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Comparative Effects of Conjugated Linoleic Acid (CLA) and Linoleic Acid (LA) on the Oxidoreduction Status in THP-1 Macrophages

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ABSTRACT: The aim of this study was to investigate the effect of conjugated linoleic acids (CLAs) on macrophage reactive oxygen species synthesis and the activity and expression of antioxidant enzymes, catalase (Cat), glutathione peroxidase (GPx), and superoxide dismutase (SOD). The macrophages were obtained from the THP-1 monocytic cell line. Cells were incubated with the addition of *cis*-9,*trans*-11 CLA or *trans*-10,*cis*-12 CLA or linoleic acid. Reactive oxygen species (ROS) formation was estimated by flow cytometry. Enzymes activity was measured spectrophotometrically. The antioxidant enzyme mRNA expression was estimated by real-time reverse transcriptase polymerase chain reaction (RT-PCR). Statistical analysis was based on nonparametric statistical tests [Friedman analysis of variation (ANOVA) and Wilcoxon signed-rank test]. *cis*-9,*trans*-11 CLA significantly increased the activity of Cat, while *trans*-10,*cis*-12 CLA notably influenced GPx activity. Both isomers significantly decreased mRNA expression for Cat. Only *trans*-10,*cis*-12 significantly influenced mRNA for SOD-2 expression. The CLAs activate processes of the ROS formation in macrophages. Adverse metabolic effects of each isomer action were observed.

KEYWORDS: Conjugated linoleic acids, macrophages, reactive oxygen species, antioxidant enzymes, catalase, glutathione peroxidase, superoxide dismutase

INTRODUCTION

Conjugated linoleic acids (CLAs) are classified as polyunsaturated fatty acids, being geometrical and positional isomers of linoleic acid (18:2 *n*-6, LA).[1] The most important biologically active isomers include *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA. The predominant isomer in food products, *cis*-9,*trans*-11 CLA represents about 90% of all CLAs present in food.[1] The second most common isomer is *trans*-10,*cis*-12 CLA.[1] Considerable focus has been recently placed on the potential CLA health benefits, which resulted in widespread use of CLA synthetic forms marketed as diet supplements.

Unfortunately, some negative effects of CLAs on the human body also exist and are related to the induction of lipid peroxidation in cells.[1] Widespread use of CLA-based products, especially among obese (predisposed to atherosclerosis) patients, is the strongest reason why the CLA isomer effects on humans should be investigated.[1]

In activated macrophages, intensive production of reactive oxygen species (ROS) occurs. In phagocytes, ROS are produced as a response to pro-inflammatory factors.[2] Intensified oxidation or a weakened antioxidant system in macrophages often results in a state of imbalance between the processes of production and neutralization of free radicals.[2]

Macrophages are protected against the various adverse effects of excessive oxidation by compounds with antioxidative properties. Among the antioxidants, a particular role is ascribed to antioxidant enzymes, including catalase (Cat), glutathione peroxidase (GPx), and superoxide dismutase (SOD).[3] Cat is a peroxisomal enzyme that catalyzes H_2O_2 decomposition into molecular oxygen and water.[3] In human cells, GPx is present in cytosole (GPx-1).[3] This selenoprotein protects against hydrogen peroxide and certain organic peroxides (e.g., fatty acid peroxides).[3]

For effective GPx-mediated catalysis, restoration of the reduced glutathione (GSH) molecule is necessary.[3] SOD protects against superoxide anion radicals. In humans, three SOD isoforms exist: cytoplasmic (Cu/ZnSOD, SOD-1), mitochondrial (MnSOD, SOD-2), and extracellular (EC-SOD). Hydrogen peroxide is the product of a dismutation reaction catalyzed by this enzyme.[3]

The purpose of this study was to investigate the effect of CLAs on macrophage ROS synthesis and the activity and expression of the selected antioxidant enzymes, namely, Cat, GPx, and SOD.

MATERIALS AND METHODS

Reagents. The following products from Sigma-Aldrich (Poland) were used: medium RPMI-1640, delipidated bovine serum albumin (BSA), penicillin, streptomycin, glutamine, a reagent for protein determination using the Bradford method (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine), NaCl, 2′,7′-diacetate dichlorofluorescein (DCFH-DA), phorbol myristate acetate (PMA), inhibitors of the respiratory chain (rotenone and antimycin), apocynin [oxidase inhibitor of NAD(P)H], reagents for spectrophotometric determination of the concentration of GSH [metaphosphoric acid (MPA)], and GSH. Some reagents for the determination of enzyme activity were also obtained from Sigma-Aldrich (Poland). The following products from POCH (Poland) were used: phosphate-buffered saline (PBS), ethanol (98%), an inhibitor of the respiratory chain (KCN), and reagents for spectrophotometric measurement of the activity of the following enzymes: Cat, GPx, and SOD.

