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Effect of Conjugated Linoleic Acids on the Activity and mRNA Expression of 5- and 15-Lipoxygenases in Human Macrophages

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Lipoxygenases are a family of non-heme enzyme dioxygenases. The role of lipoxygenases is synthesis of hydroperoxides of fatty acids, which perform signaling functions in the body. Studies on conjugated linoleic acids (CLAs) as fatty acids with a potential anti-atherosclerotic function have recently been initiated. The aim of the study was to test the effect of CLAs and linoleic acid on 5- and 15-lipoxygenase (5-LO, 15-LO-1) enzyme activity, their mRNA expression, and concentration in the cells. It was also desired to determine whether the CLAs are substrates for the enzymes. For the experiments monocytic cell line (THP-1) and monocytes obtained from human venous blood were used. Monocytes were differentiated to macrophages: THP-1 (CD14+) by PMA administration (100 nM for 24 h) and monocytes from blood (CD14+) by 7-day cultivation with the autologous serum (10%). After differentiation, macrophages were cultured with 30 μM CLAs or linoleic acid for 48 h. The 15- and 5-lipoxygenase products were measured by HPLC method. mRNA expression and protein content were analyzed by real-time PCR and Western blot analysis. The in vitro studies proved that both CLA isomers are not substrates for 15-LO-1; in ex vivo studies hydroxydecadienoic acid (HODE) concentration was significantly reduced ($p = 0.019$). The *trans*-10,*cis*-12 CLA isomer reduced HODE concentration by 28% ($p = 0.046$) and the *cis*-9,*trans*-11 CLA isomer by 35% ($p = 0.028$). In macrophages obtained from THP-1 fatty acids did not change significantly mRNA expression of the majority of the investigated genes. CLAs did not change the content of 5-LO and 15-LO-1 proteins in macrophages obtained from peripheral blood. Linoleic acid induced 15-LO-1 expression (2.6 times, $p < 0.05$). CLAs may perform the function of an inhibitor of lipoxygenase 15-LO-1 activity in macrophages.

KEYWORDS: 15-Lipoxygenase; 5-lipoxygenase; macrophages; conjugated linoleic acids

INTRODUCTION

Lipoxygenases constitute a heterogeneous family of non-heme enzyme dioxygenases (EC 1.13.11) that oxidize polyunsaturated fatty acids (*1*). The role of lipoxygenases is synthesis of hydroperoxides of fatty acids, which perform signaling functions in the body. In human macrophages, two isoforms of 15-lipoxygenase may be isolated, which are the equivalents of murine 12/15-LO: 15-lipoxygenase-1 (15-LO-1) and 15-lipoxygenase-2 (15-LO-2). Both 15-LO isoforms may oxidize free arachidonic acid or arachidonic acid esterified in phospholipids and cholesterol esters (at C12 or C15), but only isoform 1 (15-LO-1) uses linoleic acid (LIN) as a substrate, oxidizing it at C9 or C13 (*2*−*4*). LIN is transformed by the enzyme to 13-*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13*S*-HpODE) and in a smaller quantity to 9-*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9*S*-HpODE). On the other hand, arachidonic acid is transformed to 15-*S*-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15*S*-HpETE) and in a smaller quantity to 12-*S*-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12*S*-HpETE) (*4*). HpODE and HpETE are unstable compounds that are rapidly reduced in the cell (with participation of glutathione peroxidase) to hydroxyl derivatives—13*S*- or 9*S*-hydroxyoctadecadienoic acid (HODE) and 15*S*- or 12*S*-hydroxyeicosatetraenoic acid (HETE), respectively (*4*). Hydroperoxides of arachidonic acid and linoleic acid act as signaling molecules (*4*); for example, endogenous HpETE and HETE may be very effective with the participation of receptors or channels on the cell surface (*4*), and HODE may activate nuclear receptors PPARγ (*6*, *7*). Human macrophage lipoxygenase 15-LO-1 is responsible for the formation of oxidized lipids (with LDL) at the initial stages of atherosclerotic plaque formation (*4*). The expression of 15-lipoxygenase appears to be highly regulated

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by regulatory protein (e.g., lipoxygenase mRNA binding protein LOX BP) or certain cytokines, for example, IL-4 and IL-13 (*4*).

