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Conjugated linoleic acids regulate triacylglycerol and cholesterol concentrations in macrophages/foam cells by the modulation of CD36 expression

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Atherosclerosis is an inflammatory disease characterised by the accumulation of lipids and their metabolites in the artery wall. During inflammation circulating LDL are taken up by macrophages through two major scavenger receptors: CD36 and scavenger receptor A (SRA). Fatty acids that are common in food, e.g. linoleic acid and *n-3* unsaturated fatty acids can modulate expression of CD36 on the macrophage surface. Conjugated linoleic acid isomers (CLA) that originate from the human diet, have demonstrated antiatherogenic properties in several experiments. Animal study evidenced that CLA could induce resolution of plaque by activation of peroxisome proliferator activated receptors and down-regulation of pro-inflammatory genes. Less unequivocal results were obtained in human studies (on the CLA effects on the inflammatory process). Therefore in this study we investigated the influence of CLA on CD36 expression and lipid accumulation in human macrophages. Macrophages were incubated with 30 μM *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA or linoleic acid for 48 h. After that, expression of CD36 as well as accumulation of lipids were measured by flow cytometry, microscopy and a spectroscopic method. We demonstrate that both *cis*-9,*trans*-11 C 18:2 CLA and linoleic acid slightly elevated expression of CD36, whereas second isomer — *trans*-10,*cis*-12 CLA — did not. Nevertheless, only *trans*-10,*cis*-12 CLA triggered delipidation of macrophages, that is decreased triacylglycerols concentration. Also in human adipocytes, *trans*-10,*cis*-12 CLA causes cell delipidation by reduction of PPAR receptor expression. We propose a similar mechanism for human macrophages/foam cells.

Keywords: CLA, CD36, macrophages, foam cells, triacylglycerols, cholesterol, PPAR

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INTRODUCTION

Conjugated linoleic acids (CLA) are an extraordinary category of stereoisomers of linoleic acid (Park *et al.,* 2002; Badigna *et al.*, 2006). CLA isomers — *cis*-9,*trans*-11 C18:2 and *trans*-10,*cis*-12 C18:2 are common in the diet (from food or popular dietary supplements) (AbuGhazaleh *et al.,* 2003). Animal studies have evidenced antiatherosclerotic, anticancer and antidiabetic properties of CLA (Park *et al.,* 2002; Evans *et al.*, 2001a; 2001b). CLA inhibits the atherosclerotic process by reducing inflammation, and the concentration of triacylglycerols and total and LDL cholesterol (Evans *et al.,* 2001a; 2001b; Park *et al.,* 2002). Less unequivocal results were obtained in studies on the effects of CLA in humans (Lee *et al.,* 1994; Lee *et al.,* 2005; 2006a; 2006b; Whigham *et al.,* 2001).

Atherosclerosis is a progressive chronic inflammatory disease characterised by deposition of triacylglycerols, cholesterol, calcium and other substances within the artery wall. The accumulation of lipids and their metabolites can induce chronic inflammation by promoting macrophage infiltration and activation (Prieur *et al.,* 2009). During inflammatory disorders, macrophages accumulate within the arterial neointima and become a major contributor of the atherosclerotic plaque (Rahman *et al.,* 2006). The first stage of this process is the appearance of dysfunctional endothelial cells whose activated adhesion molecules and expressed chemokines recruit circulating monocytes and a subpopulation of lymphocytes (CD4/CD8) into the intima (Collot-Texiera *et al.,* 2007).

In this pathological setting, a high level of LDL may accumulate in the arterial wall, where it undergoes modifcation by macrophages (Prieur *et al.,* 2009). The modification of LDL increases lipoprotein uptake by macrophages through the overexpression of two major scavenger receptors: CD36 and scavenger receptor A (SRA) (Prieur *et al.,* 2009). CD36 is a multi-ligand scavenger receptor present on the surface of a number of cells such as monocytes/macrophages, platelets, and endothelial cells. Monocyte/macrophage CD36 has been shown to play a key role in the development of atherosclerotic lesions by its capacity to bind and endocytose oxidised low density lipoproteins (ox-LDL), and its implication in the formation of foam cells (Collot-Texiera *et al.,* 2007). Expression of CD36 in macrophages is controlled by peroxisome proliferator activated receptors type γ (PPAR γ) (Toomey *et al.,* 2003). PPAR γ also plays a role in maintaining the balance between lipid influx and efflux in macrophages. Although PPAR γ activation induces the fatty acid transporter CD36, recent work shows that this effect is not associated with foam cell formation. CD36 can promote lipid efflux by inducing ATP binding