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Table 1. Probe and Primer Sequences for Target Genes

variant	primers (5′ → 3′)	probe	
Cat	CCTGAGAGAGTTGTGCATGCTAAA GCCTTGGAGTATTTGGTAATGTCG	FAM sequence MGB	CCAAAGGCCCCTGCTCC
GPx	CCCGTGCAACCAGTTTGG GGACGTACTTGAGGGAATTCAGAAT	FAM sequence MGB	TCTTGGCGTTCTCCTGATGC
SOD-1	CACGGTGGGCCAAAGGAT AGCAGTCACATTGCCCAAGT	FAM sequence MGB	CTCCAACATGCCTCTCTTC
SOD-2	TGAACGTCACCGAGGAGAAGTA TGCAGGCTGAAGAGCTATCTG	FAM sequence MGB	TTGGCCAAGGGAGATGT

Fatty Acids. *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and LA (98% purity) were obtained from Nu-Chek Prep (Elysian, MN). A kit for measuring the intracellular concentration of GSH using the colorimetric method, Bioxytech GSH-400 assay, was obtained from Oxis International, Inc. (Beverly Hills, CA). Fetal bovine serum (FBS) was obtained from Gibco, Invitrogen (The Netherlands). RNA isolation kit (RNeasy mini kit) was obtained from Qiagen (Germany). Reagents for reverse transcription, dNTP mix, 10 mM each, oligo $(dT)_{18}$ primer, RNase inhibitor RiboLock, reverse transcriptase M-Mulv, and a buffer (5× buffer), were obtained from Fermentas (Lithuania). A kit for DNA digestion, Turbo DNA-free kit, was obtained from Ambion, Inc., Applied Biosystems/Ambion (Austin, TX). Reagents for real-time polymerase chain reaction (PCR), GPx TaqMan probes, SOD-1, SOD-2, Cat (Assay-by-Design), and a probe for glyceraldehyde phosphate dehydrogenase (GAPDH) [Assay-by-Demand, human GAPD (GAPDH) endogenous control (VIC/MGB Probe, Primer Limited)], Gene Expression MasterMix, were purchased from Applied Biosystems (Foster City, CA). THP-1 cells were obtained from the American Type Culture Collection. Minor laboratory equipment came from Sarstedt (Germany), Becton Dickinson (Franklin Lakes, NJ), and Applied Biosystems (Foster City, CA).

Monocytes of the THP-1 cell line were cultivated in RPMI-1640 medium enriched with 10% FBS, penicillin (100 units/mL), streptomycin (100 mg/mL), and glutamine (2 mmol/mL). The cultivation was conducted at 37 °C in an environment of 5% CO_2 and 90% humidity in an incubator manufactured by Assab Kebo Lab (Sweden). In this study, macrophages derived from the THP-1 monocyte cell line were used. Cell viability was determined by Trypan Blue exclusion. Cells were counted using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA).[4] Viability of the cells used was more than 95%.[4] The cells were suspended in warm (36 °C) RPMI-1640 medium, without FBS. Then, 100 nmol/L PMA was added, and incubation was performed for 24 h. The obtained macrophage cells were adherent.[5]

Fatty acids (*cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and LA) were prepared as stock solutions (4 mmol/L), dissolved in a solution of 1 mmol/L delipidated BSA.[6] Macrophages were cultured with BSA (control) and fatty acids for 48 h in RPMI culture medium. The final concentration of fatty acids was 30 μmol/L. BSA was used at the final concentration of 8 μmol/L. Before the experiment, the influence of a range of CLA concentrations on the macrophages was checked and the concentration and final concentration (30 μmol/L) of fatty acids were chosen. Cultivation was carried out in 6-well incubation plates, with 1.2 million to 3 million culture cells in a single well. Cells were collected with a rubber scraper. The suspension containing macrophages, and the medium was centrifuged (800*g* for 10 min at 4 °C). The resulting precipitate was suspended in PBS. For protein determination, 30 μL of the precipitate and PBS suspension was collected. The remaining part was used for research or frozen at −80 °C. For the determination of mRNA expression, the cell pellet after centrifugation was suspended in 200 μL of RNAlater solution.

Measurement of the Intracellular Synthesis of ROS. Oxidative stress in cells was investigated by measuring intracellular oxidation of DCFH-DA according to the methods described in the literature.[7] Fluorescence measurement was made using a FAC scan flow cytometer (Becton Dickinson). To determine to what extent intracellular sources of ROS were responsible for the synthesis of ROS, a respiratory chain inhibitor or NAD(P)H oxidase inhibitor was used. To block the respiratory chain, the following compounds were used: antimycin A (5 μg/mL), rotenone (10 μmol/L), and KCN (1 mmol/L);[8] to block NAD(P)H oxidase, apocynin at a concentration of 50 μmol/L[9] was used.

Determination of the Activity of Antioxidant Enzymes in Macrophages. Enzyme activity was measured using a Perkin-Elmer λ 400 UV/vis spectrophotometer (Waltham, MA). Activity of all enzymes was determined for macrophages incubated with CLAs: *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, or LA. The determination of GPx activity was measured using the method proposed by Wendel.[10] SOD activity was determined by the spectrophotometric method according to Misra and Fridovich,[11] while Cat activity was determined by the method proposed by Aebi.[12] The result was corrected for the amount of protein in the sample, with the final protein concentration in the cells determined by the Bradford method.[13]

Determination of GSH Content. Analyses were performed by means of the Bioxytech GSH-400 assay. The cells were collected using the procedure described above. The spectrophotometry was used to measure the GSH concentration at a wavelength of 400 nm. The result was corrected for the amount of protein in the sample, with the final protein concentration in the cells determined by the Bradford method.[13]