Another lipoxygenase responsible for the stimulation of the atherosclerotic process of blood vessels is 5-lipoxygenase (5-LO), which is present in macrophages (Alox 5; EC 1.13.11.34) (*8*, *9*). Evidence confirming the direct role of 5-LO in cardiovascular diseases was supplied by genetic studies conducted in large groups of subjects (*10*−*12*). In these studies, polymorphisms in genes associated with leukotriene synthesis (5-LO and FLAP) were proven to be responsible for individual predisposition to myocardial infarction/stroke (*10*−*12*). 5-LO is a cytoplasmic enzyme initiating the transformation of arachidonic acid to leukotriene A_4 and its derivatives. 5-LO acts in the presence of 5-lipoxygenase activating protein (FLAP). As a consequence of an appropriate stimulus action (e.g., inflammation) on the cell, 5-LO moves from the cytoplasm to the nuclear membrane, where it binds to FLAP. The inflammatory stimulus, which is a signal for 5-LO transfer, is also the stimulator for phospholipase A_2 ($cPLA_2$) movement into the nuclear membrane. Arachidonic acid (released from the nuclear membrane by $cPLA_2$) is transformed by 5-LO to 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which may be further transformed to hydroxyeicosatetraenoic acid (5-HETE) by glutathione peroxidase or to leukotriene A_4 (LTA_4) by 5-LO (*13*, *14*). LTA_4 may be then metabolized via two pathways: transformed to leukotriene B_4 (LTB_4) by cytoplasmic hydrolase or transformed to cysteinyl leukotrienes (*13*) by LTC_4 synthase. The presence of 5-lipoxygenase and other enzymes associated with the 5-lipoxygenase pathway (LTC_4 hydrolase, LTC_4 synthase) may be discovered in cells participating in the inflammatory process (in macrophages, foam cells, neutrophils, granulocytes) and in atherosclerotic arteries and atherosclerotic plaques. 5-LO expression was proven to significantly increase in advanced atherosclerotic lesions (*15*). However, in contrast to 15-LO-1, the pro-atherosclerotic function of 5-LO is not associated with LDL oxidation but with the synthesis of proinflammatory leukotrienes (e.g., LTB_4) modulating the immune response (*9*, *16*) and with the regulation of matrix metalloproteinases 2 and 9 (MMP-2, MMP-9) (*16*). Macrophage activation was proven to be related to an increased synthesis of one of the most potent chemoattractants—leukotriene B_4, the anti-atherosclerotic function of which consists of the enhancement of leukocyte ability to chemotaxis and adhesion in the area of the atherosclerotic plaque (*17*). On the other hand, metalloproteinases, the expression of which increases in macrophages along with the increase in expression of 5-lipoxygenase, are a group of proteolytic enzymes (metal-dependent Zn^{2+} and Ca^{2+}-endopeptidases) degrading connective tissue stroma and thus provoking the instability of atherosclerotic plaques (*16*).

In medicine, nutritional therapy may be important for immunoregulation, inflammation, and vascular diseases (*18*). Conjugated linoleic acids (CLAs) can trigger modification of immune cell functions and other physiological functions, for example, anti-atherosclerotic (*18*). In atherosclerosis prophylaxis, high importance is attached to the use of products containing substances that limit the intensity of inflammation processes involving lipids and their derivatives (*19*). Studies on CLAs as fatty acids with potential anti-atherosclerotic properties have recently been initiated (*20*). Conjugated linolic acid dienes are fatty acids found in food (*21*, *22*). The most common isomers are *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA (*21*). So far, few studies have been published to explain whether CLA isomers may modify lipoxygenase activity in cells (*23*−*25*). The influence of CLA on the eicosanoid metabolism in macrophages—cells that play a crucial role in the atherosclerotic process—has not been estimated. Therefore, the aim of the study was to test the effect of CLAs and linoleic acid on 5- and 15-LO enzyme activity, their mRNA expression, and concentration in the cells. We also wanted to test whether the CLAs are substrates for the enzymes. In vitro investigations aimed to improve the understanding of the mechanisms of effects of CLA dienes on arachidonic acid and linoleic acid metabolism in macrophages obtained from a cell line and from peripheral blood.

MATERIALS AND METHODS

Reagents. Cell culture media (RPMI) and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Grand Island, NY); antibiotics (penicillin and streptomycin) were purchased from Sigma (Sigma-Aldrich, Poznan, Poland). Phosphate-buffered saline (PBS) and Lymphozyten separation medium were from PAP Laboratories (Linz, Austria). CD14+ MicroBeads MS separation columns, nylon filers (30 μm), and a MACS separator system were obtained from Miltenyi Biotec (Auburn, CA). Conjugated dienes of linoleic acid (98% purity) were from Nu-Chek Prep (Elysian, MN). The fatty acids for cell culture were dissolved in fatty acids-free albumin from Sigma. A Bradford-based protein concentration measurement kit was from Sigma.

Reagents for Western blot analysis were from Pierce (Woburn, MA), Sigma, and Bio-Rad (Hercules, CA). Antibodies against human 15-LO-1 (rabbit polyclonal) were from Cayman Chemical (Ann Arbor, MI) and against human 5-LO (mouse monoclonal) from BD Biosciences (Franklin Lakes, NJ). Secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against human β-actin (mouse monoclonal) were from Sigma.

Cell Culture and Treatment. *(a) Cell Culture Line.* THP-1 cells (American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% fatty acid-free FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C in 5% CO_2. THP-1 monocytes were seeded at a density of 2×10^6 cells/well in six-well plates and then were differentiated to macrophages by administration of 100 nM phorbol myristate acetate (PMA) for 24 h (*26*). After incubation with PMA, adherent cells (macrophages) were washed three times with phosphate-buffered saline (PBS) and incubated with 30 μM fatty acids (final concentration) for 48 h at 37 °C. Incubation time and fatty acid concentration were selected on the basis of results obtained in preliminary experiments. Fatty acids were added as 4 mM stock solution dissolved in 1 mM fatty acid-free bovine serum albumin (BSA) as described in detail (*27*, *28*). Control cells after PMA treatment were incubated for 48 h with 30 μM BSA solution. The cells were harvested by trypsinization, and pellet was obtained by centrifugation (250*g* for 5 min). The pellet was resuspended in 100 μL of PBS, and then 10 μL of cell suspension was combined with 90 μL of trypan blue and viewed on a hemocytometer. The percent of the living cells that excluded trypan blue was used to determine cell viability. Cell cultures with viability of >97% were used for experiments.

(b) Monocytes (CD14+) were isolated from the blood of healthy donors. Blood sampling was performed in accordance with the principles outlined in the Declaration of Helsinki (*Cardiovasc. Res.* **1997**, *35*, 2−3). Monocytes were isolated from peripheral blood of 15 healthy donors, Caucasian white males, aged 20−25 years. Donors with a history of hypertension, diabetes, or smoking were excluded from the study. An average standard of living was found for all donors, and there were no cases of malnutrition.

