JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Conjugated Linoleic Acid Regulates Phosphorylation of PPARγ by Modulation of ERK 1/2 and p38 Signaling in Human Macrophages/Fatty Acid-Laden Macrophages

Ewa Stachowska,[*,†] Jacek Kijowski,[∥] Violetta Dziedziejko,[‡] Aldona Siennicka,[§] and Dariusz Chlubek[‡]

Departments of [†]Biochemistry and Human Nutrition, [‡]Biochemistry and Medical Chemistry, and [§]Laboratory Diagnostics and Molecular Medicine, Pomeranian Medical University, Szczecin, Poland

[∥]Department of Transplantation, Jagiellonian University Medical College, Cracow, Poland

ABSTRACT: Stimulation of macrophages by a variety fatty acids causes activation of MAP kinases (MAPKs). The consequences arising from down-regulation of MAPKs may be a limitation in the activity of PPARγ, which is modulated by a modification catalyzed by these kinases. Phosphorylation of MAP kinases-ERK1/2 and p38 as well as PPARγ was determined by real-time polymerase chain reaction and Western blotting in human macrophages cultured with conjugated linoleic acids (CLAs). We demonstrated that CLA isomers alter MAP kinase phosphorylation and PPARγ activation. Phosphorylation of ERK1/2 was diminished in cells cultivated with *cis*-9,*trans*-11 CLA, whereas phosphorylation of p38 was reduced by *trans*-10,*cis*-12 CLA. PPARγ was phosphorylated mainly by ERK1/2, and consequently, PPARγ phosphorylation was suppressed mainly by *cis*-9,*trans*-11 isomer. In human adipocytes, *cis*-9,*trans*-11 C 18:2 raised the activation of PPAR and several of its downstream target genes. We suggest that a similar process may also occur in human macrophages.

KEYWORDS: CLA, fatty acid-laden macrophages, MAPK, p38, PPARγ

INTRODUCTION

Conjugated linoleic acids (*cis*-9,*trans*-11 C 18:2 and *trans*-10, *cis*-12 C 18:2) are stereoisomers of linoleic acid (LA) (*cis*-9,*cis*-12 C 18:2).[1,2] In the body, conjugated linoleic acids (CLAs) originate mainly from food, although they may also be synthesized endogenously by conversion of some *trans* isomers of fatty acids, for example, desaturation of vaccenic acid (*trans*-11 C18:1) to *cis*-9,*trans*-11 CLA isomer.[3] The most common CLA isomers in food are *cis*-9,*trans*-11 C18:2 and *trans*-10,*cis*-12 C18:2.[4] Many studies have shown the positive role of these fatty acids as antiatherosclerotic, anticancer, anti-inflammatory,[5] and antidiabetic substances in animals.[6,7] Studies in humans have shown a less unequivocal role for CLAs.[8−13]

One of the effects of CLA intake is the embedding of these fatty acids in cell membrane phospholipids.[13,14] The fact that CLAs are built-in cell structures suggests that they may be modulators of cellular responses to the action of external inflammatory factors.[15−17]

Natural fatty acids are known activators of peroxisome proliferator activated receptors (PPARs),[18,19] transducer proteins from the nuclear receptor superfamily that control expression of various genes crucial for lipid and glucose metabolism. PPARγ was shown to have antiatherosclerotic effects in different cells: In endothelial cells, PPARγ agonists inhibit vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1) expression, resulting in the reduction of monocyte accumulation in the arterial intima.[18] However, these receptors also exhibited potential pro-atherogenic effects by the overexpression of oxidated low-density lipoprotein (ox-LDL) scavenger receptors, increasing the formation of foam cells.[18]

Stimulation of macrophages by a variety of agents (e.g., growth factors, cytokines, and hormones) causes activation of mitogen-activated protein kinases (MAPKs). MAP kinases are major components of pathways controlling, for example, cell differentiation, cell proliferation, and cell death. The extracellular signal-regulated protein kinases 1 and 2 (ERK) pathway was one of the first signaling pathways for which the link between the extracellular ligand and the nucleus was described. The signaling module of the ERK pathway is composed of ERK1/2, the dual-specificity kinase MEK1/2, and isoforms of Raf and is principally activated by hormones and growth factors.[20−22] In human cells, there are three members of the MAPK family, including ERK 1/2 (p44/p42), c-Jun-NH 2-terminal kinase (JNK), and p38 MAPK kinase.[23,24] In macrophages, ox-LDL induces phosphorylation of MAP kinases (ERK1/2 and p38 as well as JNK kinases)[18,20,24,25] and PPARγ (phosphorylated by MAP kinases);[20,21] therefore, we investigated that CLAs may influence on MAP kinase phosphorylation and PPARγ activation in macrophage/foam cells.

MATERIALS AND METHODS

Cell culture media and fetal bovine serum (FBS) were from Gibco, Invitrogen (Carlsbad, CA). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO). Isomers of CLA (+98% pure) were from Nu-CheK Prep (Elysian, MN). PD 9805, ERK1/2 inhibitor; SB 203580, an inhibitor of p38 kinase phosphorylation; and anisomycine (AN), an activator of p38 phosphorylation, were from Calbiochem (Darmstadt, Germany). The Oligotex Direct mRNA Mini Kit from Qiagen (Hilden, Germany) was used for isolation of mRNA from

Received: April 13, 2011
Accepted: August 21, 2011
Revised: August 20, 2011
Published: August 22, 2011

dx.doi.org/10.1021/jf2014233 | *J. Agric. Food Chem.* 2011, 59, 11846–11852

macrophages. Reagents for real-time polymerase chain reaction (PCR): that is, 5× First Strand Buffer, oligo (dT) and DTT, were by Promega (Madison, WI) and Superscript II reverse transcriptase was from Invitrogen. Monoclonal antibodies were used for β-actin (Sigma), PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), and phospho p38 MAPK, ERK1/2 MAP kinase, total ERK1/2 MAP kinase (Cell Signaling, Danvers, United States); polyclonal antibodies were used for phospho PPARγ (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated goat antimouse IgG or goat antirabbit IgG secondary antibodies were from Santa Cruz Biotechnology. Bradford reagent was from Sigma-Aldrich (St. Louis, MO).