Analysis of RNA Expression Using the PCR with the Detection of Fluorescence in Real Time (Real-Time PCR). Total RNA was isolated from macrophages using a non-enzymatic method with a RNeasy mini kit (Qiagen, Germany). Reverse transcription was carried out with dNTP mix, 10 mM each, oligo $(dT)_{18}$ primer, a RNase RiboLock inhibitor, M-MULV reverse transcriptase, and a buffer, (5× buffer) (Fermentas, Lithuania), according to the standard protocol provided by the kit manufacturer. After review of the published data for analysis of gene expression, the following inducible antioxidant genes were selected: GPx, CAT, SOD-1, and SOD-2.[14] GAPDH was selected as the reference gene with stable expression.[15] BSA was chosen as the calibrator. Analyses were performed using Taq-Man assays (Applied Biosystems, Foster City, CA) either produced on demand (antioxidant genes) or ready made (GAPDH) with Gene Expression MasterMix (Applied Biosystems, Foster City, CA) according to the protocol of the manufacturer on a Mastercycler EP Realplex S (Eppendorf, Germany). Sequences of primers and fluorescent probes are presented in Table 1.

Statistical Calculations. For statistical analysis, nonparametric tests were used. For the dependent variables, a Friedman analysis of variation (ANOVA) test was implemented with statistically significant results, re-analyzed by a Wilcoxon signed-rank test. All calculations were performed using Statistica 7.0 software (Statsoft Poland). p values of ≤ 0.05 were considered statistically significant. Results were expressed as the mean ± standard deviation (SD).

Table 2. ROS Synthesis [Expressed as Dichlorofluorescein (DCF) Fluorescence Intensity] in Macrophages Cultured with CLAs and LA

experimental conditions	fluorescence intensity of DCF (n = 12)	percent of control (%)
BSA	410.5 ± 18.3	
cis-9,*trans*-11 CLA	451.7 ± 41.05[a]	110
trans-10,*cis*-12 CLA	481.1 ± 23.5[a]	117
LA	440.1 ± 43.9[b]	107

[a] $p < 0.01$, versus the corresponding control, BSA (8 μmol/L for 48 h). [b] $p < 0.05$, versus the corresponding control, BSA (8 μmol/L for 48 h).

Table 3. Effect of Respiratory Chain Inhibitors on ROS Synthesis in Macrophages Cultured with CLAs and LA

experimental conditions	DCF fluorescence intensity (n = 8) macrophages cultured with respiratory chain inhibitors	DCF fluorescence intensity (%) macrophages cultured without respiratory chain inhibitor addition in comparison to macrophages cultured with respiratory chain inhibitors
BSA (control)	27.8 ± 5.7	
cis-9,*trans*-11 CLA	65.3 ± 35.6[a]	185
trans-10,*cis*-12 CLA	105.3 ± 51.8[a]	178
LA	28.1 ± 7.6[a]	192

[a] $p < 0.01$, versus the corresponding cells cultured with fatty acids but without respiratory chain inhibitor addition [antymycin A (5 μg/mL), rothenone (10 μmol/L), and KCN (1 mmol/L)].

RESULTS

ROS Synthesis in Macrophages. ROS production was higher in macrophages stimulated by CLA isomers and LA (Table 2). Stimulation with *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA increased the amount of produced ROS by 10% ($p < 0.01$; n = 12) and 17% ($p < 0.01$; n = 12), respectively, while LA increased the synthesis of ROS by 7% ($p < 0.05$; n = 12) (Table 2) (Friedman ANOVA and Wilcoxon signed-rank test). ROS generation was 9% higher in macrophages cultured with *trans*-10,*cis*-12 CLA if compared to LA ($p < 0.02$; n = 12). For *cis*-9,*trans*-11 CLA, only an upward trend was observed (Table 2). (Friedman ANOVA and Wilcoxon signed-rank test).

The addition of the respiratory chain inhibitors significantly decreased ROS generation in macrophages ($p < 0.02$; n = 8). An 85% reduction of ROS production was observed for *cis*-9,*trans*-11 CLA; a 78% reduction of ROS production was observed for *trans*-10,*cis*-12 CLA ($p < 0.01$; n = 8); and a 92% reduction of ROS production was observed for LA ($p < 0.01$; n = 8), in comparison to the cells cultivated without respiratory chain inhibitors (Tables 2 and 3) (Friedman ANOVA and Wilcoxon signed-rank test). In macrophages cultured with respiratory chain inhibitors, *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA increased ROS generation relatively by 132% ($p < 0.05$; n = 8) and 274% ($p < 0.02$; n = 8) if compared to LA (Table 3) (Friedman ANOVA and Wilcoxon signed-rank test).

No significant inhibition of ROS synthesis in macrophages cultured with apocynin was observed (Tables 2 and 4) (Friedman ANOVA and Wilcoxon signed-rank test). No significant differences between ROS generation in macrophages cultivated with CLAs (n = 8) and apocynin were observed in comparison to ROS generation in macrophages cultured with LA and apocynin (Table 4) (Friedman ANOVA and Wilcoxon signed-rank test).