Cells obtained from donors were used for quantification of LTB_4 concentration, real-time PCR, and the Western blot analysis, respectively. In each single experiment the cells obtained from one donor were cultured with BSA, LIN, or CLA isomers.

Peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated blood using Lymphozyten separations media as described (*29*). Then cells were passed through 30 μm nylon filters to remove clumps and magnetically labeled with CD14+ MicroBeads. After incubation (15 min, 6 °C), cells were separated on a column, which was placed in the magnetic field of a MACS separator. The magnetically

labeled CD14+ cells were retained in the column, whereas 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 separation buffer (phosphate-buffered saline, pH 7.2, supplemented with 0.5% BSA and 2 mM EDTA). CD14+ cells were seeded at a density of 3×10^6 cells/well in 24-well plates and cultured at 37 °C in 5% CO_2 humid atmosphere in RPMI medium containing 2 mM glutamine, antibiotics, and 10% autologous human serum for 7 days (*6*, *29*). Differentiation of monocytes to macrophages was estimated by flow cytometry with anti-CD68 antibody. After 7 days of incubation, the percentage of CD68 cells was assessed by flow cytometry (FACScan) using CellQuest software as previously described (*30*), and the fatty acids were added for 48 h.

Measurements of Concentrations of 15-Lipoxygenase Products ex Vivo. Macrophages obtained from the THP-1 cell line were used in 15-LO-1 activity studies. Lipoxygenase-15-LO-1 is an enzyme metabolizing in macrophages mainly linoleic acid to 9- and 13-hydroperoxyoctadecadienoic acids (9- and 13-HPODE) and to a lesser extent arachidonic acid to hydroperoxyeicosatetraenoic acids (e.g., 15-HPETE). The effect of fatty acids on 15-LO-1 activity was determined by a quantitative measurement of 9- and 13-HODE and 5-, 12-, and 15-HETE concentrations in studies conducted ex vivo and in vitro. In ex vivo studies, the concentration of HODE and HETE contained in homogenates obtained from cells previously incubated with fatty acids was measured. On the other hand, in in vitro studies, the concentration of HODE created in the course of incubation of the enzyme obtained from homogenate of cells (incubated previously with CLA) and exogenously added CLA or LIN isomers (30 μmol/L) was measured.

An experiment in which cells were incubated in four different conditions (with *trans*-10,*cis*-12 CLA, *cis*-9,*trans*-11 CLA, LIN, and BSA as control) was replicated six times. In ex vivo measurements the obtained cellular suspension (from THP-1 macrophages and from a single well) was suspended in 1 mL of the mixture of methanol with an addition of 1% of acetic acid and added to the previously collected cultivation medium. Equal volumes (~3 mL) of cold Folch mixture (methanol/chloroform 2:1, v/v) were added to test tubes, which were vigorously mixed (3 min, vortex). The samples were cooled for 10 min at −20 °C and then centrifuged (at 3200*g* for 10 min), and the lower layer was collected carefully and transferred to new test tubes and dried in a nitrogen stream. After drying, 0.8 mL of 1 mol/L KOH in methanol was added to the precipitate and the samples were blown with nitrogen and closed with a stopper. Hydrolysis was conducted (60 °C, 30 min), then the solution pH was adjusted to pH 4.0 with 2 M CH_3COOH, and 2.4 mL of H_2O was added to the test tubes. The samples were cooled (−20 °C, 10 min) and centrifuged to remove the potential sediment (3200*g* for 10 min, 4 °C). The pH value of the samples was adjusted to 3.0 with the use of concentrated CH_3COOH. The equivalent volume of cool ethyl acetate was added to the samples, and the mixture was agitated, cooled (−20 °C, 10 min), and centrifuged (3200*g* for 10 min, 4 °C). The upper layer was carefully collected and evaporated to dryness in a nitrogen stream. The samples were stored until analysis at −80 °C. The precipitate was dissolved in 200 μL of mobile phase (65% methanol with an addition of 0.01% CH_3COOH) (*31*, *32*), and HODE concentration was determined by HPLC.

Separations were performed with the use of the liquid chromatograph Hewlett-Packard 1050/1100 on the column LiChrospher 100-RP18 (250 × 4 mm, 5 μm) (Merck, Darmstadt, Germany) at 25 °C, using the gradient of methanol content in the mobile phase. The flow rate was 1 mL/min. The buffers contained the methanol/water/acetic acid mixture at proportions of 50:50:0.1 (v/v/v) for buffer A and 100:0:0.1 for buffer B. The percent content of buffer B in the mobile phase was 30% at 0.0 min of separation, increased linearly to 80% at 20 min, was 98% between 20.1 and 23.9 min, and was 30% between 24 and 28 min. Fifty microliter samples were injected into the 100 μL loop every 28 min. Detection was performed at a wavelength of 235 nm. For peak identification and quantitative analysis a mixture of 5-HETE, 12-HETE, and 15-HETE and 9-HODE and 13-HODE reference standards was used (the two latter were eluted as one peak).