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Abbreviations: CD36, scavenger receptor A; CLA, conjugated linoleic acid; FC, foam cells; LA, linoleic acid; ox-LDL, oxidated low density lipoprotein; PPAR, peroxisome proliferator activated receptors; SRA, scavenger receptor A

cassette protein A1 (ABCA1) (Taketa *et al.,* 2008). Fatty acids and their derivatives 9-HODE, 13 -HODE, can modulate CD36 gene expression in human macrophages (Collot-Texiera *et al.,* 2007; Prieur *et al.,* 2009). *n-3* fatty acids of fish oil origin: docosahexaenoic (DHA) and eicosapentaenoic (EPA) that are considered as preventive factor against inflammatory diseases reduced the expression of both mRNA and protein of CD36 in monocytic cells (Pietsch *et al.,* 1995; Collot-Texiera *et al.,* 2007). Linoleic acid (*cis*-9,*cis*-12 C 18:2) was reported to increase CD36 expression in human macrophages (Vallve *et al.,* 2002).

Previously we demonstrated a pro-atherogenic activity of CLA (Stachowska *et al.,* 2007; 2008). Since CD36 has been implicated in atherosclerotic plaque development, we decided to investigate the influence of CLA on the expression of CD36 and concentrations of triacylglycerols and cholesterol in human macrophages/foam cells.

MATERIALS AND METHODS

Materials. Cell culture media and fetal bovine serum (FBS) were obtained from Gibco (UK). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or Sigma (Sigma-Aldrich, Poland), unless stated otherwise. Isomers of CLA (+98% pure) were purchased from Nu-CheK Prep (USA). CD36 antibody was purchased from BD Pharmingen (USA). Commercial kits for determination of triacylglycerols and total cholesterol were from Biolabo (France). GW 6471, a PPAR α antagonist, was obtained from Calbiochem (USA).

Cell culture and treatment. The human monocytic cells (THP-1) were purchased from American Type Culture Collection (ATCC, Rockville MD, USA) and 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. The cells were differentiated to macrophages by administration of phorbol myristate acetate (PMA).

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 (Collot-Texiera *et al.,* 2007). After incubation with PMA, adherent cells (macrophages) were washed three times with phosphate-buffered saline (PBS) and incubated with 30 μM fatty acids (*cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA or linoleic acid) for 48 h at 37 °C as described in detail (Stachowska *et al.,* 2007). Foam cells were obtained after additional macrophage incubation with ox-LDL (50 μg/ml) for 24 h at 37 °C. In some experiments, after incubation with fatty acids the cells were cultured for 24 h with cholesterol (ethanolic solution; 50 μg/ml).

Preparation of fatty acids. Both isomers of CLA were complexed to fatty acid-free (>98%) bovine serum albumin (BSA) at a 4:1 molar ratio using as described in detail (Evans *et al.,* 2001).

Preparation of ox-LDL (induced by Cu^{2+}). LDL was isolated from blood plasma obtained from patients with a normal lipid profile. The plasma was centrifugation (112 000 × *g* for 24 h at 16 °C) in a Beckman L8-80M ultracentrifuge fitted with a Ti 80 rotor (Baillie *et al.,* 1996). The disc containing LDL obtained after centrifuging was transferred to an Econo-Pac 10 DG Sephadex G-25 column (Pharmacia) to remove impurities. The obtained LDL suspension was kept with EDTA (1 mg/ml) added in nitrogen atmosphere at 4 °C until the time of oxidation (not longer than one week). LDL suspension (1 ml) was transferred to an Econo-Pac 10 DG column washed with PBS and the filtrate was collected. After each LDL portion, the column was washed with 5 ml of PBS and then the next LDL portion was applied. Protein concentration in the filtrate was measured with the Bradford reagent; the filtrate was diluted with PBS to 100 μg of protein/ml, $Cu\,SO_4$ was added (10 μM) and the total content was incubated for 6 h at 37 °C. Oxidation was inhibited at 4 °C by adding EDTA (200 μM) and butylated hydroxytoluene, BHT (40 μM). The total content was dialysed. After dialysis, 200 μl of the suspension was taken for measurement of protein and thiobarbituric acid reactive substances (TBARS) content (Havel *et al.,* 1955). The ox-LDL received was frozen at –80 °C for not more than 3 months. A freezing-unfreezing cycle was avoided. ox-LDLs were added to macrophages to reach LDL final protein concentration in the cultivation well of 50 μg/ml.

