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PROSTAGLANDIN E_2 (PGE_2) AND THROMBOXANE A_2 (TXA_2) SYNTHESIS IS REGULATED BY CONJUGATED LINOLEIC ACIDS (CLA) IN HUMAN MACROPHAGES

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Conjugated linoleic acid (CLAs) are positional and geometric isomers of linoleic acid with have a potential anti-atherosclerotic and anti-inflammation properties. Metabolites of arachidonic acid - prostaglandins and thromboxans are endogenous mediators of inflammation. Prostaglandin E_2 and thromboxan A_2 which are a products of two izoformes of cyclooxygenases (COX-1 and COX-2) in macrophages, play an important role in this process. COX - 1 is a constitutive enzyme, whereas the COX - 2 is inducible and its amount in the cell rapidly increases during inflammation (e.g. *via* NF κB pathway). The aim of the study was to test the effect of CLAs on cyclooxygenases (COX-1 and COX-2) activity, their mRNA expression and protein content in macrophages. Additionally the active form of the κB (NF κB) transcription factor was measured. For the experiments monocytes from monocytic cell line (THP-1) and from human venous blood were used. Monocytes were differentiated to macrophages and cultured with 30 μM CLAs or linoleic acid for 48 h. The COX-1 and COX-2 products - PGE_2 and TXB_2, were measured by ELISA method. The enzymes (COX-s) activity were estimated by spectroscopic method. mRNA expression and protein analysis were analysed by real-time PCR and Western blot technique. In macrophages cultured with CLAs reduction of TXB_2 and PGE_2 concentration was observed. Significant change in COX-2 expression in cells cultured with *trans*-10, *cis*-12 CLA (in macrophages obtained from peripheral blood) was observed. COX-1 inhibition was resulting from competition of CLA and linoleic acid with arachidonic acid.

Keywords: *cyclooxygenase-1, cyclooxygenase-2, conjugated linoleic acid, macrophages/monocytes, PGE-2, eicosanoids, TXA_2, THP-1*

INTRODUCTION

Conjugated linoleic acid (CLAs), are produced naturally by bacterial hydrogenation and isomerization in the gut of ruminant animals, or they can be generated chemically by isomerization of linoleic acid (LA). In the human diet, CLAs are consumed in milk fat and in meats derived from ruminant animals (1). Data from a number of studies and trials have shown that different conjugated linoleic acids may produce beneficial effects on cancer, hypertension, diabetes and atherosclerosis (2).

Atherosclerosis is assumed as a complex endothelial dysfunction induced by elevated and modified low-density lipoproteins (LDL) and other factors (*eg*. free radicals, infectious microorganisms, shear stress, hypertension) that lead to a compensatory inflammatory response (3). Primordial event in atherogenesis is a recruitment of monocytes from the peripheral blood to the intima of the vessel wall. Through the expression of scavenging receptors monocytes (when differentiating into macrophages) acquire the ability to recognise and remove from the circulation oxidatively transformed low-density lipoproteins (mm-LDL, ox-LDL) (4). The activation of monocytes/macrophages is rapidly followed by the generation of eicosanoids (prostanoids and leukotrienes) (4). Prostanoids are cyclooxygenase-dependent products of arachidonic acid (*n*-6, 20:3 and 20:4) metabolism, comprising prostaglandin (PGD_2, PGE_2, PGF_2), prostacyclin (PGI_2) and tromboxanes (TxA_2) (4). Prostanoids exert a variety of actions in various tissues and cells. The most typical actions are the relaxation and contraction of various types of smooth muscles, but prostanoids are involved in vascular homeostasis and hemostasis (5). The prostanoids and thromboxane generation depends on two distinct enzymes with cyclooxygenase activity, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), respectively (4). COX-1 is constitutively expressed and is responsible for the biosynthesis of prostaglandins involved in vascular homeostasis (6) whereas COX-2 may be induced and expressed in a sustained manner during severe inflammatory reactions (4). COX-2 is induced in response to growth factors, cytokines, and phorbol esters, suggesting that this enzyme is involved in the generation of prostaglandins in inflammatory diseases (6). COX-2 is the principal enzyme providing a mechanism for the generation of proinflammatory prostanoids (4). Both isoforms of cyclooxygenases are present in macrophages (7, 8). Induction (and activation) of COX-2 significantly increases during of monocyte differentiation (9, 10). A number of factors participating in this process eg. AP2, STAT-1, STAT-3 (10). Nuclear transcription factor (NF κB) is one of the main regulators of the inflammatory process, that participates in the activation of about 160 genes playing a key role in the atherosclerotic process (11-12). Prostaglandins and fatty acids were shown to be able to regulate gene expression through NF κB activation (12).