Cell Culture and Treatment. THP-1 cells from American Type Culture Collection (ATCC, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% fatty acid-free FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in 5% CO_2. THP-1 human monocytic cells were differentiated to macrophages by administration of phorbol myristate acetate (PMA).[26] THP-1 monocytes were seeded at a density of 2×10^6 cells/well in 6-well plates and incubated with 100 nM PMA for 24 h. The viability of the cells treated with ox- LDL and fatty acids was quantified by by Trypan Blue exclusion. Cells were counted using a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). The viability of cells used was more than 95%. After incubation with PMA, adherent cells (macrophages) were washed three times with phosphate-buffered saline (PBS) and incubated with 30 μM (to stimulate macrophage final concentration) fatty acids (*cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and LA) for 48 h at 37 °C. Fatty acid-laden macrophages were obtained after additional macrophage incubation with ox-LDL (50 μg/mL) for 24 h at 37 °C.[27] In some experiments (after incubation with fatty acids), cells were cultured with phosphorylation process modulators: PD 98059 (50 μM), SB 203580 (10 μM), or AN (1 μg/mL). The modulators were added to the medium 48 h before measurement.

Preparation of Fatty Acids. Both isomers of CLA and LA were complexed to fatty acids-free at a 3:1 molar ratio using 1 mmol/L BSA stocks according to a previously published method.[28] Briefly, 10 mg of CLA was mixed with ethanol and NaOH in a volume adjusted so that the molar ratio of FA to NaOH was 1:1. The mixture was dried under nitrogen gas until CLA sodium salt was obtained and then dissolved in sterile water (stock solutions of CLA). Stock solutions of CLA complexed to BSA were made by combining CLA and 5 mM BSA in a volume adjusted so that the CLA to BSA molar ratio was 3:1. The CLA−BSA solutions were sterile-filtered and used fresh.[28]

Preparation of ox-LDLs (Induced by Cu^{2+}). LDLs were isolated from blood serum obtained from patients with a normal lipid profiles as described previously.[29−31] The group comprised 52 white Caucasian male patients aged between 25 and 30 years. Twenty patients were smokers, 10 were ex-smokers, and the rest had never smoked tobacco. An average standard of living was found for all patients, and there were no cases of malnutrition. Individuals with dyslipidemia or a history of hypertension or diabetes were excluded from the study. Patients were fully informed as to the study objectives and benefits and provided written consent prior to enrollment. The study protocol complied with the ethical standards laid down in the Declaration of Helsinki and was approved by the Committee on Human Research at the Pomeranian Medical University. Venous blood for lipid and lipoprotein analyses was collected into tubes (without anticoagulant) after an overnight fast. The plasma was then obtained by centrifugation (1200*g* for 10 min). Next, the plasma was centrifuged (112000*g* for 24 h at 16 °C).[32] The disk containing LDL obtained after centrifugation was then transferred to an Econo-Pac 10 DG Sephadex G-25 column (Pharmacia) to remove impurities created during LDL preparation. After that, the LDL suspension was transferred to an Econo-Pac 10 DG column and washed with PBS, solution and the protein concentration (with the use of a Bradford reagent) was measured in the filtrate. The final filtrate was diluted with PBS to 100 μg of protein/mL, Cu^{2+} was added (10 μM), and the total was incubated for 6 h at 37 °C. Oxidation was inhibited at 4 °C by EDTA (200 μM) and BHT (40 μM). The total was dialyzed, and 200 μL of the suspension was taken for measurement of protein and TBARS content.[33] The ox-LDLs obtained were frozen at −80 °C for not more than 3 months. A freezing−thawing cycle was avoided. The ox-LDLs were added to the macrophages in such a quantity as to obtain a protein concentration in the cultivation well of 50 μg/mL.

PCR Reaction with an Analysis of Real-Time Product Quantity Increase (Real-Time PCR). A Qiagen Oligotex Direct mRNA Mini Kit was used for the isolation of mRNA from macrophages. The mRNA content was determined by measuring the optical absorbance ratio at 260/280 nm after the sample was dissolved in water. RNA was stored at −70 °C before RT-PCR. To confirm enzymatic activity regulation, a quantitative expression analysis was performed by real-time PCR using GAPDH as the reference gene, as previously described.[34] Contamination of DNA was avoided throughout by locating the primers in two different exons. For cDNA synthesis, 1 μg of total RNA was reverse transcribed at 42 °C for 50 min in a total volume of 40 μL of reaction buffer containing 5 × First Strand Buffer, oligo (dT), DTT, deoxy-NTPs, and 200 units of SUPERSCRIPT II reverse transcriptase. cDNA was subjected to real-time PCR in a reaction mixture containing the QuantiTect SYBR Green PCR mix and primers. The sequences of primers used in this study were as follows: PPARγ—forward primer, 5′-ATGACAGCGACTTGGCAA-3′; reverse primer, 5′-TCAATGGGCT-TCACATTC-3′; GAPDH—forward primer, 5′-GCCAGCCGAGCCA-CATC-3′; reverse primer, 5′-GCGCCAATACGACCAAA-3′. All real-time PCR reactions were performed on a 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 the PCR amplification, melting curve analysis was performed with a temperature profile slope of 1 °C/s from 35 to 95 °C. A negative control (NC) without a cDNA template was run with every assay to ensure overall specificity. The expression rates were calculated as previously described.[35,36]

Analysis of Phosphorylation of MAP Kinases ERK1/2, p38 and PPARγ (Total and Phospho) with the Use of Western Blotting. The cells were cultivated for 48 h with fatty acids, collected, and washed twice with warm (37 °C) PBS. The cells were then "quieted" by cultivating them for 24 h in an incubator in RPMI 1640 cultivation medium with 0.5% BSA added. Following this, the cells were centrifuged (250*g* for 10 min, 25 °C), and the obtained cell pellet was suspended in 1 mL of RPMI with 0.5% BSA added and incubated at 37 °C for 30 min. For the analysis of protein phosphorylation, the ox-LDLs were added to test tubes (50 μg/mL), very delicately mixed, and (after the test tube was closed) kept for 10 min in the incubator (stimulation).[37] After 10 min, the cell suspension was centrifuged (14000*g* for 15 s at 4 °C), the supernatant was removed, and the cell pellet obtained was placed on ice. In parts of the experiments, PD98059 (50 μM), SB 203580 (10 μM), or AN (1 μg/mL) was added to the cells 30 min prior to the addition of ox-LDLs. In some experiments, lysates from macrophages cultured only with buffer and FBS (as NC) were obtained. The cell pellet was lysed for 10 min on ice with the use of an M-Per buffer containing protease and phosphatase inhibitors. In the examined lysates, the total protein concentration was assessed by the Bradford method. Ten micrograms of total protein was added to each well. Proteins were assessed using primary mouse mAb or rabbit polyclonal antibodies: antiphospho ERK1/2 kinases (Thr 202/Tyr 204); anti-ERK1/2 MAP kinases (Thr 202/Tyr 204); antiphospho PPARγ; anti PPARγ; antiphospho p38 MAPK; and anti β-actin and subsequently detected with horseradish peroxidase-conjugated goat antimouse IgG or goat antirabbit IgG secondary antibodies. Equal loading in the lanes was evaluated by stripping the blots and reprobing with appropriate antibodies for nonphosphorylated forms of the proteins. The membranes were developed with an ECL reagent (Amersham Life