Table 4. Effect of NAD(P)H Oxidase Inhibitor on the ROS Synthesis in Macrophages Cultured with CLAs and LA

experimental conditions	DCF fluorescence intensity (n = 8) macrophages cultured with apocynin	DCF fluorescence intensity (%) macrophages cultured without apocynin addition in comparison to macrophages cultured with apocynin
BSA (control)	441.5 ± 7.3	107
cis-9,*trans*-11 CLA	447.4 ± 28.5	99.9
trans-10,*cis*-12 CLA	438.2 ± 28.1	91.0
LA	475.1 ± 99.2	108

Effects of CLAs on Cat Activity. The *cis*-9,*trans*-11 CLA isomer increased enzyme activity by 110% in comparison to the control (BSA) ($p < 0.05$; n = 20). Incubation with *trans*-10,*cis*-12 CLA did not exert any effect on enzyme activity, while the addition of LA increased Cat activity by approximately 40% (n = 20) (Figure 1a) (Friedman ANOVA and Wilcoxon signed-rank test). The *cis*-9,*trans*-11 CLA increased Cat activity if compared to LA by 35% ($p < 0.05$; n = 20). For the *trans*-10,*cis*-12 CLA (n = 20), a strong downward trend in activity of Cat was observed if compared to LA (Figure 1a) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on Cat mRNA Expression. All fatty acids used in experiments decreased Cat mRNA expression: *cis*-9,*trans*-11 CLA by about 63% ($p < 0.02$; n = 9), *trans*-10,*cis*-12 CLA by approximately 65% ($p < 0.02$; n = 9), and LA by approximately 70% ($p < 0.02$; n = 9) (Figure 1b) (Friedman ANOVA and Wilcoxon signed-rank test). If compared to LA, *trans*-10,*cis*-12 CLA increased Cat mRNA expression by 50% ($p < 0.02$; n = 9). For the *cis*-9,*trans*-11 CLA (n = 9), a strong upward trend was observed (Figure 1b) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on SOD Activity. *cis*-9,*trans*-11 CLA added to the incubation medium reduced SOD activity by 78% ($p < 0.05$; n = 27). For the *trans*-10,*cis*-12 CLA and LA, a strong downward trend was observed in the activity of SOD (Figure 2a) (Friedman ANOVA and Wilcoxon signed-rank test). In comparison to LA, *cis*-9,*trans*-11 CLA decreased SOD activity by 62.5% ($p < 0.05$; n = 27). For *trans*-10,*cis*-12 CLA, only a downward trend in SOD activity was observed (Figure 2a) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on SOD-1 mRNA Expression. *cis*-9,*trans*-11 CLA (n = 6) slightly decreased expression of SOD-1 (Cu/ZnSOD), while a 15% decrease was observed with *trans*-10,*cis*-12 CLA (n = 6) incubated samples compared to the calibrator (BSA). LA reduced the expression of SOD-1 by about 40% ($p < 0.05$; n = 6) (Figure 2b) (Friedman ANOVA and Wilcoxon signed-rank test). In comparison to LA, both *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA increased SOD-1 mRNA expression respectively by 42% ($p < 0.05$; n = 6) and 33% ($p < 0.05$; n = 6) (Figure 2b) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on SOD-2 mRNA Expression. *cis*-9,*trans*-11 CLA (n = 6) insignificantly increased the expression of SOD-2 mRNA. The other investigated isomer (*trans*-10,*cis*-12 CLA) and LA decreased the SOD-2 (MnSOD) mRNA expression by approximately 20% ($p < 0.05$; n = 6) compared

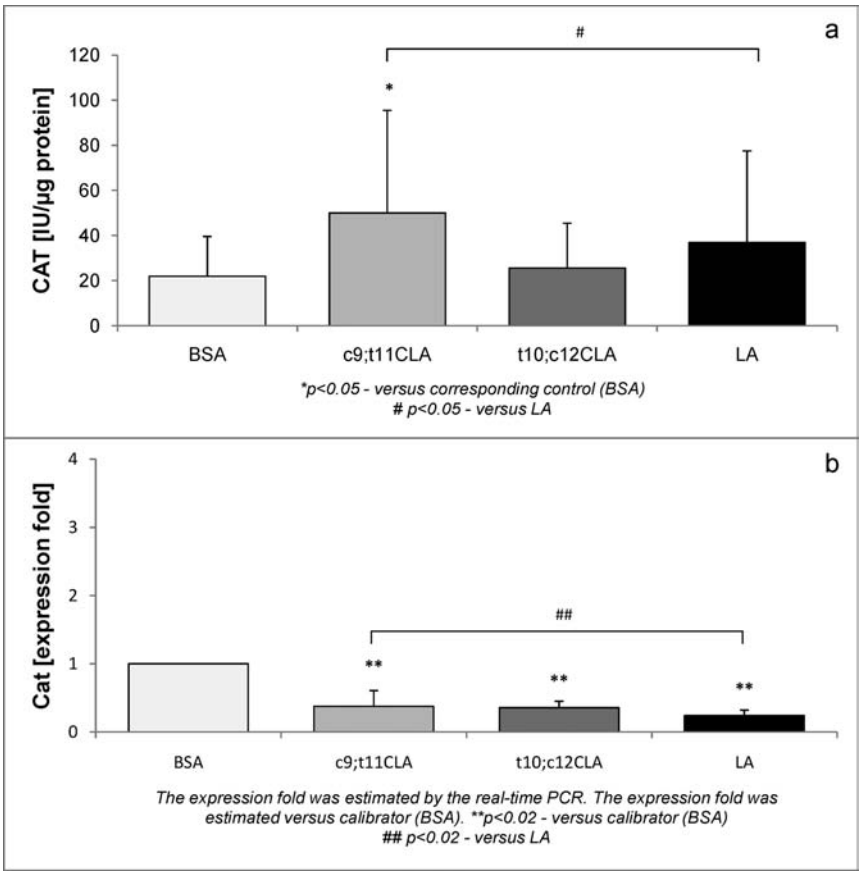

Figure 1. (a) Effect of CLAs (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the Cat activity in THP-1 macrophages. (b) Effect of CLAs (*cis*-9, *trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the Cat mRNA expression in macrophages. The expression fold was estimated by the real-time PCR. The expression fold was estimated versus the calibrator (BSA).