The objective of this study is to explain whether cyclooxygenases (particular COX-1) activity and expression may be regulated by CLA in human macrophages and in this way may change the eicosanods biosynthesis.

MATERIAL AND METHOD

Cell culture and treatment

THP-1 cells were cultured as described in details (13). After incubation with PMA adherent cells were incubated with 30 μM fatty acids or with 30 μM BSA (control) for 48 hr at 37°C. Incubation time and fatty acids concentration were selected on the basis of results obtained in preliminary experiments. The cells were harvested by trypsinization and pellet was obtained by centrifugation (250 g for 5 min). The percent of the living cells was determined by trypan blue. Cell cultures with viability more than 97 % were used for experiments.

Monocytes were isolated from blood of healthy donors in accordance with the principles outlined in the Declaration of Helsinki (Cardiovascular Research 1997; 35:2-3). Peripheral blood mononuclear cells (PBMCs) were isolated using by Lymphozyten separations media as described (14). Cells were cultured at 37°C in 5 % CO_2 in RPMI medium containing 2 mM glutamine, antibiotics and 10% autologous human serum for 7 days (14). After 7 days incubation the percentage of CD 68 cells was assessed by flow-cytometry (FACScan) using CellQuest software as previously described (15) and the fatty acids 30 μM were added for 48h. Cells (macrophages after differentiation) cultured without fatty acids in medium were used as negative control.

In vivo measurements of concentration of cyclooxygenase products: COX-1 and COX-2

Cyclooxygenase activity was measured *in vivo* by quantitative measurement of cyclooxygenase products: prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) (16). The cells were incubated for 48 hours with fatty acids, as described above. PGE_2 or TXB_2 were extracted from the cells with the use of Bakerbond columns, as described. The immunoenzymatic sets of R&D Systems were used for measurements of PGE_2 and TXB_2, in accordance with the manufacturer's instructions.

Measurement of cyclooxygenase activity on the basis of reference enzymes COX-1 and COX-2

Cyclooxygenase activity was measured by using the method proposed by Gierse (17, 18). The selected CLA or linoleic acid (LA) was added to the reference

COX-1 or COX-2 enzyme at a concentration of 20 or 40 µmol/L. Then they were incubated in the Tris/HCl buffer (pH 8.0, 37°C) for 1, 5 or 30 minutes. Then the 0.11 mol/L haematin solution (dissolved in the Tris/HCl buffer, pH 8.0) and the mixture of N,N,N',N'-tetramethyl-p-phenyldiamine (TMPD) and arachidonic acid (with a final concentration of 85 µmol/L or 50 µmol/L) were added. The initial reaction rate was determined by extinction measurement at a wavelength of 590 nm for 30 seconds. Measurements were performed with the use of the Lambda 40 (Perkin Elmer) spectrophotometer equipped with the PTP-1 Peltier System.

The values of initial reaction rates were compared with the initial rate of reaction of the reference enzyme COX-1 or COX-2 to which 0.11 mol/L of Tris/HCl buffer at pH 8.0 was added and incubated for 1 and 5 (or 30 for COX-2) minutes at 37°C (blind sample).

Quantity measurement of the active form of the κB (NF κB) transcription factor

NF κB activation was measured in the nuclear extract with the use of the immunoenzymatic set measuring the activity of the p65/RelA subunit of the κB factor (NF κB p65/RelA Transcription Factor Assay kit, Active-Motif, Belgium).

PCR reaction with the analysis of real-time product quantity increase (Real-Time PCR)

In order to confirm the regulation of the enzymes 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 details (19). Subsequently, c DNA 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:

COX-1-forward primer: 5'-CAGTGGCTCGTATCCCAAAT -3'; reverse primer: 5'-AGGCACAGATTCAGGGAATG -3'; COX-2-forward primer: 5'-CAGCACTTCACGCATCAGTT -3'; reverse primer: 5'-CGCAGTTTACGCTGTCTAGC -3' GAPDH forward primer: 5'-GCCAGCCGAGCCACATC-3'; reverse primer: 5'-GCGCCAATACGACCAAA-3'.