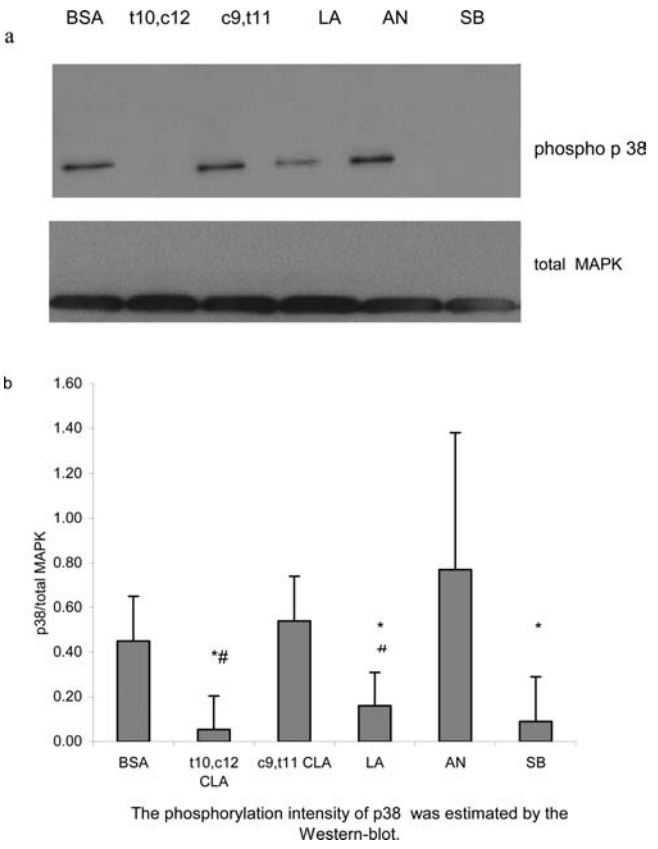

Figure 1. Effect of CLA on phosphorylation intensity of p38 (a). Cells were cultured with BSA or fatty acids as described in detail in the Materials and Methods. Afterward, the cells were stimulated for 10 min (37 °C, with oxidated LDL 50 μg/mL), and then, protein lysates were stained with antibodies against phosphorylated form p38. Mean values ± SDs of triplicate determinations of p38 protein to total MAPK ratio are presented in the diagram under the photograph (b). Each bar represents mean ± SD, $^{*}p < 0.05$ versus BSA control. $^{\#}p < 0.05$ versus *cis*-9,*trans*- 11 isomer. The data shown represent one of the three separate experiments of similar results.

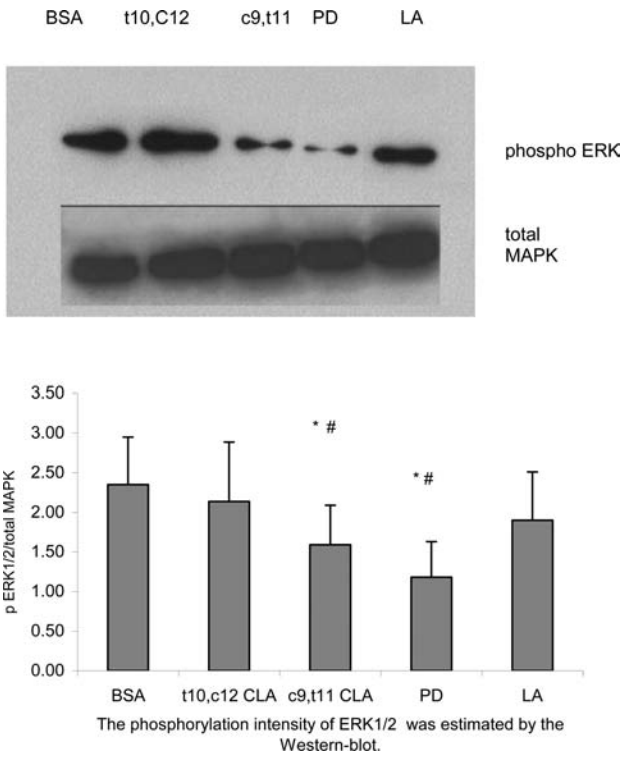

Figure 2. Effect of CLA on phosphorylation intensity of ERK1/2 (a). Cells were cultured with BSA or fatty acids as described in detail in the Materials and Methods. Afterward, the cells were stimulated for 10 min (37 °C, with oxidated LDL 50 μg/mL), and then, protein lysates were stained with antibodies against phosphorylated form ERK1/2. Total MAP was used to control the amount of protein loaded. Mean values ± SDs of triplicate determinations of ERK1/2 protein to total MAPK ratio are presented in the diagram under the photograph (b). Each bar represents mean ± SD, $^{*}p < 0.05$ vs BSA control. $^{\#}p < 0.05$ vs *trans*-10,*cis*-12 isomer. The data shown represent one of the three separate experiments of similar results.

Sciences; Little Chalfont, United Kingdom) and subsequently exposed to film (HyperFilm, Amersham). Densitometric analysis was performed using exposures that were within the linear range of the densitometer (Personal Densitometer SI, Molecular Dynamics; Sunnyvale, CA) and ImageQuant software (Molecular Dynamics). The protein concentration was estimated by the Bradford method. The results, normalized with β-actin, represent the means ± standard deviations (SDs) of triplicate determinations from three independent experiments.

Statistical Analysis. All results are expressed as means ± SDs. As the distribution in most cases deviated from normal (Shapiro-Wilk test), nonparametric tests were used. For related samples, significance was first determined with Friedmann's analysis of variance, and significant results were subjected to the Wilcoxon matched-pair test. The software used was Statistica 6.1, Statsoft (Poland); $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

***trans*-10,*cis*-12 CLA Decreased p38 Phosphorylation.** p38 phosphorylation (induced by the administration of ox-LDL) underwent a nearly total reduction in macrophages cultivated with isomer *trans*-10,*cis*-12 CLA ($p < 0.05$ vs BSA control, $n = 3$) and LA (Figure 1). p38 phosphorylation was reduced in the presence of *trans*-10,*cis*-12 CLA and LA to an extent similar to the effect of the p38 inhibitor SB 230580 ($p < 0.05$ vs BSA control, $n = 3$). In macrophages cultivated with isomer *cis*-9,*trans*-11 CLA, the intensity of p38 phosphorylation was almost identical to phosphorylation in the presence of AN, an activator of p38 phosphorylation (Figure 1). Differences at p38 phosphorylation intensity were observed between both CLA isomers and *cis*-9, *trans*-11 CLA and LA.

