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Inhibition of phospholipase A_2 activity by conjugated linoleic acids in human macrophages

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Abstract The objective of this study was to assess the effect of conjugated linoleic acid isomers (CLAs) on the expression and activity of phospholipases A_2 (PLA_2) in human macrophages.

Macrophages were incubated with 30 μM *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLAs for 48 h. After incubation, the total activity of phospholipases as well as the expression of mRNA for cytosolic ($cPLA_2$) and secretory ($sPLA_2$) phospholipases and activity of $sPLA_2$ were measured.

Both CLA isomers reduced the total activity of PLA_2 (by 30.2%, $P < 0.01$ for *cis*-9, *trans*-11 CLA and by 30%, $P < 0.001$ for *trans*-10, *cis*-12 CLA). *Trans*-10, *cis*-12 CLA isomer downregulated the expression of mRNA of $sPLA_2$ and decreased the enzymatic activity of this enzyme (by 23%, $P = 0.02$) in macrophages.

Conjugated linoleic acid isomers can significantly reduce the activity of PLA_2 in macrophages and downregulate $sPLA_2$ expression. The consequence of this effect may be reduction of releasing the arachidonic acid (AA) from the cellular membranes of macrophages.

Key words phospholipase A_2 – $cPLA_2$ – $sPLA_2$ – conjugated linoleic acid isomers – arachidonic acid – macrophages – inflammation – atherosclerosis

Introduction

Phospholipases A_2 (PLA_2) represent a diverse group of enzymes that catalyze the hydrolysis of ester bonds at the *sn-2* position of membrane phospholipids and release fatty acids (e.g., arachidonic or linoleic) and lysophospholipids [1, 2]. According to an updated classification, phospholipases can be subdivided into several groups based upon their structures, localization, and enzymatic characteristics [3]. Mammalian cells contain diverse forms of PLA_2 including cytosolic ($cPLA_2$) and secretory phospholipases ($sPLA_2$). Cytosolic PLA_2 is highly specific for arachidonic acid (AA) and participates in the release of this fatty acid in various cell types [4]. Secretory PLA_2 is assumed to release AA and other unsaturated fatty acids [1].

Secretory phospholipases are associated with the development of the atherosclerotic process. Expression of the $sPLA_2$ isozymes increases dramatically during inflammation [4, 5] and very high levels of them are found in human atherosclerotic, macrophage-rich arterial walls [6].

Both classes of phospholipases participate in the regulation of physiological and pathological processes in the cell, including the release of pro-inflammatory mediators and stimulation of inflammatory processes [2].

Few reports are available on the effect of fatty acids on the regulation of activity of macrophage phos-

pholipases. So far, the effects of arachidonic, eicosapentaenoic and oleic (C18:1) acids on $sPLA_2$ expression in guinea pig macrophages have been extensively investigated [4, 7, 8]. These investigations showed that both polyunsaturated and monounsaturated fatty acids downregulate enzyme expression by inhibiting the transcription of the $sPLA_2$ gene [7].

Conjugated linoleic acids (CLAs) is a term used to describe positional and geometric isomers of linoleic acid with the presence of conjugated double bonds. CLAs are formed especially during the biohydrogenation in the rumen, thus the major sources of CLAs are ruminant meat and milk products [9]. Commercial sources of CLA predominantly contain *cis*-9, *trans*-11 (~40%) and *trans*-10, *cis*-12 (~40%) CLA isomers [9, 10]. In rats, CLAs display anti-carcinogenic, anti-atherosclerotic and anti-diabetic properties [11]. In murine macrophages, CLAs inhibit inflammatory processes [11]. In human dietary supplementation of the *trans*-10, *cis*-12 CLA isomer contributed to an increase in the oxidation stress (measured by the quantity of 8-iso-$PGF_{2\alpha}$ in urine) [12]. These phenomena are associated, according to the authors, with the intensification of lipid peroxidation induced by the CLA isomer [12].

Numerous studies have demonstrated the implication of both phospholipases in the release of AA, which can be converted into eicosanoids, potent inflammatory mediators. In this study, we investigated the regulation of PLA_2 expression and activity by CLAs in human THP-1 macrophages.