Expression of CD36 measurement. The percentage of CD36+ cells was assessed by flow cytometry (FACScan) using CellQuest software. CD36+ cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde and incubated with fluorochrome-conjugated monoclonal PE CD36 antibody for 30 min. Mouse IgM was used as an isotype control antibody (Becton Dickinson, Oxford, UK). Macrophages cultured without BSA and fatty acids were used in this study as a negative control.

Measurement of total cholesterol and triacylglycerol content in macrophages/foam cells. THP-1-derived macrophages (2.5×10^6 cells/well) were cultured with 30 μM fatty acids and with cholesterol (50 μg/ml). Incubation at 37 °C lasted for 48 h. After that, in macrophages which were differentiated to foam cells, incubation with ox-LDL (50 μg/ml for 24 h at 37 °C) was additionally conducted. After incubation the cells were scraped, washed three times with PBS (10 min, 850 × *g*), and 1 ml isopropyl alcohol was added, the suspension was sonicated for 30 s and used for lipid determinations. Triacylglycerols and total cholesterol were determined by an enzymatic method using a commercial kit (Biolabo, France) with spectroscopic reading (Perkin Elmer Lambda 40) and referred to the protein concentration in the cells. Protein concentration was estimated by the Bradford method with commercial reagents (Sigma-Aldrich).

Oil Red O (ORO) staining and confocal microscopy. Macrophages (2.5×10^6 cells/well) were cultured with 30 μM fatty acids for 48 h (37 °C) as described above. In macrophages after incubation with fatty acids, stimulation with ox-LDL (50 μg/ml for 24 h) was conducted. After incubation with fatty acids, cells were washed three times with PBS to remove suspended apoptotic cells. The phagocytic cells were fixed with 10% formalin for 40 min. After a wash with PBS, the cells were stained with saturated ORO solution in isopropanol/water (3:2, v/v) for 15 min. Then, the cells were washed with 70% ethanol for 5 s to remove background staining. Finally, the cells were rinsed in tap water, counterstained with Harris hematoxylin (10 s), and mounted in glycerol/PBS (9:1, v/v) for observation. Cells were viewed *in situ* in 35 mm diameter tissue culture plates under a bright-field using an Olympus IMT-2 inverted phase–contrast microscope with ×100 objective (Cynshi *et al.,* 1994).

Confocal microscopy is a powerful imaging technique for this task, due to its capability to perform high resolution imaging and optical sectioning in both reflectance and fluorescence modes. Macrophages (2.5×10^6 cells/well) were cultured with 30 μM fatty acids for 48 h