Intensity of Phosphorylation of ERK1/2 Kinase. In cells cultivated with *cis*-9,*trans*-11 CLA, phosphorylation of ERK1/2 decreased ($p < 0.05$ vs BSA control, $n = 3$) similar to the decrease observed in cells cultivated with PD 98059, an ERK1/2 inhibitor ($p < 0.05$ vs BSA control, $n = 3$). In macrophages cultivated with *trans*-10,*cis*-12 CLA phosphorylation of ERK1/2 was elevated as compared to *cis*-9,*trans*-11 CLA but not for BSA. LA not changed phosphorylation of ERK1/2 as compared to BSA or *cis*-9,*trans*-11 CLA (result is on the border of statistical significance).

CLA Isomers Have a Different Effect on ox-LDL-Induced PPARγ Phosphorylation. Both CLA isomers reduced the ox-LDL-induced PPARγ phosphorylation in comparison to the BSA control (Figure 3). However, the phosphorylation of PPARγ was much more intense in cells cultivated with *trans*-10,*cis*-12 CLA ($p < 0.05$ vs BSA control, $n = 3$) than in macrophages cultivated with *cis*-9,*trans*-11 CLA ($p < 0.05$ vs BSA control, $n = 3$) (Figure 3). LA was not changed PPARγ

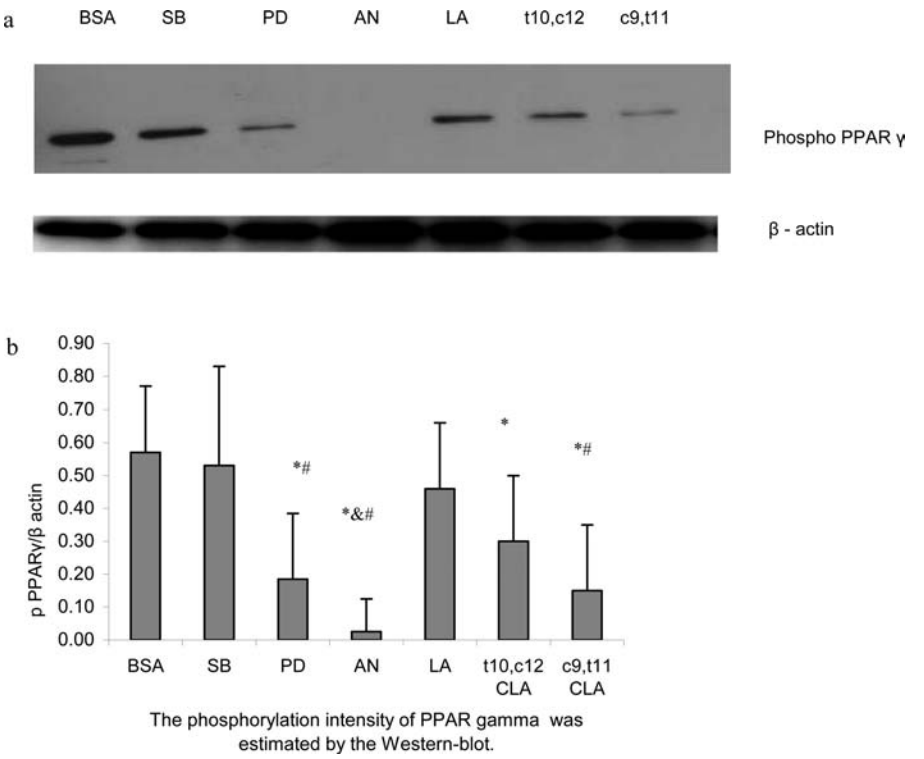

Figure 3. Effect of CLA on PPARγ phosphorylation (a). Cells were cultured with BSA or fatty acids as described in detail in the Materials and Methods. Afterward, the cells were stimulated for 10 min (37 °C, with oxidated LDL 50 μg/mL), and then, protein lysates were stained with antibodies against phosphorylated form of PPARγ. The data shown represent one of the three separate experiments of similar results. Mean values ± SDs of triplicate determinations of PPARγ protein to β-actin ratio are presented in the diagram under the photograph (b). Each bar represents mean ± SD > (n = 3), $^{*}p$ < 0.05 vs BSA control, $^{\&}p$ < 0.01 vs BSA control, and $^{\#}p$ < 0.05 vs LA.

phosphorylation as compared to BSA, but there was elevated intensity of PPARγ phosphorylation in comparison to *cis*-9,*trans*-11 CLA isomers (p < 0.05). To determine the impact of MAPK activation and PPARγ phosphorylation on ox-LDL-induced foam cell formation, we used SB 203580, a specific inhibitor of p38, and PD98059, a specific inhibitor of ERK-1/2 (Figure 3). The ERK1/2 inhibitor (PD98059) reduced phosphorylation of PPARγ more effectively (p < 0.05 vs BSA control, n = 3) than SB 203580 (p = NS, n = 3) (Figure 3). Phosphorylation of PPARγ was practically abolished in cells cultivated with AN (p < 0.01 vs BSA control, n = 3).

When cells were cultivated with combinations of CLA and inhibitors (SB 203580 or PD98059), the intensity of PPARγ phosphorylation was different. This phenomenon was elevated in cells cultured with *trans*-10,*cis*-12 CLA in comparison to *cis*-9, *trans*-11 isomer (Figure 4a,b) and was decreased in cells cultured with the combination *trans*-10,*cis*-12 CLA with PD98059 (p < 0.05 vs *trans*-10,*cis*-12 CLA control, n = 3) (Figure 4a,c). The second isomer of CLA affected PPARγ phosphorylation more effectively as compared to *trans*-10,*cis*-12. PD98059 did not change PPARγ phosphorylation as compared to cells cultivated with *cis*-9,*trans*-11 (p = NS vs *cis*-9,*trans*-11 CLA control, n = 3), whereas SB 203580 tended to slightly elevate this phenomenon of PPARγ (p = NS *cis*-9,*trans*-11 CLA control, n = 3) (Figure 4b,d). In macrophages cultivated with CLA, the expression of mRNA PPARγ and the content of PPAR did not change (Figure 5) and (Figure 6).

