flow cytometry. Results are expressed in DCF fluorescence intensity and percentage of control (BSA taken as 100%). Results are shown as mean fluorescence ± SD from eight separate experiments.

† P < 0.05, significant difference versus corresponding control.

The reduction of the ROS synthesis was also observed in macrophages cultivated with the mixture of the respiratory chain inhibitors: antimycin A (5 µg/mL) and rotenone (10 µM; Table 2).

In THP-1 macrophages, both CLA isomers increased the concentration of 8-epi-PGF₂α by 150% (compared with the BSA control, P ≤ 0.05; Table 4).

In THP-1 monocytes and peripheral blood mononuclear cells; Fig. 4 and Fig. 5A,B).

CLA changes intracellular concentration of adenylates and value of AEC

Both isomers of CLA significantly increased the concentration of ATP and other adenylates (adenosine and AMP) in macrophages obtained from THP-1 (Table 3).

In the cells cultivated with CLA, the AEC values also increased.

Influence of CLA on 8-epi-PGF₂α concentration

In THP-1 macrophages, both CLA isomers increased the concentration of 8-epi-PGF₂α by 160% (P ≤ 0.05) and cis-9, trans-11 CLA isomer increased this value by 120% (compared with the BSA control, P ≤ 0.05; Table 4).

Table 2: Effect of inhibitors of respiratory chain and PPAR-α on ROS synthesis in macrophages cultured with fatty acids

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DCF fluorescence intensity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA with inhibitors</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>Trans-10, cis-12 CLA</td>
<td>74 ± 2</td>
</tr>
<tr>
<td>Cis-9, trans-11 CLA</td>
<td>88 ± 5</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>BSA untreated inhibitor</td>
<td>402 ± 89</td>
</tr>
</tbody>
</table>

BSA, bovine serum albumin; CLA, conjugated linoleic acid diene; DCFH-DA, 2',7'-dichlorofluorescein diacetate; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species

*Macrophages (THP-1) were cultured with fatty acids for 48 h and then incubated with a mixture of antimycin A (5 µg/mL) and rotenone (10 µM) or with KCN (1 mM). In some experiments macrophages were cultured with GW 6471 (48 h/50 µM). Afterward, cells were incubated with DCFH-DA (25 µmol/L) for 30 min and the final fluorescence intensity was measured by flow cytometry. Results show tendency and are expressed as fluorescence intensity (mean fluorescence ± SD) from three or five separate experiments.

† P < 0.05, significant difference versus corresponding control.
CLAs reduce PLA2 activity in macrophages from blood

It is estimated that ionophore A12387 is a stimulator of activity of cytoplasmic PLA2 [46]. In our experiment, AA released by PLA2 (as a result of cell stimulation by ionophore A12387) was extracted and separated from the remaining total lipids with the use of thin-layer chromatography. Then the concentration of AA methyl esters was measured by gas chromatography.

In macrophages from peripheral blood, a trend was observed toward lower secretory PLA2 activity in the CLA environment (Fig. 6).

A similar phenomenon was noticed in THP-1 macrophages, as described previously [30].
CLA in differentiating monocytes can probably increase the number of apoptotic cells

To investigate whether increased ROS synthesis might have activated the apoptotic process, the cytometric measurement of the early apoptotic phase was performed (with the use of an annexin V kit). In macrophages incubated with CLA, a slight reduction in the number of viable cells was observed ($P < 0.005$, macrophages from THP-1; $P < 0.012$, macrophages from blood). In macrophages originating from THP-1, both CLA isomers reduced the content of viable cells in the culture by about 10% ($P < 0.05$) compared with the BSA control. In macrophages from peripheral blood, the isomers reduced this content by about 6% ($P < 0.05$; Table 5).

Discussion

Long-chain non-esterified fatty acids are the factors that increase ROS synthesis in vivo and in vitro in many types of cells [49–54]. As shown in this study, fatty acids may increase ROS production in parallel with a partial inhibition of electron transport in the respiratory chain, most likely at the level of complexes I and III, and by changing membrane fluidity [55].

In this report we show for the first time that CLA increases ROS synthesis in macrophages. We believe that, in macrophages cultivated with CLA, the mitochondrial respiratory chain is a source of ROSs, and free radicals are produced as a result of the CLA-induced processes of mitochondrial oxidation.

Reduced nicotinamide adenosine dinucleotide (created in the $\beta$-oxidation process and Krebs cycle) must be oxygenated by the respiratory chain enzyme system [56]. Activation of respiratory chain enzymes results in increased synthesis of ROSs in the cell.