All real-time PCR reactions were performed on the DNA Engine Option II (MJ Research). 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. Following 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 cDNA template was run with every assay to ensure overall specifity. The expression rates were calculated as described (19-20).

Analysis of the content of other proteins with the use of the Western-blot method

The procedure was consistent with the generally applied methodologies (21). The cell pellet was freeze-dried for 10 mins on ice, with the use of a buffer containing protease and phosphatase inhibitors. In the lysates examined, protein concentration was assessed and the lysates were mixed (with Laemmli Sample Buffer containing b-mercaptoethanol) and incubated in a dry bath at 70°C for 10 mins. Such quantity of coloured lysate was added to well to make them contain 10 µg of protein each. Electrophoresis was conducted under a stable voltage of 150 V for about 1.5 hours. Then transfer to PVDF membrane was performed for 1 hour, at a stable voltage of 100V.

Membranes were incubated for 1 h with antibodies direct against COX-1 (1:500), COX-2 (1:1000) or with a monoclonal anti-β actin (clone AC-74, Sigma). Bound antibody was detected by using appropriate horseradish peroxidase conjugated antibody. Signals were visualized by chemiluminescence (Amersham, Buckinghamshire, UK).

Statistical analysis

All results are expressed as mean ± standard error. As 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 ANOVA, then significant results were subjected to the Wilcoxon matched-pair test (22). The software used was Statistica 6.1, Statsoft, Poland. $p<0.05$ was considered significant.

RESULTS

CLA effect on activity of macrophage cyclooxygenase (COX-1 and COX-2) and of reference COX-1 and COX-2

CLAs and linoleic acid were limited TXB_2 synthesis depending on the cultivation conditions ($p \le 0.004$ for macrophages with THP-1 and $p \le 0.002$ for macrophages originating from the blood) (*Table1)*.

63% reduction of TXB_2 concentration for the *trans*-10, *cis*-12 CLA isomer was observed in THP-1 macrophages (compared with BSA) ($p \leq 0.01$), whereas 52% for *cis*-9, *trans*-11 CLA ($p \leq 0.01$) and 53% for linoleic acid ($p \leq 0.05$) - *Table1*. In macrophages obtained from the peripheral blood, near 55% reduction of TXB_2 was noticed for both CLA isomers ($p \leq 0.01$) and 45% for linoleic acid ($p \leq 0.05$) (compared with the BSA). Differences in TXB_2 concentration (in blood macrophages) were also noted between both CLA isomers ($p \leq 0.05$) - *Table 1.*

The activity of reference COX-1 incubated with fatty acids was reduced in proportion to the incubation time and concentration of fatty acids. Prolongation of incubation time (from 1 min to 5 mins) and elevation of fatty acid concentration to 40 μmol/L enhanced inhibition of COX-1 (p = ns) - *Fig.1*.

In order to determine whether fatty acids may affect COX-2 activity, concentration of the main product of this enzyme - prostaglandin E_2 (PGE_2) was performed. Additionally, activity of the reference COX-2 was analysed. Throughout application of the selective COX-2 inhibitor - NS 386, the main enzyme's source of PGE_2 in the macrophages was determined - as COX-2 (addition of NS 386 caused a reduction of PGE_2 concentration by 90% - data not shown).

Incubation of cells with fatty acids led to reduction of PGE_2 concentration (dependent on the fatty acid $p \leq 0.002$). Concentration of PGE_2 was reduced by 15% in THP-1 macrophages incubated with the *trans*-10, *cis*-12 CLA ($p \leq 0.05$), and by 20% for the *cis*-9, *trans*-11 CLA isomer ($p \leq 0.05$) - *Table 2*. Differences between both CLA isomers ($p \leq 0.01$) and between the *trans*-10, *cis*-12 CLA

Table.1. Effect of fatty acids on thromboxane A_2 (determined as TXB_2) concentration measured in macrophages by immunoezymatic method.