Measurements of Concentrations of 15-Lipoxygenase Products in Vitro. An experiment in which cells were incubated in four different conditions (with *trans*-10,*cis*-12 CLA, *cis*-9,*trans*-11 CLA, LIN, and BSA as control) was replicated six times. The enzyme was released from macrophages (THP-1, incubated with fatty acids) with the use of a double freezing/thawing cycle of the cell suspension at −80 °C/10 min. LIN was added to the suspension at a final concentration of 100 μmol/L, and the mixture was incubated at 37 °C for 15 min. In some experiments the *trans*-10,*cis*-12 CLA or *cis*-9,*trans*-11 CLA (30 μmol/L) was added to the suspension (**Figure 1**). Then, 2 M CH_3COOH and H_2O were added to the test tubes, which were cooled, centrifuged, acidified, and extracted with ethyl acetate as described above. The precipitate was dissolved in 200 μL of the mobile phase, and 9- and 13-HODE (LIN oxidation products) concentrations were determined by HPLC as described above.

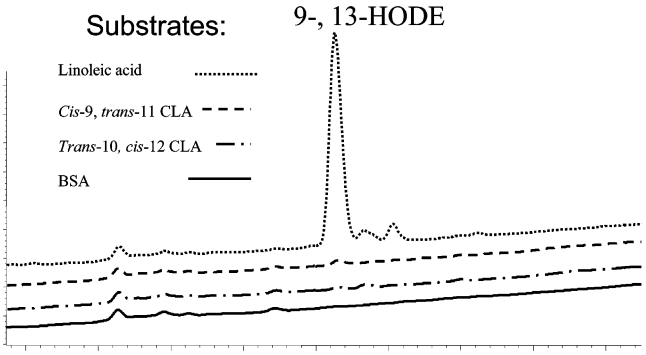

Figure 1. Effect of CLA on the synthesis of hydroxyoctadecadienoic acids (HODEs) in macrophages in in vitro assays. Fatty acids were added to the homogenates prepared based on the macrophages cultured previously with the same fatty acids: 100 μmol/L *trans*-10,*cis*-12 CLA (to the cells cultured before with this isomer) and 100 μmol/L *cis*-9,*trans*-11 CLA (to the cells cultured before with this isomer) as well as 100 μmol/L LIN (to the cells cultured before with BSA). HODEs concentration was measured by HPLC. The figure represents one of three replicates of the experiment with similar results.

Measurement of Concentration of 5-Lipoxygenase Product Leukotriene B_4 (LTB_4). An experiment in which cells were incubated in four different conditions (with *trans*-10,*cis*-12 CLA, *cis*-9,*trans*-11 CLA, LIN, and BSA as control) was replicated five times. 5-Lipoxygenase activity was determined by a measurement of concentration of the main product of this enzyme—(LTB_4) (*33*, *34*). Adherent macrophages (THP-1 and from blood) were incubated for 48 h with fatty acids as described. LTB_4 was extracted from the cell suspension (with the use of Bakerbond columns) consistently with the instruction enclosed with the measurement kit made by R&D Systems. The procedures of preparation of LTB_4 from macrophages and the measurement of LTB_4 concentration (by immunoenzymatic method) were conducted consistently with the kit instruction.

PCR with the Real-Time Analysis of Product Quantity Increase (Real-Time PCR). To investigate the mechanism of regulation of the enzyme activity (from THP-1 and from blood macrophages), the quantitative expression analysis was performed by real-time PCR using GAPDH as the reference gene as described in detail (*35*). After a reverse transcription step, cDNA was subjected to real-time PCR in a reaction mixture containing QuantiTect SYBR Green PCR (Qiagen) mix and primers. The sequences of primers used in this study were as follows: 5-LO forward primer, 5′-GGGCATGGAGAGCAAAGAAG-3′; 5-LO reverse primer, 5′-ACCTCGGCCGTGAACGT-3′; 15-LO-1 forward primer, 5′-ACTGAAATCGGGCTGCAAGGG-3′; 15-LO-1 reverse primer, 5′-GGGTGATGGGGGCTGAAATAA-3′; GAPDH forward primer, 5′-GCCAGCCGAGCCACATC-3′; GAPDH reverse primer, 5′-GCGCCAATACGACCAAA-3′.

All real-time PCR reactions were performed on the DNA Engine Option II (MJ Research) according to the method given in ref *35*. Following PCR amplification, melting curve analysis was performed with a temperature profile slope of 1 °C/s from 35 to 95 °C. A negative

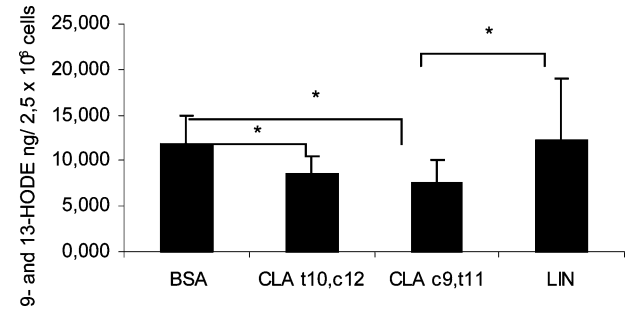

Figure 2. Effect of CLA on the concentration of hydroxyoctadecadienoic acids (HODEs) in ex vivo assays. In macrophages (THP-1) the concentration of HODEs was measured with HPLC. Data are expressed as ng/2.5 $\times$ 10^6 cells and shown as mean concentration $\pm$ SD from six replicates. Friedman ANOVA: $p = 0.019$; $*$, $p < 0.05$.