While the anti-inflammatory properties of CLAs seem to be unequivocal in animal models,[5,38−40] their role during inflammation in human cells (e.g., macrophages) is still unclear.[41] Recent investigations have demonstrated that MAPKs are activated by ox-LDL stimulation. PPARγ is phosphorylated by MAPK family members.[22] In human macrophages, activation of PPARγ was shown to have anti-inflammatory effects. In these cells, PPARγ regulated the expression of genes involved in lipid metabolism and homeostasis by inducing expression of several key genes involved in cholesterol concentration.[16,27,40,42] The phosphorylation status of PPARγ is mediated by MAP kinase activation, which subsequently decreases PPARγ target gene expression.[24] Synthetic activators of PPARγ are capable of reducing macrophage foam cell cholesterol accumulation through the activation of genes involved in cholesterol efflux.[43] CLA induced resolution of the atherosclerosis process in animals by the negative regulation of pro-inflammatory genes and activation of apoptosis in the atherosclerotic lesion. In macrophages, ox-LDL-induced phosphorylation of both ERK1/2 and p38 MAP kinase, and consequently, PPARγ led to repression of PPARγ activation and finally to inhibition of ox-LDL uptake.[21,34] In the current study, the influence of CLA on the human body and atherosclerotic processes was not assessed; we studied only the molecular mechanism of PPARγ activation in cell line macrophages. Therefore, any physiological and functional conclusions on human health were not expressed. We evaluated whether CLAs could change the phosphorylation status of PPARγ (similarly to human adipocytes).[42] We speculate that CLAs may act as ligands and activators of PPARγ in macrophages in an isomer-specific manner. As compared to *cis*-9,*trans*-11, *trans*-10,*cis*-12 CLA isomer increased phosphorylation of ERK 1/2

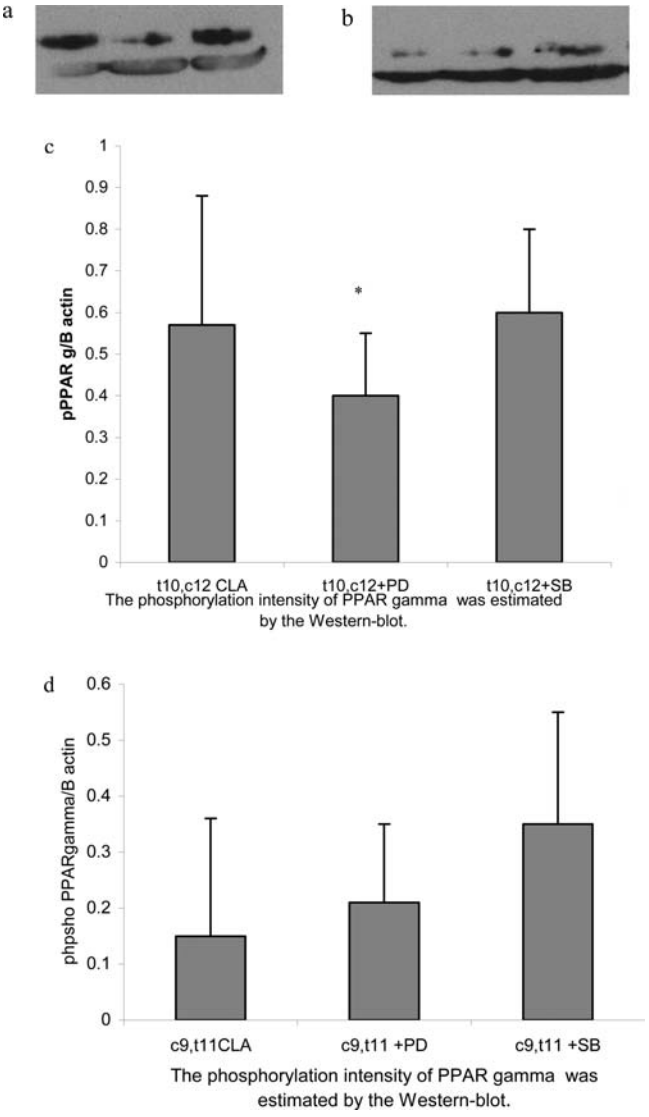

Figure 4. Effect of CLA and inhibitors on PPARγ phosphorylation (a). Cells were cultured with *trans*-10,*cis*-12 CLA or/and with inhibitors (a) as well as with *cis*-9,*trans*-11 CLA or/and with inhibitors (b) as described in detail in the Materials and Methods. Afterward, the cells were stimulated for 10 min (37 °C, with oxidated LDL 50 μg/mL), and then, protein lysates were stained with antibodies against phosphorylated form of PPARγ. Ten micrograms of total protein was loaded per lane. β-Actin was used to control the amount of protein loaded. The data shown represent one of the three separate experiments of similar results. Mean values ± SDs of triplicate determinations of PPARγ protein to β-actin ratio are presented in the diagram under the photograph (c and d). *p < 0.05 vs *trans*-10,*cis*-12 CLA control.

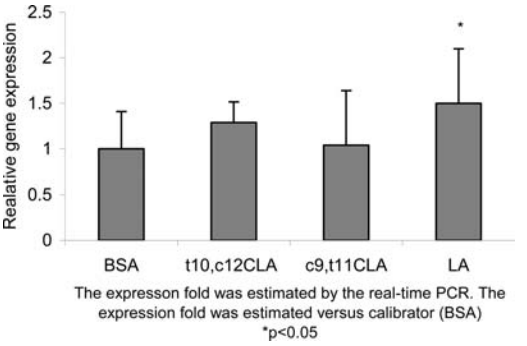

Figure 5. Expression of genes measured by quantitative real-time PCR in macrophages from macrophages. Cells were cultured with fatty acids for 48 h as described in the Materials and Methods. Data are expressed as the relative gene expression ratio. Each bar represents mean ± SD of triplicate determinations from three independent experiments, *p < 0.05 vs BSA control.

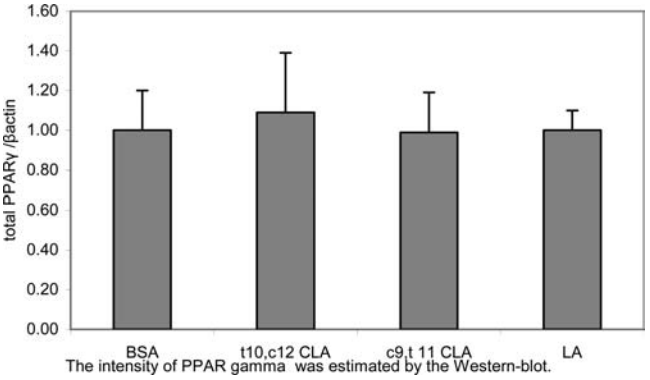

Figure 6. Effect of CLA on PPARγ expression (a). Cells were cultured with BSA or fatty acids as described in detail in the Materials and Methods. Afterward, the cells were stimulated for 10 min (37 °C), with oxidated LDL (50 μg/mL), and then, protein lysates were stained with antibodies against of PPARγ. Mean values ± SDs of PPARγ protein to β-actin total ratio are presented in the diagram under the photograph (b). The results represent the mean ± SD of triplicate determinations from three independent experiments. *p < 0.05 vs BSA control.