to the calibrator (BSA) (Figure 2c) (Friedman ANOVA and Wilcoxon signed-rank test). In comparison to LA, *cis*-9,*trans*-11 CLA ($n = 6$) significantly increased SOD-2 mRNA expression by 144% ($p < 0.02$; $n = 6$), while *trans*-10,*cis*-12 CLA significantly decreased SOD-2 mRNA expression by 14% ($p < 0.05$; $n = 6$) (Figure 2c) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on GPx Activity. A 250% activity increase was observed in macrophages incubated with *trans*-10,*cis*-12 CLA isomer compared to the control (BSA) ($p < 0.02$; $n = 3$). The *cis*-9,*trans*-11 CLA isomer and LA did not affect GPx activity (Figure 3a) (Friedman ANOVA and Wilcoxon signed-rank test). In comparison to LA, only *trans*-10,*cis*-12 CLA significantly increased the GPx activity ($p < 0.05$; $n = 3$). *cis*-9,*trans*-11 CLA ($n = 3$) increased GPx activity, but the differences were not significant (Figure 3a) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on GPx mRNA Expression. mRNA GPx expression in cells decreased by 30% when cultured with *cis*-9, *trans*-11 CLA ($p < 0.05$; $n = 4$), decreased by 40% when cultured with *trans*-10,*cis*-12 CLA ($p < 0.05$; $n = 4$), and decreased by 50% when cultured with LA ($p < 0.05$; $n = 4$) (Figure 3b) (Friedman ANOVA and Wilcoxon signed-rank test). In comparison to LA, both *cis*-9,*trans*-11 CLA ($n = 4$) and *trans*-10,*cis*-12 CLA ($n = 4$) increased GPx mRNA expression, but the differences were insignificant (Figure.3b) (Friedman ANOVA and Wilcoxon signed-rank test).

Effects of CLAs on Macrophage GSH Concentration. Both isomers of LA (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA reduced intracellular GSH concentration by 35, 28, and 27%, respectively. The differences were statistically significant compared to the control (BSA) ($p < 0.05$; $n = 6$) (Figure 3) (Friedman ANOVA and Wilcoxon signed-rank test). In comparison to LA, both *cis*-9,*trans*-11 CLA ($n = 6$) and *trans*-10,*cis*-12 CLA ($n = 6$) increased GSH concentration in macrophages, but the differences were insignificant (Figure 4) (Friedman ANOVA and Wilcoxon signed-rank test).

DISCUSSION

The effects of CLAs on the oxidoreduction cell equilibrium have yet to be fully elucidated. Contradictory research results have been published to date indicating: (1) antioxidant action of both the CLA isomers investigated here, (2) antioxidant properties of *cis*-9,*trans*-11 CLA and pro-oxidant properties of *trans*-10, *cis*-12 CLA, and (3) pro-oxidant action of both isomers.[16−18] These mentioned inadequacies have prompted current research to determine the effects of *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA isomers on ROS synthesis in macrophages. Additionally, it has stimulated the analysis of indirect CLA influence, by alteration in ROS production, on both antioxidant enzyme activity and mRNA expression.

The results of this study confirm the pro-oxidant activity of both CLA isomers (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA)