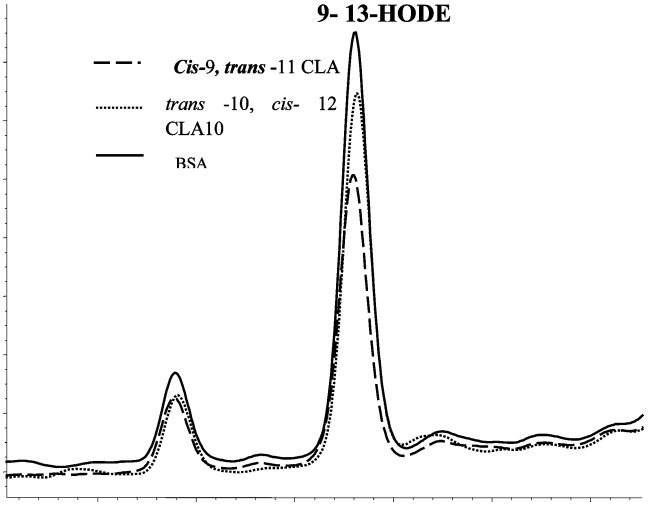

Figure 3. Representative chromatography analysis obtained from macrophages (THP-1) cultured with CLA (ex vivo). The lines show HODE peak in macrophages cultured with *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and BSA (control).

control without cDNA template was run with every assay to ensure overall specificity. The expression rates were calculated as described (*35*, *36*).

Analysis of the Content of Other Proteins with the Use of the Western Blot Method. An experiment in which cells were incubated in four different conditions (with *trans*-10,*cis*-12 CLA, *cis*-9,*trans*-11 CLA, LIN, and BSA as control) was replicated three times. The procedure was consistent with the generally applied methodologies (*37*, *38*). After incubation with fatty acids, the cells were collected to test tubes and washed twice with cool PBS (250*g* $\times$ 10 min, 4 °C). In the meantime, lysing buffer was prepared on ice, with the following composition: M-PER, aprotinin (10 mg/mL in PBS), pepstatin A (1 mg/mL of methanol), sodium orthovanadate (1 mol/L), sodium fluoride (1 mol/L), sodium pyrophosphate (100 mmol/L), leupeptin (10 mg/mL), EDTA (500 mmol/L), PMSF (10 mg/mL of isopropanol—due to its short half-life, PMSF was added to lysing buffer immediately before addition to cells). One hundred and twenty microliters of lysing buffer was added to washed cells. The cellular precipitate was lysed on ice for 10 min and then centrifuged for 15 min (14000*g*, 4 °C). The supernatant was collected, 20 μL was used for protein determination (using Bradford's method), and the remaining lysate was frozen at −80 °C until analysis.

The volume of lysate containing 10 μg of protein was mixed with Laemmli sample buffer containing β-mercaptoethanol and incubated in a dry bath at 70 °C for 10 min. The colored lysate was applied to gel. Electrophoresis was conducted under a stable voltage of 150 V for about 1.5 h. Then transfer to PVDF membrane was performed for 1 h, at a stable voltage of 100 V.

Table 1. Effect of CLA on the 5-, 12-, and 15-Hydroxyeicosatetraenoic Acid Concentrations Measured ex Vivo in Macrophages by HPLC[a]

treatment	5-HETE	12-HETE ($p < 0.05$, test ANOVA Friedman)	15-HETE ($p < 0.04$, test ANOVA Friedman)
BSA	13.60 ± 4.12	13.75 ± 3.34	10.24 ± 4.82
trans-10,*cis*-12 CLA (30 μM)	12.51 ± 3.15	7.17 ± 2.9[b]	11.74 ± 2.04
cis-9,*trans*-11 CLA (30 μM)	11.45 ± 3.4	8.38 ± 3.25[b]	5.73 ± 3.92[c,d]
LIN (30 μM)	12.58 ± 3.44	12.83 ± 4.13	10.33 ± 5.79

[a] Data are expressed as ng/2.5 $\times$ 10^6 cells and shown as mean concentration $\pm$ SD from six replicates. [b] $p < 0.05$ compared to BSA. [c] $p < 0.05$ compared to *trans*-10,*cis*-12 CLA. [d] $p < 0.05$ compared to LIN.

Table 2. Effect of Fatty Acids on Leukotriene B_4 (LTB_4) Concentration Measured ex Vivo in Macrophages by Immunoenzymatic Method[a]

treatment	concentration of LTB_4 macrophages from THP-1	concentration of LTB_4 macrophages from blood
BSA	17.25 ± 1.10	31.34 ± 9.89
trans-10,*cis*-12 CLA	15.94 ± 2.94	22.61 ± 7.62
cis-9,*trans*-11 CLA	14.75 ± 1.56	24.91 ± 5.24
LIN	18.56 ± 2.91	30.05 ± 6.31
negative control	16.98 ± 1.53	30.87 ± 7.25

[a] Data are expressed as pg/μg of protein and shown as mean concentration $\pm$ SD from five replicates.

Membranes were incubated for 1 h with antibodies against 15-LO-1 (1:2000) or 5-LO (1:250) and β-actin as control (clone AC-74, Sigma). Bound antibody was detected by using appropriate horseradish peroxidase conjugated antibody. Signals were visualized by chemiluminescence (Amersham, Buckinghamshire, U.K.).

Statistical Analysis. All results are expressed as mean $\pm$ standard deviation. As the distribution in most cases deviated from normal (Shapiro−Wilk test), nonparametric tests were used. For related samples significance was first checked with Friedman ANOVA, and then significant results were subjected to the Wilcoxon matched-pair test. The software used was Statistica 6.1 (Statsoft, Poland). $P < 0.05$ was considered to be significant.

RESULTS

Effect of CLAs on 15-LO-1 Activity Measured ex Vivo and in Vitro. The in vitro studies proved that both CLA isomers are not substrates for 15-LO-1 (**Figure 1**).