Materials and methods

Cell culture media and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Grand Island, USA) and Sigma (Sigma-Aldrich, Poznan, Poland). Fatty acids-free albumin (BSA); ionophore A 23187, solvents for thin-layer chromatography (TLC) (petroleum ether, diethylether, acetic acid) and trypan blue were purchased from Sigma (Sigma-Aldrich, Poland). Conjugated linoleic acids (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA) (98% purity) were from Nu-CheK Prep (Nu-CheK Prep, Elysian,USA). High-purity standards, solvents, and reagents for gas chromatography (GC) were obtained from Sigma or Fluka (Sigma-Aldrich, Poznan, Poland). Silica gel plates were from Merck (Germany). dATP, dTTP, dCTP, dGTP were purchased from Promega (Promega, Madison, USA); random hexamers, reverse transcriptase, PCR buffer, *Taq* polymerase were from Boehringer (Mannheim, Ingelhaim, Germany). Kits for measurement of $sPLA_2$ activity were from R&D (R&D Systems, Minneapolis, UK).

Cell culture and treatment

THP-1 were purchased from American Type Culture Collection (ATCC, Rockville, USA) and cultured in RPMI 1640 medium supplemented with 10% fatty acid free FBS, penicillin (100 U/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 6 well plates, then were differentiated to macrophages by administration of 100 nM phorbol myristate acetate (PMA) for 24 h [13]. 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 [14]. Fatty acids were added as 4 mM stock solution dissolved in 1 mM fatty acid free bovine serum albumin (BSA) as described in details [15–17]. 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 PBS, and then 10 μl cells suspension was combined with 90 μl of trypan blue and viewed on a hemocytometer. The percent of the living cells which excluded trypan blue was used to determine cell viability [18]. Cell cultures with viability more than 97% were used for experiments.

Phospholipase A2 activity measurement

The influence of CLAs on PLA_2 activity was determined with the use of gas chromatography. Macrophages were incubated with 30 μM fatty acids for 48 h. After that 5 μM (final concentration) ionophore (A 23187) was added to the cells, which were then incubated for 1 h at 37°C with gentle agitation [19]. Ionophore A 23187 stimulates PLA_2 to hydrolysis of 300–400 ng of AA per 1.5×10^7 monocytes over 15 min [19]. Next, the cells were collected (with the medium) and used for the extraction of total lipids with the Folch mixture 2:1 (v/v) chloroform/methanol containing 0.01% (w/v) butylated hydroxytoluene as antioxidant [20]. Care was taken to minimize exposure of samples to air. The extract obtained was evaporated to dryness under nitrogen flow and was then suspended in 150 μl of Folch mixture. A total of 100 μl portions of the extract were applied to thin-layer chromatography plates. The plates were developed with petroleum ether/diethylether/acetic acid mixture 90/10/1 (v/v/v) [21]. The fraction of free fatty acids obtained as a result of chromatographic separation was scraped off from the plate, then methylated with 20% (w/v) boron trifluoride–methanol, and extracted using hexane. A Perkin–Elmer gas chromatograph (model 8500) equipped with a flame ionization

detector FID, PTV injector (in splitless mode), and 60 m capillary column SP™ 2340 (Sigma-Aldrich) was used to analyze AA methyl ester. The oven temperature raised from 180 to 200°C at 2°C/min, and was held at 200°C for 40 min. AA was identified by comparison of its retention times with pure standard and an electronic integrator was used to measure peak areas (Chromed, Medson, Poland). AA content in cells was divided by the number of cells in the well and expressed as ng/3 × 10^6 cells.