Treatment	**Concentration of TXB_2 (pg/μg protein)** **$p \leq 0.004$ (ANOVA Friedman, n=5)** **Macrophages from THP-1**	**Concentration of TXB_2 (pg/μg protein)** **$p \leq 0.002$ (ANOVA Friedman, n=5)** **Macrophages from blood**
BSA	4.22 ± 0.15	6.31 ± 0.89
Trans-10, cis-12 CLA	1.58 ± 0.17 **	2.83 ± 0.62 **
Cis-9, trans -11 CLA	2.04 ± 1.18 **	3.06 ± 0.24 **,$^{\#}$
Linoleic acid	1.99 ± 0.16 *	3.50 ± 0 .31*
Negative control	4.00 ± 0.24	6.14 ± 1.08

Data are expressed as pg/μg protein and shown as mean concentration ± SD from five replicates.
* p < 0,04, ** p < 0,01 – compared to BSA, the Wilcoxon matched-pair test
\# p < 0,05 – compared to trans-10, cis-12 CLA, the Wilcoxon matched-pair test

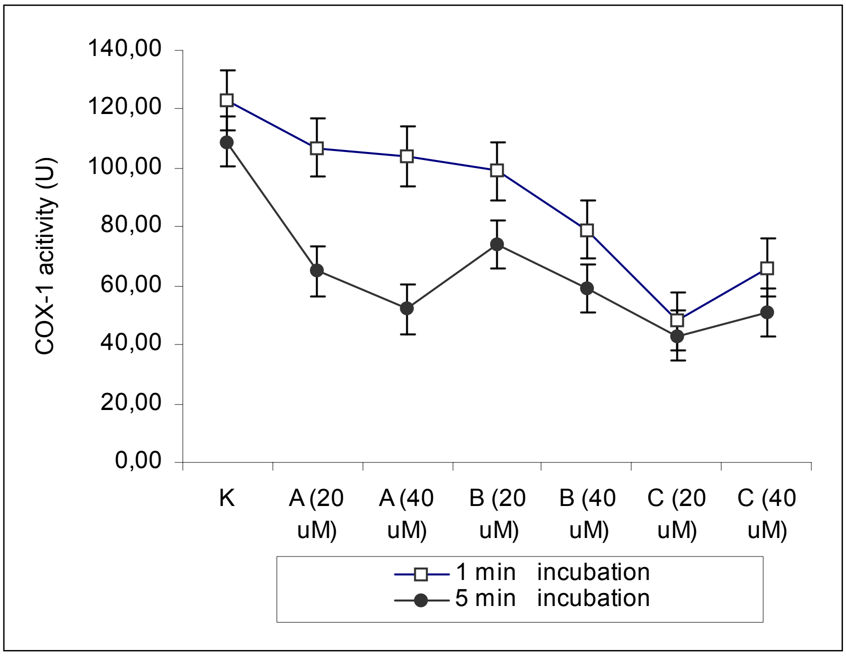

Fig. 1. Effect of CLA on the reference COX-1 activity (reference enzyme was incubated for 1 or 5 min) in assay buffer that contained fatty acids in the concentration of 20 or 40 μmol/L. Activity of COX-1 was measured with the spectrophotometric method. Data were expressed in U/μg protein and showed as mean activity ± SD from three separate experiments). P = ns. (between curves)
K – activity of enzyme measured in 0,11 mol/L buffor Tris/HCl, pH 8,0; A – activity of enzyme measured in trans-10, cis-12 CLA isomer containing buffer; B – activity of enzyme measured in cis-9, trans- 11 CLA isomer containing buffer; C – activity of enzyme measured in linoleic acid containing buffer.

Table.2. Effect of fatty acids on prostaglandin E_2 (PGE_2) concentration measured in macrophages by immunoezymatic method.