In ex vivo studies, conducted in cells incubated for 48 h with fatty acids, HODE concentration was significantly reduced ($p = 0.019$). The *trans*-10,*cis*-12 CLA isomer reduced HODE concentration by 28% ($p = 0.046$), and the *cis*-9,*trans*-11 CLA isomer reduced this concentration by 35% ($p = 0.028$) as compared with the BSA control (**Figures 2** and **3**) (sample chromatogram).

The concentration of arachidonic acid derivatives, 12- and 15-HETE, decreased after incubation with fatty acids ($p = 0.050$ for 12-HETE and $p = 0.035$ for 15-HETE) (**Table 1**). A significant fall in 12-HETE concentration (as compared with the BSA control) was noted for the *trans*-10,*cis*-12 CLA isomer ($p = 0.043$) and the *cis*-9,*trans*-11 CLA isomer ($p = 0.043$). A significant reduction in 15-HETE concentration was noted for the *cis*-9,*trans*-11 CLA isomer as compared with BSA ($p = 0.028$) and as compared with the *trans*-10,*cis*-12 CLA isomer ($p = 0.028$) (**Table 1**). A weak tendency ($p = 0.14$) to reduction

in 5-HETE concentration in macrophages incubated with *cis*-9,*trans*-11 CLA was noted (**Table 1**).

Effect of CLAs on 5-LO. To determine whether fatty acids change the activity of 5-LO, an immunoenzymatic measurement of concentration of the enzyme product, LTB_4, was performed. No significant differences were noted between LTB_4 concentrations in macrophages incubated with CLAs in both macrophage types (**Table 2**).

Effect of Fatty Acids on Changes in mRNA Expression and Protein Content in Macrophages Obtained from THP-1. In macrophages obtained from THP-1 fatty acids did not change significantly mRNA expression of the investigated genes (**Figure 4A**). Quantitative measurements of the content of 5-LO and 15-LO-1 protein in macrophages obtained from THP-1 proved that the fatty acids did not have an effect on the content of the investigated proteins (**Figure 4B**,**C**−**E**).

Quantitative measurements of mRNA in macrophages from peripheral blood showed significant changes in expression of 15-LO-1 in the fatty acid environment (**Figure 5A**). Linoleic acid induced 15-LO-1 expression (2.6 times, $p < 0.05$). CLAs did not change the content of 5-LO and 15-LO-1 proteins in macrophages obtained from peripheral blood (**Figure 5B**−**D**).

DISCUSSION

So far, few studies have been published to explain whether conjugated linoleic acid isomers may modify the activity of lipoxygenases (15-LO and 5-LO) in cells. Results showed by Hoffmann indicate that CLAs change availability of substrates (mainly arachidonic acid) for the lipoxygenase pathways generating eicosanoids (*39*), partly by inhibition of the phospholipase A_2 activity (*40*). CLA may also change content of lipoxygenases in the cells. The mixture of CLAs (*cis*-9,*trans*-11 and *trans*-10,*cis*-12, a 50:50 ratio, 150 μM) decreased significantly the levels of mRNA and protein of 5-lipoxygenase. *cis*-9,*trans*-11 CLA at high concentrations (100−150 μM) significantly reduced 5-LO mRNA levels, whereas *trans*-10,*cis*-12 CLA did not show any significant effect on the expression of this enzyme (*41*). In the human breast tumor cells MDA-MB-231, the *trans*-10,*cis*-12 isomer did not affect expression of 5-LO but reduced the 5-lipoxygenase activating protein (FLAP) expression, and both isomers decreased the production of 5-HETE by the competition with arachidonic acid (*42*). Interestingly, in murine mammary tumor cells only the *trans*-10,*cis*-12 isomer reduced the production of the 5-HETE (*23*).

For this time, only one pioneering study by Cho et al. (*43*) described the influence of CLA on the 15-lipoxygenase activity: the *cis*-9,*trans*-11 (but not the *trans*-10,*cis*-12) isomer was found to be a substrate for the enzyme in rat. Our results show that CLAs may act as an inhibitor of lipoxygenase 15-LO-1 activity in macrophages.

15-LO-1 is an enzyme responsible for the progression of atherosclerotic lesions, participating in the oxidation of low-density lipoproteins (LDL) (*11*) and in the synthesis of hydroperoxides that are PPAR-receptor ligands (*4*, *5*). In the normal arterial wall 15-LO-1 is not expressed. However, in atherosclerotic plaque 15-LO-1 has been detected in macrophage-derived foam cells (*4*, *44*). Because macrophages derive from peripheral blood monocytes that do not express the 15-lipoxygenase activity, this enzyme must be induced during monocyte−macrophage differentiation or during foam cell formation (*4*). What is the function of macrophage 15-LO-1 in the atherosclerotic process? There is evidence that 15-LO-1 is directly involved in the oxidation of LDL to strongly atherogenic oxidized LDLs (oxLDLs). Monocytes are attracted by oxLDL,