Semiquantitative reverse transcription—rt PCR

Total RNA was extracted from cell cultures using the Qiagen kit (Gibco, UK). Total RNA concentrations were calculated after spectrophotometric measurements at 260 nm wavelength. RNA (500 ng) was dissolved in a reaction mixture containing 2.5 mM of dNTP, 20 U of RNAsin, 100 pM of random hexamers, and 20 U of MMLV Reverse Transcriptase. Incubation was carried out at 37°C for 60 min, then the reaction temperature was raised to 94°C for 5 min to inactivate the enzyme, and finally decreased to 4°C An aliquot of cDNA was dissolved in a reaction mixture containing 10× PCR buffer, 2.5 mM of dATP, dTTP, dCTP, dGTP, 10 pM of up- and down-stream primers ($cPLA_2$, $sPLA_2$, GAPDH) and 1 U *Taq* polymerase [22]. For semi-quantitative analysis, the linearity of amplification of $cPLA_2$, $sPLA_2$, and GAPDH cDNAs depending on PCR cycle number was established in preliminary experiments. About 25 cycles for $cPLA_2$, 35 for $sPLA_2$, and 24 for GAPDH, resulted to be the best amplification profile to recognize differences among the samples. The following sets of primers were used in PCR amplification: **GAPDH:** sense 5′-GAG TCA ACG GAT TTG GTC GT-3, antisense 5′-GTT GTC ATG GAT GAC CTT GG-3, **$cPLA_2$ (PLA2G4A):** sense 5′-TCC CCT TTT ACT TCT CAC ACC ACA, antisense 5′-GAA CCA GAA ACG CCC AAA ACT C, **$sPLA_2$ (PLA2G2A):** sense 5′-GAA GTT GAG ACC ACC CAG CA, antisense 5′-GTT GCA TCC TTG GGG GAT CCT CTG. Amplification products obtained in PCR were electrophoretically separated on 3% agarose gel. Images of ethidium bromide-stained bands for $cPLA_2$, $sPLA_2$, and GAPDH were photographed using DS-34 Polaroid camera. Band intensity was measured densitometrically. $cPLA_2$ and $sPLA_2$ signals were normalized to cDNA levels of the housekeeping gene (GAPDH) and expressed as a ratio.

Measurement of macrophage $sPLA_2$ activity

The THP-1 derived macrophages were washed three times with phosphate-buffered saline (PBS) and incubated with fatty acids for 48 h at 37°C. After incubation macrophages were harvested and the activity of $sPLA_2$ was measured by a spectrophotometric method for determination of $sPLA_2$ activity in biological fluids. In this assay, a specific substrate for $sPLA_2$ 2-hexadecanoylthio-1-ethylphosphorylcholine (HEPC) that is converted into sulfhydryl molecule was used. The presence of this sulfhydryl product was detected colorimetrically using reagents which form a yellow product with the sulfhydryl formed. The amount of $sPLA_2$ in the sample was compared to the amount of $sPLA_2$ in the standards by comparison of the yellow color generated.

The results obtained were expressed as enzyme activity per 3 × 10^6 cells.

Statistical analysis

As the distribution of variables in most cases deviated from normal (Shapiro–Wilk test), non-parametric tests were used. For related samples significance was first checked with Friedman's ANOVA, then significant results were subjected to the Wilcoxon matched-pair test. $P < 0.05$ was considered statistically significant. For each experiment, a minimum of three independent measurements were performed and the results are reported as mean ± SD. The software used was Statistica 6.1, Statsoft, Poland.

Results

CLAs reduce PLA_2 activity

The incubation of macrophages with *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers contributed to reduction of PLA_2 activity. Addition of *cis*-9, *trans*-11 CLA to the cells led to a 30.2% decrease of AA concentration in free fatty acid fraction compared to the control (cells incubated with BSA) ($P < 0.01$, Wilcoxon matched-pair test, $n = 5$). For *trans*-10, *cis*-12 CLA the decrease was 30% ($P < 0.001$, Wilcoxon matched-pair test, $n = 5$)—Fig. 1.

The *trans*-10, *cis*-12 CLA isomer reduced the expression of $sPLA_2$

In macrophages incubated with *trans*-10, *cis*-12 CLA a tendency to a slight reduction (26%) of expression of mRNA for $sPLA_2$ was observed. In the cells incubated with *cis*-9, *trans*-11 CLA isomer the level of expression of mRNA for $sPLA_2$ did not change ($n = 3$)—(Fig. 2).