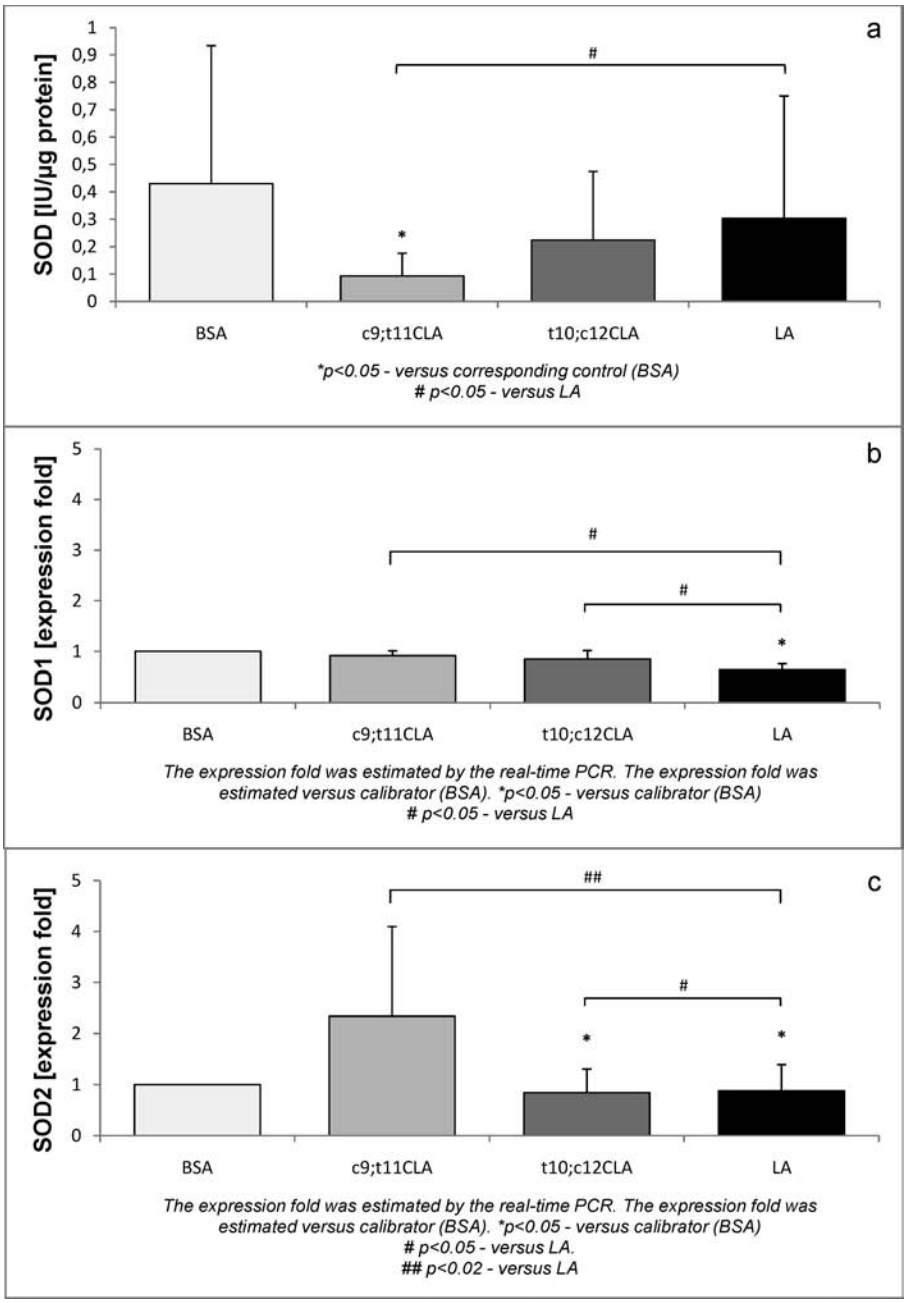

Figure 2. (a) Effect of CLAs (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA (30 μmol/L for 48 h) on the SOD activity in THP-1 macrophages. (b) Effect of CLAs (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the Cu/ZnSOD (SOD-1) mRNA expression in macrophages. The expression fold was estimated by the real-time PCR. The expression fold was estimated versus the calibrator (BSA). (c) Effect of CLAs (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the MnSOD (SOD-2) mRNA expression in macrophages. The expression fold was estimated by the real-time PCR. The expression fold was estimated versus the calibrator (BSA).

(Table 2). Both isomers increased the amount of ROS generated. The stronger pro-oxidant effects were observed for *trans*-10,*cis*-12 CLA. Such results are confirmed by the following population studies: the pro-oxidant effect of *trans*-10,*cis*-12 CLA has been reported by Riserus et al.,[1,19] with observation that, on CLA supplementation, 8-iso-PGF_2 excretion in the urine of male patients with metabolic syndrome is increased.[1,19] Similar results were observed by Tholstrup et al. in a group of postmenopausal women.[20] Although LA is an essential nutritient, the fatty acid increased the amount of produced ROS in THP-1 macrophages. That effect has already been described other authors. It was observed that LA did not exert as strong effect on ROS production as CLAs (particularly *trans*-10,*cis*-12 CLA exerted a stronger pro-oxidant effect if compared to LA).[21] The discrepancies may be related to the differences in the geometrical structure of LA and CLAs. It has already been proven that conjugated double bonds stabilize free radicals.[17]

The second stage of our research, which followed the investigation of CLA pro-oxidant properties, was identification of the intracellular ROS source in the CLA-incubated macrophages. Research data indicates that macrophage ROS sources include NAD(P)H oxidase, mitochondrial respiratory chain,