MAPK (Figure 2), whereas phosphorylation of p38 MAPK was completely absent (Figure 1). Importantly, isomer *trans*-10,*cis*-12 CLA also seems to intensified phosphorylation of PPARγ (Figures 3 and 4), while mRNA (Figure 5) and protein levels of PPARγ in these cells were unchanged (Figure 6). We suggest that phosphorylation of PPARγ in macrophages is conducted mainly by ERK1/2. When PD 98059 (inhibitor of ERK1/2) was added, the reduction in PPARγ phosphorylation was stronger than when SB230580 (inhibitor of p38) was added to the cell culture medium (Figures 3 and 4). We propose that CLA isomers changed PPARγ activity at two different intensities: *trans*-10,*cis*-12 CLA decreased phosphorylation of PPARγ (via activation by ERK 1/2 but not by phosphorylation of p38), whereas *cis*-9,*trans*-11 CLA decreased phosphorylation of PPARγ via activation of ERK1/2 MAPK (Figure 3). LA—a key fatty acid of n-6 family—did not change the phosphorylation of PPARγ (as compared to BSA control) but elevated its expression (Figures 3 and 5).

These results confirm observations by Takeda who indicated that ox-LDL induced activation of PPARγ by the activation of ERK-1/2 but not p38 MAPK. Inhibition of ERK1/2 (by PD 98059) abrogated ox-LDL-induced PPARα and PPARγ activation in cells, whereas p38 MAPK specific inhibition had no effect.[22] We hypothesized that CLA isomers antagonized ligand-dependent activation of PPAR in a different way. *trans*-10,*cis*-12 CLA was previously shown to be the isomer responsible for a reduction in body fat in animals. This isomer specifically decreased triglyceride (TG) accumulation in primary human adipocytes in vitro.[34,44] In human adipocytes, *trans*-10,*cis*-12 CLA decreased, while *cis*-9,*trans*-11 CLA increased the expression of PPAR and several of its downstream target genes.

We suggest that a similar process may occur in macrophages/fatty acid-laden macrophages, although this is only a suggestion, as we did not assess the effect of the expression of downstream genes regulated by PPARγ in this study. Our results support (but only as a supposition) the study of Yu et al. and Song et al., who showed that both CLAs or *trans*-10,*cis*-12 CLA activate PPARγ receptors and consequently activated anti-inflammatory properties in mouse RAW macrophages.[45,46] However, the consequences of PPARγ activation were not studied in this paper, and it seems that both CLA isomers slightly reduced phosphorylation of PPARγ (as compared to BSA), which may slightly increase receptor activity. What is interesting is that increased mRNA and protein levels of PPARγ were not observed in this study. Such a phenomenon—elevation of PPARγ mRNA as well as protein levels—was observed when macrophages were obtained from blood monocytes (porcine or human) and cultured with CLA isomers,[47,48] whereas in THP-1 macrophages PPARγ and genes controlled by this receptor were not expressed.[28] These contradictory results highlight the essential differences between macrophages obtained from two distinct sources (blood vs THP-1 monocytes), in addition to the findings of this study.

What do these findings mean in the context of diet and human health? It is difficult to assess. The results of previous population-based studies are inconclusive[49−53] and require further observations on large groups of subjects.

AUTHOR INFORMATION

Corresponding Author

*Tel: +48914414806. Fax: +48 91 4414805. E-mail: ewa.stachowska@pum.edu.pl.

Funding Sources

This work was supported by grant no. 15 POB 11723 from the State Committee for Scientific Research, Poland.

ABBREVIATIONS USED

AN, anisomycine—the activator of p38 phosphorylation; CLA, conjugated linoleic acid; ERK, extracellular signal-regulated protein kinases 1 and 2; LA, linoleic acid; MAPK, mitogen-activated kinase; NC, negative control; ox-LDL, oxidated low-density lipoprotein; p38, mitogen-activated kinase; PD, PD 98059—the ERK1/2 inhibitor; PPAR, peroxisome proliferator activated receptors; SB, SB 203580—the inhibitor of p38 kinase phosphorylation

REFERENCES

(1) Badinga, L.; Greene, E. S. Physiological properties of conjugated linoleic acid and implications for human health. *Nutr. Clin. Pract.* **2006**, *21*, 367–363.

(2) Park, S. J.; Park, C. W.; Kim, S. J.; Kim, J. K.; Kim, Y. R.; Park, K. A.; Kim, J. O.; Ya, Y. L. Methylation methods for the quantitative analysis of conjugated linoleic acid (CLA) isomers in various lipid samples. *J. Agric. Food Chem.* **2002**, *50*, 989–986.

(3) Kuhnt, K.; Kraft, J.; Vogelsang, H.; Eder, K.; Kratzsch, J.; Jahreis, G. Dietary supplementation with trans-11- and trans-12−18: 1 increases cis-9, trans-11-conjugated linoleic acid in human immune cells, but without effects on biomarkers of immune function and inflammation. *Br. J. Nutr.* **2007**, *97*, 1196–1205.

(4) AbuGhazaleh, A. A.; Schingoethe, D. J.; Hippen, A. R.; Kalscheur, K. F. Conjugated linoleic acid and vaccenic acid in rumen, plasma, and milk of cows fed fish oil and fats differing in saturation of 18 carbon fatty acids. *J. Dairy Sci.* **2003**, *86*, 3648–3660.

(5) Butz, D. E.; Li, G.; Huebner, S. M.; Cook, M. E. A mechanistic approach to understanding conjugated linoleic acid's role in inflammation using murine models of rheumatoid arthritis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2007**, *293*, R669–R676.

(6) Evans, M.; Park, Y.; Pariza, M.; Curtis, L.; Kuebler, B.; McIntosh, M. Trans-10,cis-12 conjugated linoleic acid reduces triglyceride content while differentially affecting peroxisome proliferator activated receptor gamma2 and aP2 expression in 3T3-L1 preadipocytes. *Lipids* **2001**, *36*, 1223–1232.

(7) Evans, M.; Geigerman, C.; Cook, J.; Curtis, L.; Kuebler, B.; McIntosh, M. Conjugated linoleic acid suppresses triglyceride accumulation and induces apoptosis in 3T3-L1 preadipocytes. *Lipids* **2001**, *36*, 899–910.