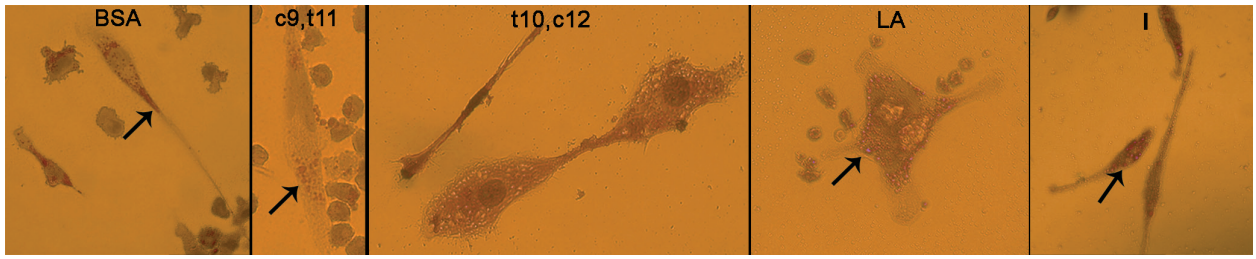

Figure 1. Cell morphology and lipid droplets in macrophages
Macrophages were treated with BSA (a vehicle control), 30 μmol/l *cis*-9,*trans*-11-C 18:2, *trans*-10,*cis*-12-C 18:2, or linoleic acid for 2 days. Cells were harvested, fixed with 4% paraformaldehyde and stained with Oil Red O. Then cells were lightly counterstained with hematoxylin. Intracellular lipid droplets were stained red (100x microscopic field). Droplets are marked by black arrows. Cells cultured with BSA are labeled BSA; with *cis*-9,*trans*-11-C 18:2 — c9,t11; with *trans*-10,*cis*-12 — t10,c12; with linoleic acid — LA; with GW 6471 — I.

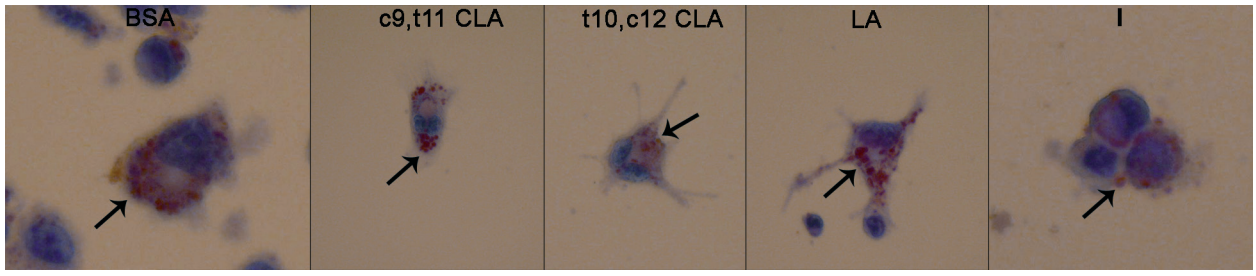

Figure 2. Cell morphology and lipid droplets in foam cells
Foam cells were treated with BSA (a vehicle control), 30 μmol/l *cis*-9,*trans*-11-C 18:2, *trans*-10,*cis*-12-C 18:2, or linoleic acid for 2 days. Foam cells were obtained after additional macrophage incubation with ox-LDL (50 μg/ml) for 24 h at 37°C. Cells were then harvested, fixed with 4% paraformaldehyde and with Oil Red O. Then cells were lightly counterstained with hematoxylin. Intracellular lipid droplets were stained in red (100x microscopic field). Droplets are marked by black arrows. Cells cultured with BSA are labeled BSA; with *cis*-9,*trans*-11-C 18:2 — c9,t11; with *trans*-10,*cis*-12 — t10,c12; with linoleic acid — LA; with GW 6471 — I.

(37°C), and then with cholesterol (50 μg/ml)/24 h. After that, cells were viewed with an Olympus FV500 laser scanning confocal microscope.

Statistical analysis. All results are expressed as mean ± standard deviation. 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. The software used was Statistica 6.1, Statsoft (Poland). $P < 0.05$ was considered significant.

RESULTS

Trans-10,*cis*-12 CLA decreases lipid content and alters morphology of lipid droplets in macrophage and foam cells

To investigate the isomer-specific regulation of macrophage/foam cell morphology (as well as triacylglycerol and cholesterol content) cells were treated with 30 μM CLA isomers (*trans*-10,*cis*-12, or *cis*-9,*trans*-11) or linoleic acid as well as BSA (control) as described in Materials and Methods. Foam cells were obtained by additional incubation with ox-LDL (50 μg/ml) for additional 24 h, at which the cell morphology and content of triacylglycerols and cholesterol were investigated. Macrophages treated with linoleic acid contained relatively large lipids droplets within each cell compared to control cells incubated with BSA (Fig. 1). Less pronounced accumulation of lipid droplets was observed in cells cultured with *cis*-9,*trans*-11 CLA — Fig. 1. Similar results were obtained for foam cells (Fig. 2).