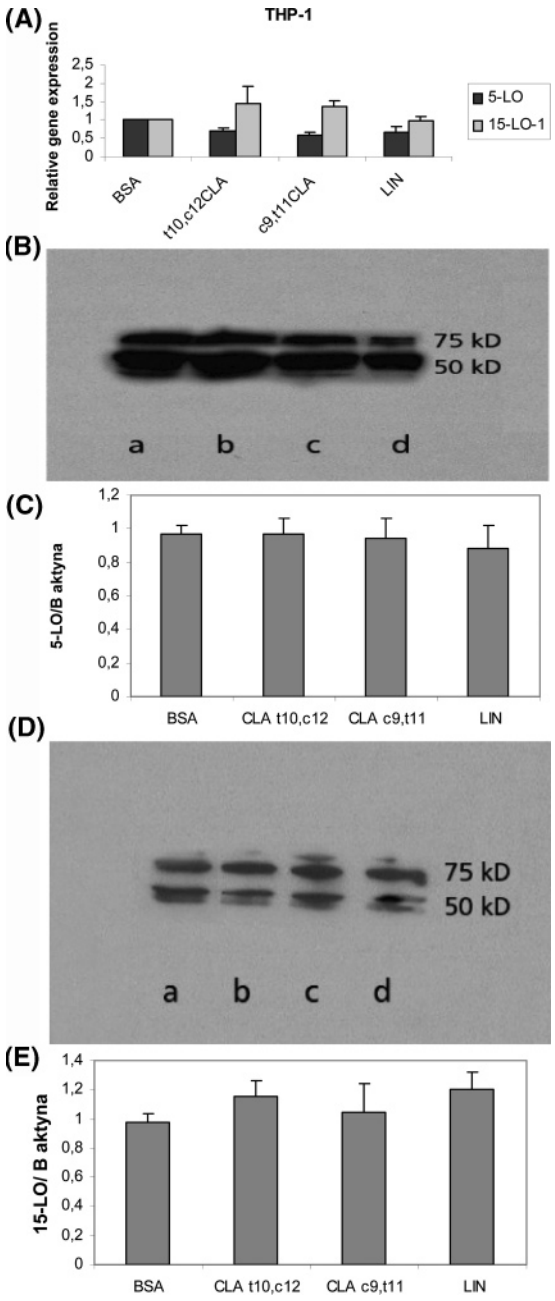

Figure 4. (**A**) Expression of 5-LO and 15-LO-1 mRNA measured by quantitative real-time PCR in THP-1 macrophages. Macrophages were cultured with fatty acids for 48 h. Data are expressed as the relative gene expression ratio. Mean values ± SD of the experiment done in triplicate are shown. (**B, C**) Content of 5-LO protein in THP-1 macrophages cultured with fatty acids. Cell lysates were obtained based on macrophages cultured with fatty acids. Membranes were incubated with antibody against specified antigen as described under Materials and Methods. The figure shows one of three replicates of the experiment with similar results. Mean values ± SD of 5-LO protein to β-actin ratio are presented in the diagram under the photograph. Letters a–d describe protein isolated from macrophages cultured with (a) BSA, (b) *trans*-10,*cis*-12 CLA isomer, (c) *cis*-9,*trans*-11 CLA isomer, or (d) LIN. Analysis of 15-LO-1 protein in THP-1 macrophages cultured with fatty acids Membranes were incubated with antibody against specified antigen as described under Materials and Methods. The figure shows one of three replicates of the experiment with similar results. Mean values ± SD of 15-LO-1 protein to β-actin ratio are presented in the diagram under the photograph. Letters a–d describe protein isolated from macrophages cultured with (a) BSA, (b) *trans*-10,*cis*-12 CLA isomer, (c) *cis*-9,*trans*-11 CLA isomer, or (d) LIN.