(8) Whigham, L. D.; Cook, E. B.; Stahl, J. L.; Saban, R.; Bjorling, D. E.; Pariza, M. W.; Cook, M. E. CLA reduces antigen-induced histamine and PGE(2) release from sensitized guinea pig tracheae. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2001**, *280*, R908–R912.

(9) Lee, K. N.; Kritchevsky, D.; Pariza, M. W. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis* **1994**, *108*, 19–21.

(10) Lee, S. H.; Yamaguhi, K.; Kim, J. S.; Elinq, T. E. Conjugated linoleic acid stimulates an anti-tumorigenic protein NAG-1 in an isomer specific manner. *Carcinogenesis* **2006**, *27*, 972–981.

(11) Lee, K. W.; Lee, H. J.; Cho, H. Y.; Kim, Y. J. Role of the conjugated linoleic acid in the prevention of cancer. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 135–144.

(12) Stachowska, E.; Chlubek, D. Conjugated linoleic acid-a safe dietary supplement? *J. Clin. Chin. Med.* **2009**, *4*, 181–185.

(13) Riserus, U.; Basu, S.; Jovinge, S.; Fredrikson, G. N.; Arnolov, V.; Vesseby, B. Supplemantation with conjugated linoleic acid causes isomer-dependent oxidative stress and elevated C-reactive protein: A potential link to fatty acid-induced insulin resistance. *Circulation* **2002**, *15*, 1925–1929.

(14) Kummerow, F. A.; Zhou, Q.; Mahfouz, M. M.; Smircky, M. R.; Grieshop, C. M.; Schaeffer, D. J. *Trans* fatty acids in hydrogenated fat inhibited the synthesis of the polyunsaturated fatty acids in the phospholipid of arterial cells. *Life Sci.* **2004**, *16*, 2707–2723.

(15) Roach, C.; Feller, S. E.; Ward, J. A.; Shaikh, S. R.; Zerouga, M.; Stillwell, W. Comparison of *cis* and *trans* fatty acid containing phosphatidylocholines on membrane properties. *Biochemistry* **2004**, *43*, 6344–6351.

(16) Pariza, M. W.; Park, Y.; Cook, M. E. The biologically active isomers of conjugated linoleic acid. *Prog. Lipid Res.* **2002**, *40*, 283–298.

(17) Stachowska, E.; Dziedziejko, V.; Safranow, K.; Gutowska, I.; Adler, G.; Ciechanowicz, A.; Machaliński, B.; Chlubek, D. Inhibition of phospholipase A 2 activity by conjugated linoleic acid in human macrophages. *Eur. J. Nutr.* **2007**, *46*, 28–33.

(18) Kota, B. P.; Huang, T. H.; Roufogalis, B. D. An overview on biological mechanisms of PPARs. *Pharmacol. Res.* **2005**, *51*, 85–94.

(19) Ahmed, W.; Ziouzenkova, O.; Brown, J; Devchand, P.; Francis, S.; Kadakia, M.; Kanda, T.; Orasanu, G.; Sharlach, M.; Zandbergen, F.; Plutzky, J. PPARs and their metabolic modulation: New mechanisms for transcriptional regulation? *J. Intern. Med.* **2007**, *262*, 184–198.

(20) Taketa, K.; Matsumura, T.; Yano, M.; Ishii, N.; Senokuchi, T.; Motoshima, H.; Murata, Y.; Kim-Mitsuyama, S.; Kawada, T.; Itabe, H.; Takeya, M.; Nishikawa, T.; Tsuruzoe, K.; Araki, E. Oxidized low density lipoprotein activates peroxisome proliferator-activated receptor-alpha (PPARalpha) and PPARgamma through MAPK-dependent COX-2 expression in macrophages. *J. Biol. Chem.* **2008**, *283*, 9852–9862.

(21) Senokuchi, T.; Matsumura, T.; Sakai, M.; Matsuo, T.; Yano, M.; Kiritoshi, S.; Sonoda, K.; Kukidome, D; Nishikawa, T.; Araki, E. Extracellular signal-regulated kinase and p38 mitogen-activated protein kinase mediate macrophage proliferation induced by oxidized low-density lipoprotein. *Atherosclerosis* **2004**, *176*, 233–245.

(22) Forman, H. J.; Torres, M. Reactive oxygen species and cell signaling: Respiratory burst in macrophage signaling. *Am. J. Respir. Crit. Care Med.* **2002**, *166*, S4–S8.

(23) Rahman, M. M.; Bhattacharya, A.; Fernandes, G. J. Conjugated linoleic acid inhibits osteoclast differentiation of RAW264.7 cells by modulating RANKL signaling. *Lipid Res.* **2006**, *47*, 1739–1748.

(24) Pearson, G.; Robinson, F.; Beers Gibson, T.; Xu, B. E.; Karandikar, M.; Berman, K.; Cobb, M. H. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr. Rev.* **2001**, *22*, 153–183.

(25) Yin, R.; Dong, Y. G.; Li, H. PPARg phosphorylation mediated by JNK MAPK: A potential role in macrophage-derived foam cell formation. *Acta Pharm. Sin.* **2006**, *27*, 1146–1152.

(26) Cohn, Z. A.; Benson, B. The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **1965**, *1*, 153–170.

(27) Fu, Y.; Luo, N.; Lopes-Virella, M. F.; Garvey, W. T. The adipocyte lipid binding protein (ALBP/aP2) gene facilitates foam cell formation in human THP-1 macrophages. *Atherosclerosis* **2002**, *165*, 259–269.

(28) Ringseirs, R.; Schulz, N.; Eder, K. Troglitazone but not conjugated linoleic acid reduces gene expression and activityy of matrix-metalloproteinases-2 and -9 in PMA—Differentiated THP-1 macrophages. *J. Nutr. Biochem.* **2008**, *19*, 596–603.

(29) Steinbrecher, U. P.; Witztum, J. L.; Parthasarathy, S.; Steinberg, D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. *Arteriosclerosis* **1987**, *7*, 135–143.

(30) Jialal, I.; Grundy, S. M. Preservation of the endogenous antioxidants in low density lipoprotein by ascorbate but not probucol during oxidative modification. *J. Clin. Invest.* **1991**, *87*, 597–601.

(31) Cynshi, O.; Takashoma, Y.; Suzuki, T.; Kawabe, Y.; Ohba, Y.; Kodama, T. Characterization of aggregated low density lipoproteins induced by copper-catalyzed oxidation. *J. Atheroscler. Thromb.* **1994**, *1*, 87–96.

(32) Havel, R. J.; Eder, H. A.; Brahdon, J. A. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **1955**, *34*, 1345–1353.