In contrast, macrophages/foam cells cultured with *trans*-10,*cis*-12 CLA contained few lipid droplets (Figs. 1 and 2). Also the morphology of macrophages cultured with *trans*-10,*cis*-12 CLA isomer was different from that of macrophages cultured with the other fatty acids (Fig. 1). Treatment of the cells for 2 days with *trans*-10 *cis*-12 CLA, but not *cis*-9,*trans*-11 CLA or LA, signifi-

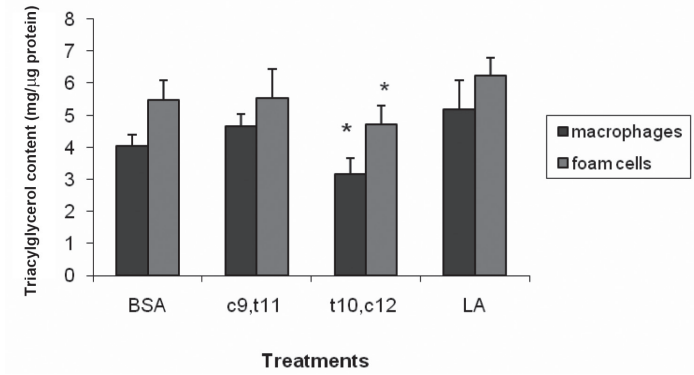

Figure 3. Effect of CLA on concentration of triacylglycerol in macrophages
Cells were grown in differentiation medium containing BSA (a vehicle control), 30 μmol/l *cis*-9,*trans*-11-C 18:2, *trans*-10,*cis*-12-C 18:2 or linoleic acid for 2 days. Triglycerides concentration was determined spectrophotometrically. The data are expressed as mean (mg/mg protein) ± S.D. from ten separate experiments. Values with different letters are significantly different ($P < 0.05$).

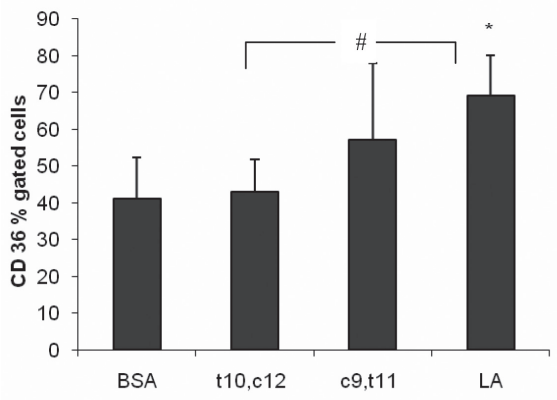

Figure 4. Effect of CLA on CD36 expression in THP-1 macrophages
Cells were grown in differentiation medium containing BSA (a vehicle control), 30 μmol/l *cis*-9,*trans*-11-C 18:2, *trans*-10,*cis*-12-C 18:2, or linoleic acid for 2 days, and with ox-LDL (50 μg/ml, 24 h). Results are expressed as percentage of CD 36 positive cells and given as mean ± S.D. from eight separate experiments as described in Materials and Methods. *Compared to BSA; #compared to linoleic acid. Values with different letters are significantly different ($P < 0.05$).

cantly reduced the triacylglycerol content in macrophages (Fig. 3) ($P < 0.05$, n = 10) as well as in foam cells $P < 0.05$, n = 10) (Fig. 3).

The inhibitor of PPAR α, GW 6471, slightly raised the triacylglycerol content compared to BSA control in both macrophages (Fig. 1) and foam cells (Fig. 2).

CD36 expression is decreased in macrophages cultured with *trans*-10,*cis*-12 CLA

Studies on the expression of CD36 were conducted in macrophages obtained from THP-1 cells cultured for 48 h with fatty acids (30 μM) and with ox-LDL for 24h. In these conditions CD36 is induced on the cell surface ($P < 005$, n = 8) — Fig. 4. Expression of CD36 was elevated in cells cultured with LA (compared to BSA, $P < 0.05$, n = 8), whereas in the presence of *cis*-9,*trans*-11 C 18:2 a strong tendency toward induction was seen. A slight increase of CD36 expression was observed in cells cultured with *trans*-10,*cis*-12 18:2 C (compared to BSA).

CLAs do not change the cholesterol level in foam cells

As shown in Figs. 5 and 6, cultivation of foam cells with the examined fatty acids did not significantly influence the cholesterol content. Only a slight tendency to reduce the cholesterol content in cells cultivated with *trans*-10,*cis*-12 CLA (compared to BSA; Fig. 6) was observed.