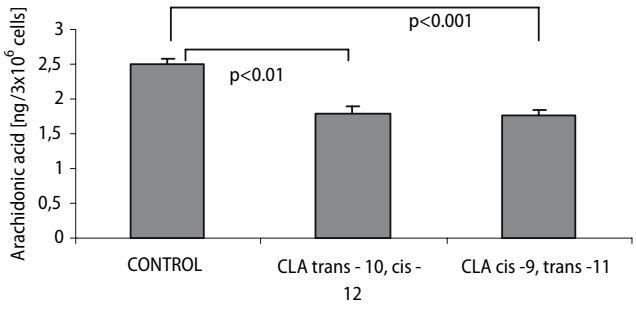

Fig. 1 Conjugated linoleic acids inhibit the activity of macrophage PLA_2. Procedure was described in Material and Methods. Data are expressed as mean ± SD from five experiments. $P < 0.01$—Friedman's ANOVA test. Comparison between control cells and cells incubated with CLAs using Wilcoxon matched-pair test

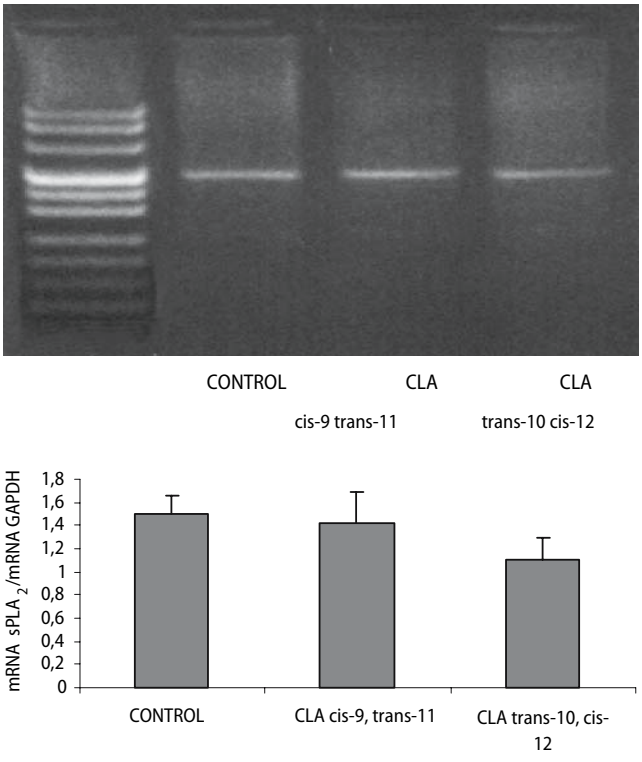

Fig. 2 The *trans*-10, *cis*-12 CLA isomer reduces the expression of mRNA for $sPLA_2$ in macrophages incubated for 48 h with CLAs. Data are expressed as $sPLA_2$/GAPDH and are given as the mean ± SD from three experiments

The expression of mRNA of $cPLA_2$, that is constitutive enzyme, in macrophages incubated with CLAs did not change ($n = 3$)—Fig. 3.

The *trans*-10, *cis*-12 CLA isomer reduces macrophage activity of $sPLA_2$

The culture of macrophages with the *trans*-10, *cis*-12 CLA isomer contributed to 23% reduction of $sPLA_2$ activity ($P = 0.027$, Wilcoxon matched-pair test, $n = 6$) as compared with the control (cells incubated with BSA). The *cis*-9, *trans*-11 CLA isomer did not change the activity of $sPLA_2$ (8% reduction, P = ns, Wilcoxon matched-pair test, $n = 6$)—Fig. 4.

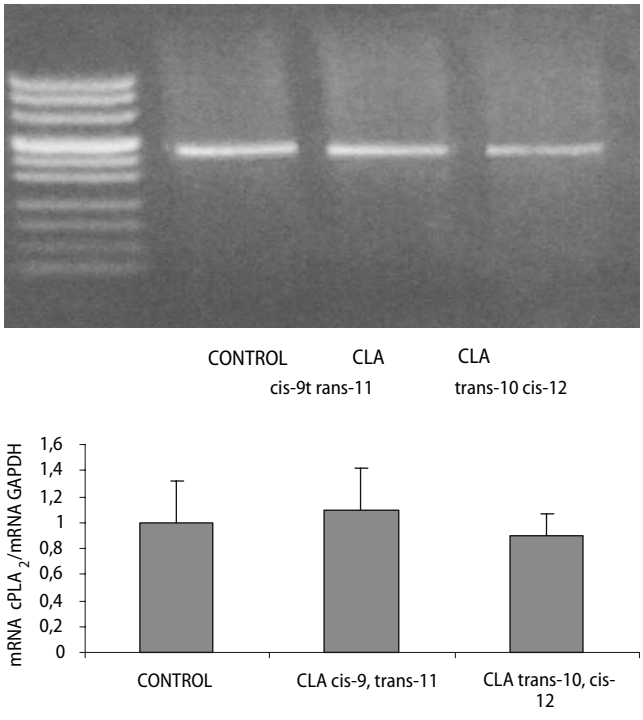

Fig. 3 The expression of mRNA for $cPLA_2$ in macrophages incubated for 48 h with CLAs. Data are expressed as $cPLA_2$/GAPDH and are given as the mean ± SD from three experiments