(33) Kojima, T.; Kikugawa, K.; Kosugi, H. Is the thiobarbituric acid-reactivity of blood plasma specific to lipid peroxidation? *Chem. Pharm. Bull.* **1990**, *38*, 414–418.

(34) Brown, J. M.; Boysen, M. S.; Jensen, S. S.; Morrison, R. F.; Storkson, J.; Lea-Currie, R.; Pariza, M.; Mandrup, S.; McIntosh, M. K. Isomer-specific regulation of metabolism and PPAR gamma signalling by CLA in human preadipocytes. *J. Lipid Res.* **2003**, *44*, 1287–1300.

(35) Dembinska-Kiec, A.; Polus, A.; Kiec-Wilk, B.; Grzybowska, J.; Mikołajczyk, M.; Hartwich, J.; Razny, U.; Szumilas, K.; Banas, A.; Bodzioch, M.; Stachura, J.; Dyduch, G.; Laider, P.; Zagajweski, J.; Langman, T.; Schmitz, G. Proangiogenic activity of beta-carotene is coupled with the activation of endothelial cell chemotaxis. *Biochim. Biophys. Acta* **2005**, *1740*, 222–229.

(36) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45.

(37) Baranova, I. N.; Vishnyakova, T. G.; Bocharov, A. V.; Kurlander, R.; Chen, Z.; Kimelman, M. L.; Remaley, A. T.; Csako, G.; Thomas, F.; Eggerman, T. L. Patterson AP.Serum amyloid A binding to CLA-1 (CD36 and LIMPII analogous-1) mediates serum amyloid A protein-induced activation of ERK1/2 and p38 mitogen-activated protein kinases. *J. Biol. Chem.* **2005**, *280*, 8031–8040.

(38) Bassaganya-Riera, J.; Reynolds, K.; Martino-Catt, S.; Cui, Y.; Hennighausen, L.; Gonzalez, F.; Rohrer, J.; Benninghoff, A. U.; Hontecillas, R. Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology* **2004**, *127*, 777–791.

(39) Jaudszus, A.; Krokowski, M.; Möckel, P.; Darcan, Y.; Avagyan, A.; Matricardi, P.; Jahreis, G.; Hamelmann, E. Cis-9,trans-11-conjugated linoleic acid inhibits allergic sensitization and airway inflammation via a PPAR gamma-related mechanism in mice. *J. Nutr.* **2008**, *138*, 1336–1342.

(40) Ringseis, R.; Wen, G.; Saal, D.; Eder, K. Conjugated Linoleic Acid Isomers Reduce Cholesterol Accumulation in Acetylated LDL-Induced Mouse RAW264.7 Macrophage-Derived Foam Cells. *Lipids* **2008**, *43*, 913–923.

(41) O'Shea, M.; Bassaganya-Riera, J.; Mohede, I. C. Immunomodulatory properties of conjugated linoleic acid. *Am. J. Clin. Nutr.* **2004**, *79*, 1199S–1206S.

(42) Tontonoz, P.; Nagy, L.; Alvarez, J. G.; Thomazy, V. A.; Evans, R. M. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. *Cell* **1998**, *93*, 241–252.

(43) Toomey, S.; Roche, H.; Fitzgerald, D.; Belton, O. Regression of pre-established atherosxclerosis in the apoE−/− mouse by conjugated linoleic acid. *Biochem. Soc. Trans.* **2003**, *31*, 1075–1079.

(44) Kennedy, A.; Chung, S.; LaPoint, K.; Fabiyi, O.; McIntosh, M. K. Trans-10, cis-12 conjugated linoleic acid antagonizes ligand-dependent PPARgamma activity in primary cultures of human adipocytes. *J. Nutr.* **2008**, *138*, 455–461.

(45) Yu, Y.; Correll, P. H.; Vanden Heuvel, J. P. Conjugated linoleic acid decreases of pro-inflammatory products in macrophages: Evidence for a PPAR gamma-dependent mechanism. *Biochim. Biophys. Acta* **2002**, *1581*, 89–99.

(46) Song, D. H.; Kang, J. H.; Lee, G. S.; Jeung, E. B.; Yang, M. P. Upregulation of tumor necrosis factor-alpha expression by trans-10-cis-12 conjugated linoleic acid enhances phagocytosis of RAW macrophages via a peroxisome proliferator-activated receptor gamma-dependent pathway. *Cytokine* **2007**, *37*, 227–235.

(47) Kang, J. H.; Lee, G. S.; Jeung, E. B.; Yang, M. P. Trans-10,cis-12 conjugated linoleic acid increases phagocythosis of porcine peripheral blood polymorphonuclear cells in vitro. *Br. J. Nutr.* **2007**, *97*, 117–125.

(48) Stachowska, E.; Baśkiewicz-Masiuk, M.; Dziedziejko, V.; Adler, G.; Bober, J.; Machaliński, B.; Chlubek, D. Conjugated linoleic acid can change phagocytosis of human monocytes/macophages by reduction in Cox-2 expression. *Lipids* **2007**, *42*, 707–716.

(49) Racine, N. M.; Watras, A. C.; Carrel, A. L.; Allen, D. B.; McVean, J. J.; Clark, R. R.; O'Brien, A. R.; O'Shea, M.; Scott, C. E.; Schoeller, D. A. Effect of conjugated linoleic acid on body fat accretion in overweight or obese children. *Am. J. Clin. Nutr.* **2010**, *9*, 1157–1164.

(50) Iwata, T.; Kamegai, T.; Yamauchi-Sato, Y.; Ogawa, A.; Kasai, M.; Aoyama, T.; Kondo, K. Safety of dietary conjugated linoleic acid (CLA) in a 12-weeks trial in healthy overweight Japanese male volunteers. *J. Oleo Sci.* **2007**, *56*, 517–525.

(51) Kuhnt, K.; Kraft, J.; Vogelsang, H.; Eder, K.; Kratzsch, J.; Jahreis, G. Dietary supplementation with trans-11- and trans-12−18: 1 increases cis-9, trans-11-conjugated linoleic acid in human immune cells, but without effects on biomarkers of immune function and inflammation. *Br. J. Nutr.* **2007**, *97*, 1196–1205.

(52) Larsen, T. M.; Toubro, S.; Gudmundsen, O.; Astrup, A. Conjugated linoleic acid supplementation for 1 y does not prevent weight or body fat regain. *Am. J. Clin. Nutr.* **2006**, *83*, 606–612.

(53) Pariza, M. W. Perspective on the safety and effectiveness of conjugated linoleic acid. *Am. J. Clin. Nutr.* **2004**, *79*, 1132S–1136S.