The proof for this is a high energy potential of mitochondria and the presence of uncoupled polarized mitochondria in cells (Figs. 1 and 2). Activation of the respiratory chain in macrophages cultivated with CLA is also confirmed by the observed increase in ATP concentration in cells cultivated with these fatty acids (Table 3). The obtained results indicate that CLAs are used in macrophages as substrates for oxidative phosphorylation, leading to ATP synthesis (Table 3).

An additional proof of the ROS mitochondrial origin in cells cultivated with CLA is the inhibition of ROS synthesis observed after application of respiratory chain inhibitors (antimycin, rotenone, and KCN; Table 2). Interestingly, complete inhibition of the respiratory chain (by a mixture of rotenone and antimycin A) did not result in complete inhibition of ROS synthesis in macrophages; this indicates the existence of extramitochondrial sources of ROS in cells cultivated with CLA. Extramitochondrial sources of ROS can be coupled with activity of 15 lipo-oxygenase-1 or cyclo-oxygenase-2, which are not inhibited by respiratory...
CLA, as strong activators of PPAR-α.

In addition, we have shown in this study that ROS synthesis (in cells cultivated with CLA) is controlled by PPAR-α; the addition of GW 6471 (PPAR-α inhibitor) significantly decreased ROS synthesis in the cells cultivated with CLA, but not with LA (Table 2). It seems that CLAs, as strong activators of PPAR-α [3,33], stimulate the processes of mitochondrial oxidation by PPAR-α. PPAR-α activation is required for the induction of β-oxidation enzymes in monocytes/macrophages [3,15].

Such a phenomenon was observed in cells cultivated with α-LA and γ-LA, docosahexaenoic acid and eicosahexaenoic acid. The fatty acids, acting as PPAR-α ligands, increased the activity and level of mRNA of enzymes associated with oxygenating fatty acids in the mitochondria. Unlike them, LA, which is preferentially used for phospholipid synthesis, did not have any significant effect on PPAR-α activity [15], and seems to be a weaker ligand for this receptor than α-LA and γ-LA [15].

Importantly, no changes in expression of mRNA of PPAR-α were noted, although activation of PPAR-α mRNA expression was discovered in cancer cells [3] and in aortas of mice receiving CLA-supplemented feed (in vivo studies) [7].

Activation of ROS production in macrophages cultivated with CLA led to an increase in the synthesis of isoprostane 8-epi-PGF$_{2α}$, a non-enzymatic (free radical) product of AA oxygenation (Table 4). Enhanced 8-epi-PGF$_{2α}$ synthesis may be explained by two concomitant phenomena: inhibition of PLA$_2$ activity and activation of ROS synthesis in cells. AA “retained” in plasmatic membrane phospholipids (caused by reduced PLA$_2$ activity) provokes the use of this acid as a substrate for free radical synthesis of isoprostanes (including 8-epi-PGF$_{2α}$) [28]. Another effect of increased ROS synthesis in macrophages cultivated with CLA was activation of the apoptotic process (Table 5) [15]. When ROSs are produced in quantities exceeding the efficiency of chain inhibitors, or decoupling proteins whose expression is controlled by PPAR-α.

### Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATP (pmol/mg protein)$^†$</th>
<th>ADP (pmol/mg protein)$^†$</th>
<th>AMP (pmol/mg protein)$^†$</th>
<th>AEC$^†$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>205.6 ± 5</td>
<td>45.1 ± 1.6</td>
<td>22.8 ± 0.15</td>
<td>0.83 ± 0.015</td>
</tr>
<tr>
<td>Trans-10, cis-12 CLA</td>
<td>214.8 ± 4.3$^¶$</td>
<td>43.4 ± 1.4$^¶$</td>
<td>21.1 ± 0.38$^¶$</td>
<td>0.85 ± 0.014$^¶$</td>
</tr>
<tr>
<td>Cis-9, trans-11 CLA</td>
<td>210.2 ± 2.9$^¶$</td>
<td>41.3 ± 0.76$^¶$</td>
<td>20.3 ± 0.22$^¶$</td>
<td>0.85 ± 0.015$^¶$</td>
</tr>
<tr>
<td>LA</td>
<td>208.1 ± 7.7</td>
<td>42.8 ± 2.47$^¶$</td>
<td>24.1 ± 1.36</td>
<td>0.83 ± 0.011</td>
</tr>
</tbody>
</table>

AEC, adenylate energy charge; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; BSA, bovine serum albumin; CLA, conjugated linoleic acid diene; LA, linoleic acid

* AEC values were calculated according to the following formula: AEC = (ATP + 0.5 ADP)/(ATP + ADP + AMP). Values are means ± SDs of eight independent experiments.