Treatment	**Concentration of PGE_2 (pg/µg protein). p<0.002 (ANOVA Friedman, n=5)** **Macrophages from THP-1**	**Concentration of PGE_2 (pg/µg protein). p<0.0002 (ANOVA Friedman, n=10)** **Macrophages from blood**
BSA	17.70 ± 1.26	41.74 ± 6.14
Trans-10, cis-12 CLA	14.98 ± 0.46 *	25.37 ± 3.25 ***
Cis-9, trans -11 CLA	14.02 ± 0.45 $^{*,\#}$	28.60 ± 4.08 **
Linoleic acid	17.98 ± 0.27 $^{\&}$	32.20 ± 5.66 $^{**,\&}$
Negative control	17. 02 ± 2.65	40.87 ± 5.67

Data are expressed as pg/µg protein and shown as mean concentration ± SD from five or ten replicates.
*p < 0,05, **p < 0,01, ***p < 0,005 – compared to BSA, the Wilcoxon matched-pair test
$^{\#}$p < 0,01, $^{\&}$p < 0,01 –compared to trans-10, cis-12 CLA, the Wilcoxon matched-pair test

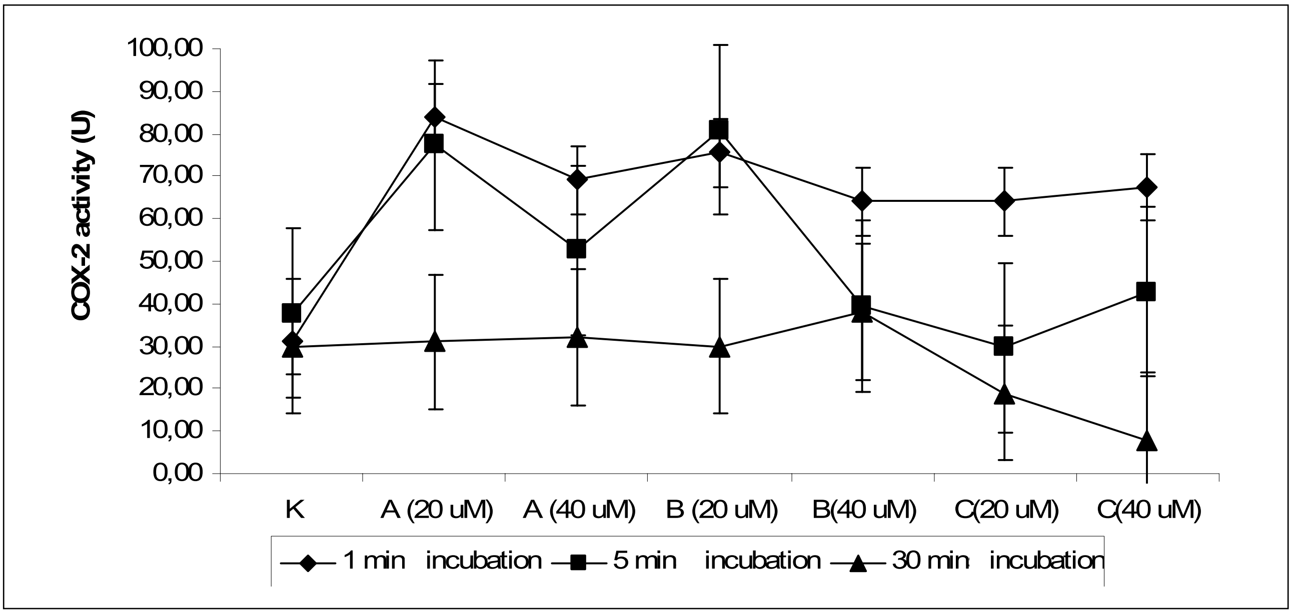

Fig. 2. Effect of CLA on the reference COX-2 activity (reference enzyme was incubated for 1 min, 5 min or 30 min) in assay buffer containing fatty acids in concentration of 20 or 40 µmol/L. Activity of COX-2 was measured with the spectrophotometric method. Data were expressed in U/µg protein and showed as mean activity ± SD (from three separate experiments).
K – activity of enzyme measured in 0,11 mol/L buffor Tris/HCl, pH 8,0; A – activity of enzyme measured in trans-10, cis-12 CLA isomer containing buffer; B – activity of enzyme measured in cis-9, trans-11 CLA isomer containing buffer; C – activity of enzyme measured in linoleic acid containing buffer.

isomer and linoleic acid (p ≤ 0.01) were also measured.

Both CLA isomers (*trans*-10, *cis*-12 12 CLA and *cis* -9, *trans*-11 CLA) and linoleic acid reduced PGE_2 concentration by 39% (p ≤ 0.005), 32% (p ≤ 0.01), and 23% (p ≤ 0.01), respectively in macrophages obtained from blood. The difference in PGE_2 concentration was significant also between the cells incubated with the *trans*-10, *cis*-12 CLA isomer and linoleic acid (p ≤ 0.01) (*Table 2*) (compared to BSA control).