Discussion

It is still unknown, whether CLAs affect inflammation in human in macrophages. Diverse PLA_2 enzymes

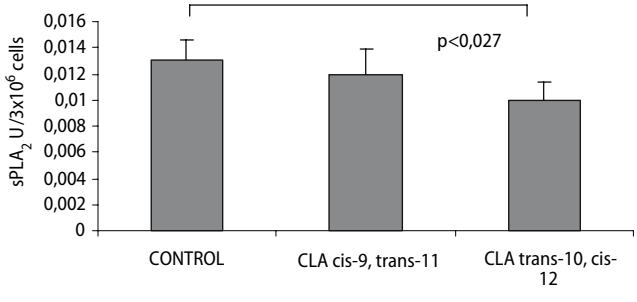

Fig. 4 CLA isomers reduce the activity of $sPLA_2$ in macrophages. The activity of $sPLA_2$ was determined by a spectrophotometric method. A statistically significant difference in $sPLA_2$ activity was noted between the control and the *trans*-10, *cis*-12 isomer; ($P = 0.027$, Wilcoxon's matched-pairs rank test). Data are expressed as mean ± SD from six experiments

hydrolyze the phospholipids releasing fatty acids, mainly AA. Its further metabolism gives rise to several types of bioactive lipids known as eicosanoids. Many of them mediate inflammation [23]. Several studies showed that CLAs reduce the formation of eicosanoids in various animal cells [24, 25]. It was shown that treating endothelial cells with high concentrations (50 μM) of both CLA isomers, reduced the release of various eicosanoids [26]. Less information concerning the effects of CLAs on the phospholipase A_2 activity is available [26–28]. The results of Eder's study suggest that CLAs reduce availability of AA in endothelial cells due to reduced activity of $sPLA_2$, which results in diminished formation of eicosanoids [26]. $sPLA_2$ is enzyme widely distributed in variety of mammalian tissues, and its concentration increases in many inflammatory processes [6]. In atherosclerotically changed vascular walls (rich in macrophage infiltrations), an increased expression of $sPLA_2$ is observed [6].

The results of our study suggest that CLA may change activity of phospholipases also in macrophage cells. We observed that both CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) inhibited the total activity of PLA_2 (measured as AA concentration) (Fig. 1) without the change in the expression mRNA of $cPLA_2$ (Fig. 3). Additionally *trans*-10, *cis*-12 CLA (not *cis*-9, *trans*-11) isomer reduced activity and mRNA expression of $sPLA_2$ in macrophages (Fig. 2). In endothelial cells both isomers of CLA at 50 μM reduced gene expression of the $sPLA_2$ [26], but in other studies (e.g. 29) *trans*-10, *cis*-12 CLA was more bioactive than *cis*-9, *trans*-11 CLA isomer. This isomer reduced expression and activity of many enzymes e.g. COX-2, stearoyl-CoA desaturase or LPL lipase [26, 29].

In the physiological conditions $cPLA_2$ is required for the regulation of $sPLA_2$ activity and the process involves the metabolites of 12/15-lipoxygenase-1 (12/15 LOX) [1]. These findings suggest that AA derivatives may play a role in the regulation of $sPLA_2$ expression in cell. The hypothetical link might involve the limitation of availability of AA and linoleic acids for oxygenases and decreased synthesis of their derivatives. Similar phenomena were observed in murine fibroblasts, where inhibitors of 12/15 LOX caused suppression of $sPLA_2$ induction [3, 30]. Thus, CLA may indirectly inhibit $sPLA_2$ induction, but the details of the mechanism remain to be determined.

Conclusions:

- The *cis*-9 *trans*-11 and *trans*-10, *cis*-12 CLA isomers reduce the activity of PLA_2
- The *trans*-10, *cis*-12 isomer diminishes the expression of $sPLA_2$ mRNA.

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