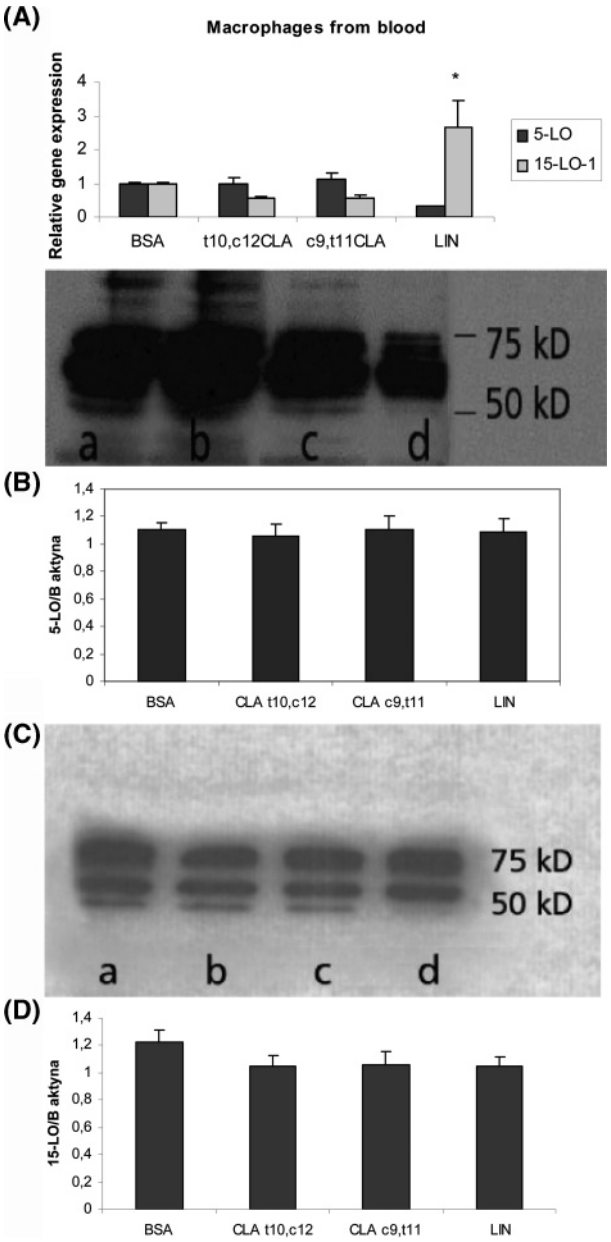

Figure 5. (**A**) Expression of 5-LO and 15-LO-1 mRNA measured by quantitative real-time PCR in macrophages obtained from blood. Macrophages were cultured with fatty acids for 48 h. Data are expressed as the relative gene expression ratio. Mean values ± SD of the experiment done in triplicate are shown. ∗, $p < 0.05$, significantly different from the corresponding control (BSA). (**B**) Content of 5-LO protein in macrophages obtained from blood monocytes cultured with fatty acids. Cell lysates were obtained based on macrophages cultured with fatty acids. Membranes were incubated with antibody against specified antigen as described under Materials and Methods. The figure represents one of three replicates of the experiment with similar results. Mean values ± SD of 5-LO protein to β-actin ratio are presented in the diagram under the photograph. Letters a–d describe protein isolated from macrophages cultured with (a) BSA, (b) *trans*-10,*cis*-12 CLA isomer, (c) *cis*-9,*trans*-11 CLA isomer, and (d) LIN. Analysis of 15-LO-1 protein in macrophages obtained from blood monocytes cultured with fatty acids. Membranes were incubated with antibody against specified antigen as described under Materials and Methods. The figure shows one of three replicates of the experiment with similar results. Mean values ± SD of 15-LO-1 protein to β-actin ratio are presented in the diagram under the photograph. Letters a–d described protein isolated from macrophages cultured with (a) BSA, (b) *trans*-10,*cis*-12 CLA isomer, (c) *cis*-9,*trans*-11 CLA isomer, or (d) LIN.

and they gather in the endothelium (with the participation of MCP-1 protein produced by endothelium and smooth muscle cells) (*43*). In the vascular wall, macrophages created as a result of monocyte differentiation become a source of free radicals synthesized by three enzymes: NADPH oxidase, myeloperoxidase, and 15-lipoxygenase (*3*). The consequence of free radical activity is generation of oxidized LDLs, which are taken up by scavenging receptors on the macrophage surface. Macrophage activation is also a signal for Th1 lymphocytes to release cytokines (e.g., IL-4), activating additionally 15-LO-1 expression (*4*, *16*). 15-LO also participates in the intracellular synthesis of hydroperoxides transferred subsequently to LDL molecules (which makes LDL more susceptible to the oxidation process) (*2*).

In this study potent expression of 15-LO-1 (**Figure 5**) was noted in macrophages from the blood incubated with linoleic acid, which may be explained by the cell reaction to the increasing concentration of substrate for 15-LO-1 in the cell. Increased substrate (LIN) concentration in the medium increases 15-LO-1 expression, whereas CLAs "unused" (not metabolized) by macrophage 15-LO-1 do not display such properties (**Figures 1** and **5**).

The results of studies by Cho et al. suggest that the *cis*-9,*trans*-11 CLA isomer (but not the *trans*-10,*cis*-12 CLA isomer) is the substrate of rat 15-LO-1 originating from the lungs, which oxidizes it to 13-HODE (*43*). In our study none of CLA isomers was the substrate for macrophage 15-LO-1 (**Figure 1**), and both of these isomers were enzyme inhibitors, contributing to the fall in HODE and HETE concentrations in the medium (**Figures 2** and **3**; **Table 1**). It cannot be ruled out that both CLA isomers, structurally close to LIN, inhibit the activity of the enzyme, competing with linoleic acid for the active site of the enzyme. A similar phenomenon was observed in prostate cancer cells, where fatty acids of the n-3 family inhibited proliferation of cancer cells, competing with n-6 acids for enzymatic pathways (15-LO-1 and COX-2) (*45*).

In these studies, no significant differences were found in 5-HETE (**Table 1**) and LTB_4 (**Table 2**) synthesis depending on the acid used for the culture. Furthermore, both CLA and LIN isomers had no effect on 5-LO expression in macrophages (**Figures 4** and **5**). Overexpression of mRNA and 5-LO protein was previously observed in prostate cancer cells cultivated in the environment of the *cis*-9,*trans*-11 CLA isomer (*41*). Furthermore, the effect of fatty acids on the activity of both lipoxygenases (15-LO-1 and 5-LO) was described in the literature. The studies were performed in neutrophils isolated from blood, to which two fatty acids of the n-6 family [linoleic and dihomo-γ-linolenic (DGLA)] were added along with ionophore A 23187. Decreased synthesis of LTB_4 (5-LO product) was noted in the environment of both fatty acids: inhibition constant was 45 μmol/L for LIN and 40 μmol/L for DGLA. The inhibition of LTB_4 synthesis was associated with increased synthesis of 15-LO-1 products of both fatty acids [13-HODE, a linoleic acid product, and 15-hydroxyeicosatrienoic acid (15-HETrE), a DGLA product]. Both 13-HODE and 15-HETrE led to non-dose-dependent inhibition of LTB_4 synthesis. A similar effect was exerted also by the 15-LO product originating from ARA: 15-HETE (*46*). Nevertheless, such a relationship was not observed in our studies.

ABBREVIATIONS USED

ARA, arachidonic acid; BSA, bovine serum albumin; CLA, conjugated linoleic acid; FBS, fetal bovine serum; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic

acid; LIN, linoleic acid; 15-LO-1, 15-lipoxygenase isoform 1; 5-LO, 5-lipoxygenase; LTB_4, leukotriene B_4.

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