Activity of the reference COX-2 increased in the buffer containing a both: CLAs and a linoleic acid. Activity of this enzyme was reduced only during incubation with linoleic acid thought 30 mins. Contradictory, CLA isomers did not change COX-2 activity (compared to the control) - *Fig.2*.

Effect of fatty acids on changes in mRNA expression, gene expression and protein content in macrophages obtained from peripheral blood

In macrophages obtained from THP-1, fatty acids did not change significantly mRNA expression of the tested genes (data not shown).

In cells incubated with the *trans*-10, *cis*-12 CLA isomer, expression of the COX-2 gene was reduced almost 8 times - *Fig. 3a*. The reduction in the expression of the COX-2 gene was also confirmed by the reduction of concentration of the enzymatic protein in cells ($p \leq 0.05$, n=3) - *Fig. 3b*. Linoleic acid reduced (more than 6 times) the expression of the COX-1 gene ($p \leq 0.05$) – *Fig.3a*. COX-1 gene expression reduction was reflected in the trend to COX-1 protein content reduction in the cells - *Fig. 3c*.

Effect of CLAs on the quantity of the active form of the κB (NF κB) transcription factor

In macrophages obtained from THP-1, a tendency to reduction in quantity of the active p65 NF κB subunit in the CLA environment was observed (*Fig. 4.)* On the other hand, in macrophages obtained from peripheral blood, the *trans*-10, *cis*-12 CLA isomer reduced the quantity of the active p65 NF κB subunit by 55% ($p \leq 0.05$, the Wilcoxon matched-pair test) and the *cis*-9, *trans*-11 CLA isomer reduced this quantity by 58% ($p \leq 0.05$, the Wilcoxon matched-pair test) - *Fig. 4*.

DISCUSSION

CLAs are fatty acids which may be used in the prophylaxis of the civilisation diseases. Anti-atherosclerotic, anti- inflammatory, anti-cancer and anti-diabetic properties of CLAs have been evidenced in both: tissue cell and animal studies (23-25). Work by Whigham *et al.* (24) demonstrated that CLAs inhibit the atherosclerotic process by reduction of the inflammatory processes (26) and by decline of the cholesterol concentration (27). Some evidence exists that CLAs have an positive effect on accumulation of macrophages, cholesterol uptake, and size of atherosclerotic plaques in apo E (-/-) mice (8, 28, 29). CLA feeding was also shown to down – regulate size of atherosclerotic plaques and number of atherogenic macrophages in aortic roots of LDL receptor double-knockout mice (apoE/LDLR-/-) (30). It was suggested that the different isomers could have different properties in mice: whereas *trans*-10, *cis*-12 CLA increased plaque size, pronounce hyperlipidemia, changed lipid metabolism, *cis*-9, *trans*-11-CLA impeded the development of atherosclerosis (31, 32).

Human epidemiologic data support the anti-atherosclerotic potential of CLA less unequivocal. Whereas both CLAs contributed to the inhibition of cancer cell growth (32-37) and lipogenesis process (23), isomer *trans*-10, *cis*-12 CLA enhanced oxidative stress and plasma concentration of C-reactive protein in obese men (38).

In this paper, we demonstrated for first time that CLA may reduce COX-1 activity in human

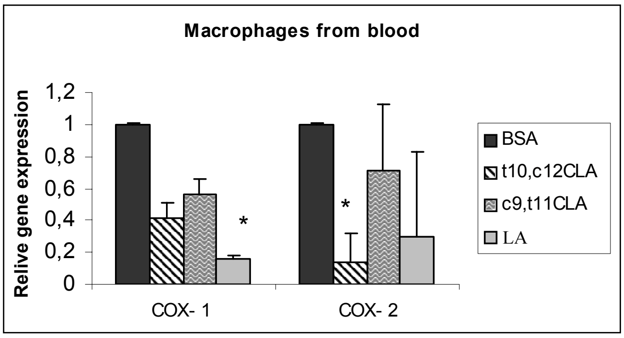

Fig. 3a. The expression of genes measured by quantitative real-time PCR in macrophages from blood (macrophages were cultured with fatty acids for 48 h. Data are expressed as the relative to GAPDH gene expression ratio. The mean values ± SD, n = 3 in triplicate are shown).