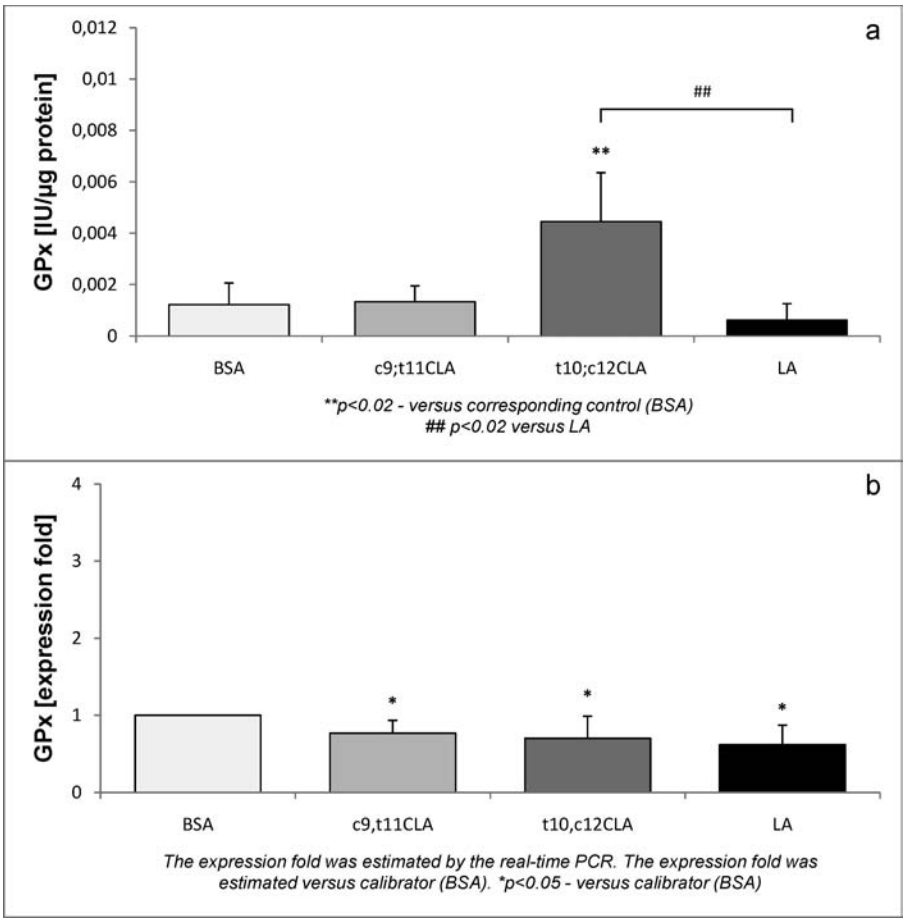

Figure 3. (a) Effect of CLAs (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the GPx activity in THP-1 macrophages. (b) Effect of CLAs (*cis*-9, *trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the GPx mRNA expression in macrophages. The expression fold was estimated by the real-time PCR. The expression fold was estimated versus the calibrator (BSA).

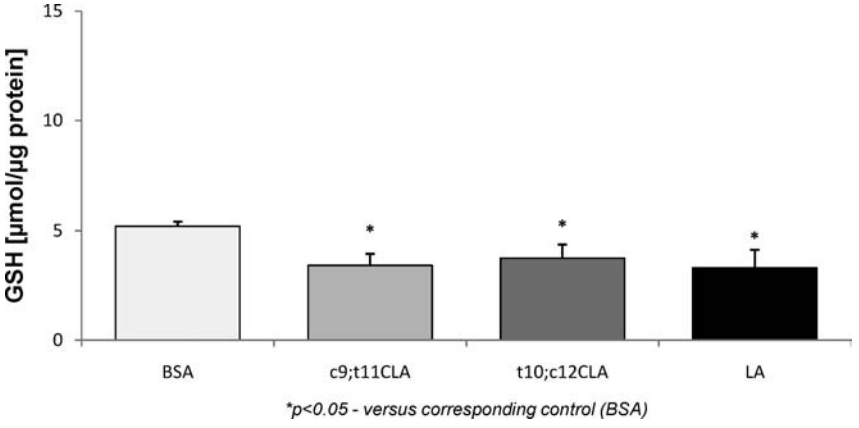

Figure 4. Effect of CLAs (*cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA) and LA on the intracellular GSH concentration in THP-1 macrophages.

cyclooxygenase, and lipoxygenase.[22] However, a question to be addressed in this study was whether the incubation of macrophages with CLAs would result in the preferential stimulation of ROS cellular sources. On the basis of a previously published report,[22] it was assumed that, in the experimental model, either mitochondrial respiratory chain or NAD(P)H oxidase might be of the greatest importance for ROS generation. Previous studies on macrophages cultured with CLAs indicated that increased oxidative stress is induced by β-oxidation and stimulation of the respiratory chain.[16] The results obtained in this study confirmed the respiratory chain to be the most important ROS source (Table 3). NAD(P)H oxidase was proven insignificant in the CLA- and LA-dependent ROS formation in macrophages (Table 4). The predominant role of the respiratory chain in ROS generation in CLA-treated macrophages was also reported by other authors,[23] which confirms predominant

mitochondrial origin of the CLA- and LA-dependent ROS synthesis.

CLA and LA metabolite the pro-oxidant, and the effect has already been reported, stimulating the progression of inflammation. Atherosclerosis is a typical example of an inflammatory disease with an initial stage of endothelial damage and subsequent accumulation of oxidized lipoprotein (oxLDL) in the vascular intima.[24]

In this study, we also analyzed if increased ROS quantity triggers a corresponding antioxidative response and intensification of free-radical scavenging, ROS production-related activity of the key macrophage antioxidant enzymes, Cat, GPx, and SOD, following CLA and LA incubation was examined. Protein activity of these enzymes was analyzed and complemented with the mRNA expression assay for each of them. It was assumed that CLA, through changes in secondary mediator synthesis, would modulate both protein activity and mRNA expression of antioxidant enzymes with possible involvement of cellular transcription factors [e.g., nuclear factor-κB (NF-κB)].[25]

Because Cat is an enzyme involved in the decomposition of hydrogen peroxide (H_2O_2) with activity stimulated by this substrate,[26] it was expected that an increase in ROS production would also increase the activity of Cat.