$¹$ $P < 0.003$, Friedman’s analysis of variance (n = 8).

$²$ $P < 0.016$, Friedman’s analysis of variance (n = 8).

$³$ $P < 0.0006$, Friedman’s analysis of variance (n = 8).

$¶$ $P < 0.0003$, Friedman’s analysis of variance (n = 8).

$§$ $P < 0.05$, significant difference versus corresponding control.

### Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>8-epi-PGF$_{2α}$ (ng/μg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macrophages from THP$-1$</td>
</tr>
<tr>
<td>BSA</td>
<td>0.021 ± 0.02</td>
</tr>
<tr>
<td>Trans-10, cis-12 CLA</td>
<td>0.047 ± 0.03$^¶$</td>
</tr>
<tr>
<td>Cis-9, trans-11 CLA</td>
<td>0.045 ± 0.04$^¶$</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.021 ± 0.01</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.019 ± 0.01</td>
</tr>
</tbody>
</table>

BSA, bovine serum albumin; CLA, conjugated linoleic acid diene; 8-epi-PGF$_{2α}$, 8-epi-prostaglandin F$_{2α}$

* In macrophages the concentration of 8-epi-PGF$_{2α}$ was measured by an immunoenzymatic method. Units are shown as mean ± SD from eight or six separate experiments.

$¹$ $P < 0.02$, Friedman’s analysis of variance (n = 8).

$²$ $P < 0.05$, Friedman’s analysis of variance (n = 6).

$¶$ $P < 0.05$, significant difference versus corresponding control.
cellular antioxidant systems (such as glutathione, anti-oxidant enzymes), the structures important for correct functioning of the cell are damaged and the apoptotic process is activated [17,19,21]. Thus far, proapoptotic properties have been demonstrated for polyunsaturated long-chain fatty acids (e.g., γ-linolenic acid and eicosehexaenoic acid) [57], trans-isomers of fatty acids [58], and the trans-10, cis-12 CLA isomer [18]. An enhanced apoptotic process was observed also in aortas of mice receiving CLA-supplemented feed [7].

In the prophylaxis of ischemic heart disease, great importance is attached to the use of products containing substances that limit the intensity of inflammatory processes. They also include fatty acids appropriately selected in the diet [59].

The phenomenon of inhibition of inflammatory reactions after food supplementation with CLAs suggested that these acids might be used in the prophylaxis of ischemic heart disease in humans [10]. However, the intensity of free radical reactions in monocytes cultivated with CLAs, noted in these studies, warrants a critical view of the possibility of including CLAs in prophylaxis of ischemic heart disease. This opinion is also supported by the existing results of tests including CLAs in prophylaxis of ischemic heart disease in humans [10]. However, the intensity of free acids might be used in the prophylaxis of ischemic heart disease suggested that these CLAs are effective in the prophylaxis of ischemic heart disease in humans [10].

Table 5: Effect of fatty acids on content of living (non-apoptotic) macrophages cultured with CLA*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Macrophages from THP-1</th>
<th>Macrophages from blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-apoptotic cells</td>
<td>Percent of control</td>
</tr>
<tr>
<td>BSA</td>
<td>69.05 ± 22.05</td>
<td>100</td>
</tr>
<tr>
<td>Trans-10, cis-12 CLA</td>
<td>63.53 ± 22.72</td>
<td>−8</td>
</tr>
<tr>
<td>Cis-9, trans-11 CLA</td>
<td>64.94 ± 23.80</td>
<td>−6</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>58.57 ± 38.79</td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td>69.77 ± 16.29</td>
<td></td>
</tr>
</tbody>
</table>

BSA, bovine serum albumin; CLA, conjugated linoleic acid diene

* Macrophages were cultured with fatty acids for 48 h. Cells were then harvested, stained with annexin V/fluorescein isothiocyanate and propidium iodide, and analyzed by flow cytometry. Data show content (percentage) of non-apoptotic cells and the difference in content of non-apoptotic cells observed between control (BSA) and fatty acids (mean ± SD from five or eight separate experiments).

1 P < 0.005, Friedmann’s analysis of variance (n = 5).
2 P < 0.012, Friedmann’s analysis of variance (n = 8).
3 P < 0.05, significant difference versus corresponding control.

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