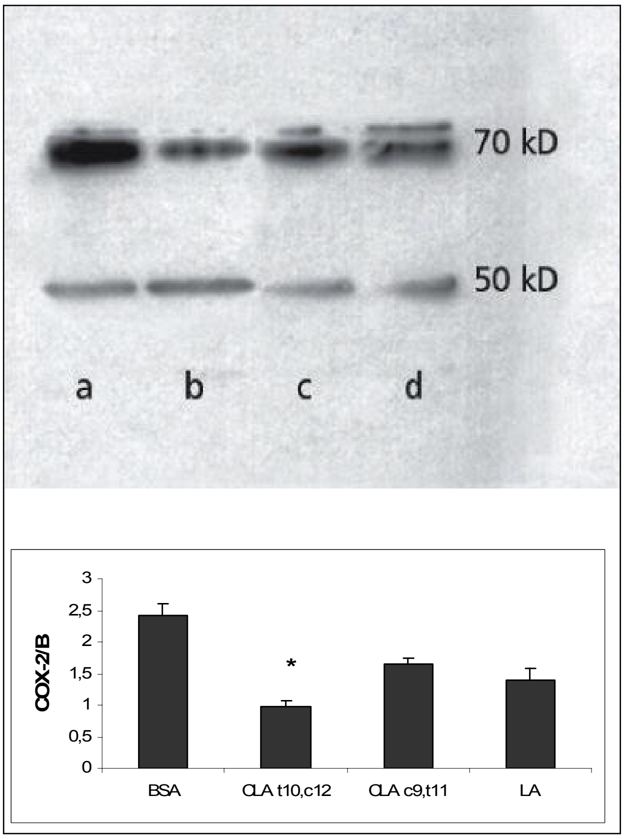

Fig. 3b. The analysis of COX-2 proteins in blood macrophages cultured with fatty acids (the cell lysates were obtained from macrophages cultured with fatty acids). Lysates were stained with antibodies against specified antigens as described in the Methods section. All blots were controlled for equal loading by actin monoclonal antibody and were scanned to calculate the fold increase in antigen level. The values, expressed as arbitrary densitometric units (a.d.u.), were obtained by the reading of blots and are means ± SD of at least three independent experiments, each performed in triplicate; bar graphs represent net intensity of protein bands using the Scion Image program. Representative gel analyses are shown. * $P < 0.05$, comparing cells in coculture with control cells grown alone, by Wilcoxon matched-pair test.
The diagrams (below the pictures) present the content of proteins compared to β-actin. On the picture from left are sequentially a) BSA; b) trans-10, cis-12 CLA isomer; c) cis-9, trans-11 CLA isomer; d) linoleic acid (LA).