In this study, however, the following results were obtained: (1) only *cis*-9,*trans*-11 CLA stimulated Cat activity; (2) despite the strongest pro-oxidant effect of *trans*-10,*cis*-12 CLA, it did not increase Cat activity (Figure 1a). Only LA increased both ROS production and Cat activity (Table 2 and Figure 1a). That opposite if compared to conjugated dienes of the LA effect is also related to differences in the geometrical structure and localization of double bonds.[17]

Both CLA isomers and LA decreased the expression of Cat mRNA (Figure.1b). The mechanism of this phenomenon remains unclear, with only a few reports on this subject. Previously, in an environment of increased ROS production, both activity and Cat mRNA expression were reported to increase.[26] As was pointed out by Nakamura and Omaye, the mechanism of the influence of CLA on the antioxidant enzyme expression is complex and depends upon, e.g., the dose of CLA.[25] Doses of CLA used in this study (30 μmol/L) are regarded by some researchers as too low to be protective against excessive oxidation (including lipid peroxidation) in the cell.[27] On the other hand, at low concentrations of CLA, activation of the NF-κB transcription factor and increased cytoplasmic expression of Cat and SOD isoforms occur.[25] Bergamo et al. showed that the use of CLAs resulted in a reduced production of ROS in macrophages.[28] Increased generation of ROS is a factor that may potentially trigger the induction of Cu/ZnSOD.[29] The activation of SOD occurs through the PPAR-γ- and NF-κB-dependent pathway.[25] In this study, however, a previously suggested positive ROS influence on SOD activity or its expression was not observed. It is difficult to explain the cause of SOD activity decrease after *cis*-9, *trans*-11 CLA administration (Figure 2a). This type of interaction has previously been observed for lower doses of the isomer[25] but not for the concentration used in this experiment (30 μmol/L).[25] On the other hand, the mitochondrial ROS origin has been proven in this experiment, after CLA and LA administration. In this case, minor importance of the Cat (cytosolic) action may be suggested. The predominating involvement of the mitochondrion-localized antioxidant enzymes has to be taken under consideration. This hypothesis may be an explanation why none of the isomers used affected the expression of SOD-1 (Figure 2b), but both reduced the SOD-2 (mitochondrial isoenzyme) mRNA expression (Figure 2c). Unambiguous interpretation of these results is difficult. On one hand, the reports that a CLA concentration of 30 μmol/L is too low for an antioxidant effect are confirmed; however, the possible cause of the SOD expression decline, being the effect of the NF-κB inhibition, was reported for lower *cis*-9,*trans*-11 CLA concentrations only.

It is possible that CLAs influence alternative, previously not investigated, transcription pathways and transmitter secretion. Another possible explanation is that neither SOD nor Cat were involved in primary cell protection against ROS. Indeed, some researchers suggest that GPx is the major protective antioxidant enzyme, which targets an array of peroxides, including hydrogen peroxide.[30] In this study, ROS-dependent fluorescence was primarily associated to hydrogen peroxide production.[31] Previous *in vitro* experiments demonstrated that protection against ROS is mainly GPx-dependent, with Cat and SOD of minor importance.[30] However, it was also observed that, in atherosclerotic vessels, GPx is often poorly active, being deactivated by the ROS (including products of lipid peroxidation) present in the atherosclerotic plaque.[32] Our results confirm the observations on ROS-associated GPx deactivation.[32] It is possible that mitochondrion-dependent ROS production could lead to apoptosis promotion. *trans*-10,*cis*-12 CLA, independent of ROS synthesis stimulation, increased the macrophage GPx expression (Table 2 and Figure 3b). Interestingly, despite the *trans*-10,*cis*-12 CLA-dependent ROS increase, no rise of GPx expression was observed (Table 2 and Figure 3b). The other CLA isomer (*cis*-9, *trans*-11 CLA) both inhibited the activity of GPx and decreased its mRNA expression (panels a and b of Figure 3). In our experiments, CLAs were associated with GSH concentrations (Figure 4). It must be noted that previous studies reported increased GSH levels in response to ROS production.[33] Reasons for this discrepancy have yet to be drawn. The hypothesis suggested by Emdin et al.[34] should be taken under consideration. According to findings of their study, the GSH concentration decrease could be the result of the GSH hydrolysis via γ-glutamyl transpeptidase. That hypothesis seems to be supported by the presence of γ-glutamyl transpeptidase as a factor increasing ROS synthesis and LDL oxidation within the atherosclerotic plaque.[35] Moreover, depletion in GSH content in excessive ROS synthesis conditions may be due to apoptosis occurrence.[36] As mentioned above, the function of antioxidant enzymes is to maintain a cellular redox balance.[37] In this study, it was assumed that an increase in the synthesis of ROS may stimulate the expression of antioxidant enzymes (SOD, Cat, and GPx), as previously suggested.[38] The question of whether the reduction of the antioxidant enzyme expression is a consequence or the cause of the increased ROS synthesis remains unanswered fully thus far.[39] The results presented here indicate that the increased ROS production in macrophages might be independent of the antioxidant enzyme expression and not always associated with its increase (Table 2 and Figures 1−4).

Thus far, the influence of CLA on the macrophage antioxidant enzyme expression has not been fully determined. The results suggest that oxidative stress is a dynamic condition associated with possible activation or inactivation of antioxidant pathways, with the former related to the mRNA expression decrease. A decrease in antioxidant enzyme gene expression for the macrophage line studied could be caused by early apoptosis. This hypothesis is based on the fact that intense

generation of ROS leads to the disruption of the mitochondrial membrane potential, with subsequent destruction of these organelles, cytochrome *c* release, and subsequent activation of apoptosis.[40]

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ABBREVIATIONS USED

BSA = bovine serum albumin; Cat = catalase; CLA = conjugated linoleic acid; GPx = glutathione peroxidase; GSH = reduced form of glutathione; ROS = reactive oxygen species; SOD = superoxide dismutase

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