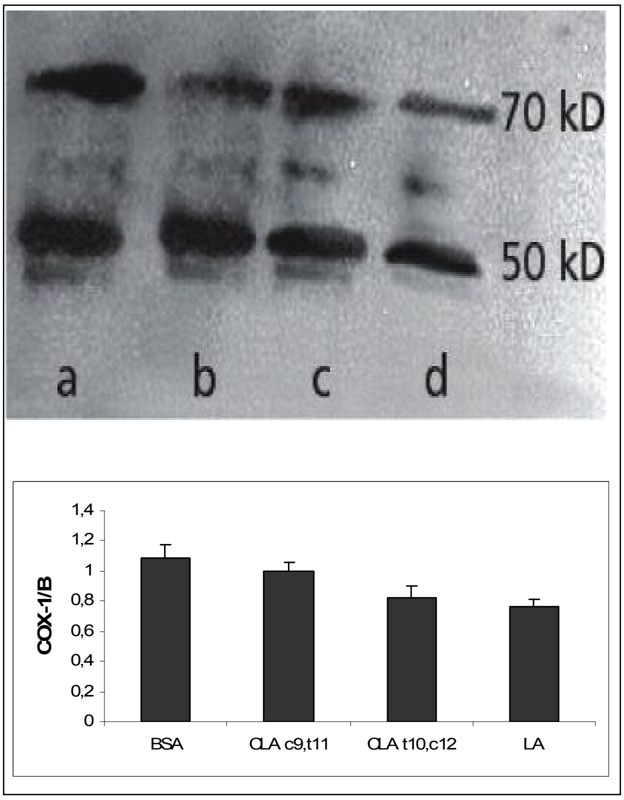

Fig. 3c. The analysis of COX-1 proteins in blood macrophages cultured with fatty acids (the cell lysates were obtained from macrophages cultured with fatty acids). Lysates were stained with antibodies against specified antigens as described in the Methods section. The pictures show one of the three separate experiments of similar results. The mean values are provided ± SD. The diagrams (below the pictures) present the content of proteins compared to β-actin (described on the picture as B).On the picture from left are sequentially a) BSA; b) trans-10, cis-12 CLA isomer; c) cis-9, trans-11 CLA isomer; d) linoleic acid (LA).

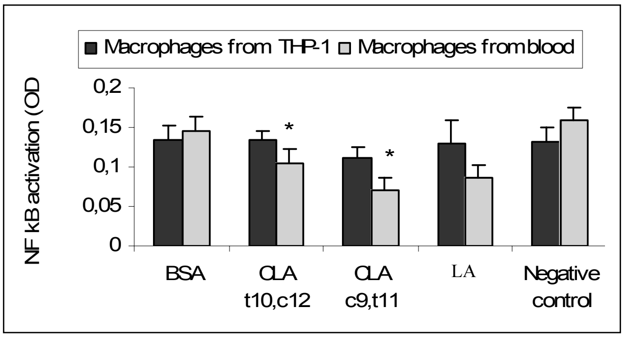

Fig. 4. Effect of CLA on the content of the active form of NK-κB in macrophages (activity of NF-κB were measured by the immunoenzymatic method. The data show content of active p65 subunit in macrophages ± SD from five separate experiments).

macrophages. We also confirmed that *trans*-10, *cis*-12 isomer inhibited COX-2 activity by the NF κB pathway (1, 24, 39, 40) what is similarly with considerable evidence from the studies (28, 29, 39) conducted on the mice macrophages (41) and smooth muscles (28). Unexpectedly, incubation of reference COX-2 with CLA or linoleic acid (LA) led to a slight elevation of the enzyme activity in this study. Only prolongation of the incubation time to 30 mins, with linoleic acid alone, reduced activity of reference COX-2, what may be explain by the characteristics of COX-2 activity. Evidence suggests, that both isoforms of COXs can metabolise other fatty acids (*e.g.* linoleic acid) than arachidonic acid (AA) (42- 46). These fatty acids, may compete with AA about the active site of cyclooxygenase, and may inhibit of enzyme activity (47-55). Opposite to linoleic acid, CLAs didn't act as competitive inhibitor of COX-2. Our studies suggest, that the mechanism by which CLA inhibits PGE_2 synthesis could involve the modulation of the quantity of arachidonic acid caused by a) reduction of AA content in phospholipids, b) inhibition of phospholipase A $_2$ activity (56), and c) down-regulation of COX-2 mRNA and protein expression (only *trans*-10, *cis*-12 CLA isomer).

Compared with the amount of work relating to CLA and COX –2 activity, the effects of CLA on COX-1 activity is practically unknown. COX-1 is an enzyme participating in the first phase of the inflammatory response (57). In some cells (*e.g.* mastocytes) COX-1 is a main source of prostaglandin for the first 30 minutes inflammatory process, then (after 2-4 hours) prostaglandin is synthesise by COX-2 (57). Therefore, inactivation of $cPLA_2$ by CLA (56) and in the consequence, reduction of arachidonic acid availability to COXs may explain the phenomenon of the decrease TXB_2 concentration observed in macrophages cultured with CLA. An additional mechanism contributing to reduction of COX-1 activity may consist in direct inhibition of the enzyme by CLAs. Such phenomenon was observed for sheep COX-1 whose activity was inhibited by both: CLA isomers and linoleic acid.

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Abbreviations:
BSA - bovine serum albumin
CLA - conjugated linoleic acid
COX -1, 2–cyclooxygenase-1, 2
IkB - inhibitory protein kB
LA - linoleic acid
NF - kB-nuclear factor kB
ox-LDL - oxidized low density lipoproteins
PG - prostaglandins
PPAR a, g – peroxisome proliferator activated receptors
TX - thromboxans

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