DISCUSSION

In animals, CLA induced the resolution of atherosclerosis by negatively regulating the expression of pro-inflammatory genes and by activation of apoptosis in the atherosclerotic lesion (Tommey *et al.,* 2003). We have previously demonstrated that CLA shows some pro-atherogenic activity *in vitro*: it stimulates reactive oxygen species (ROS) production and isoprostan PGF 2α synthesis, elevates phagocytosis and apoptosis in human monocytes/macrophages *in vitro* (Stachowska *et al.,* 2007; 2008). Pro-atherosclerotic action of CD36 is associated with its participation in the uptake of ox-LDL from circulation (Teupser *et al.,* 2008), in this study we characterised the isomer-specific effects of CLA on CD36 expression and lipid accumulation in macrophages. We demonstrated that linoleic acid (LA, *n-6*) and *cis*-9,*trans*-11 CLA increased or tended to increase expression of CD36 (compared to the BSA control). We also noticed that high amount of the lipids (triacylglycerols and total cholesterol) accumulated in cells cultured with *cis*-9,*trans*-11 CLA. Our data suggest that both *cis*-9,*trans*-11 CLA and LA can up-regulated CD36 expression and lipid deposition in human macrophages/foam cells. Other authors who studied the effect of linoleic acid and other *n-6* unsaturated fatty acids on CD36 expression showed that linoleic acid (as well as arachidonic acid) elevated the expression of CD36 in human monocytic cells (Pietsch *et al.,* 1995). In contrast *n-3* fatty acids eicosapentaenoic acid (EPA) and docahexaenoic acid (DHA) investigated in the same study, reduced both CD36 mRNA and protein (Pietsch *et al.,* 1995). In contrast, Vallve and coworkers and others observed that LA, EPA, or DHA increased CD36 expression (mRNA and protein) in macrophages (Collot-Texiera *et al.,* 2007; Vallve *et al.,* 2002).

Opposite results were obtained in this study for cells cultured with *trans*-10,*cis*-12 CLA. This isomer repressed both CD36 expression and deposition of lipids in macrophages when compared with other treatments: *cis*-9,*trans*-11 CLA, or LA. (Fig. 4). *Trans*-10,*cis*-12 CLA has been shown earlier to alter *in vitro* lipid metabolism and specifically to decrease triacylglycerol accumulation in other cells, e.g. primary human adiopcytes. This CLA isomer inhibited fatty acid desaturation by decreasing the activity of desaturases and also by disturbing glu-

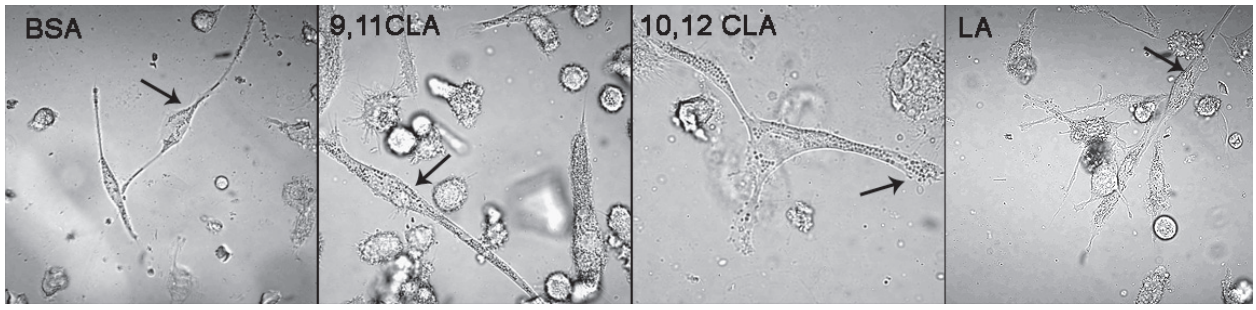

Figure 5. Cholesterol droplets in foam cells.
Macrophages were treated with BSA (a vehicle control), 30 μM *cis*-9,*trans*-11 CLA (9, 11), *trans*-10,*cis*-12 CLA (10, 12), or linoleic acid (LA) for 2 days. Foam cells were obtained after additional incubation of macrophages with ox-LDL (50 μg/ml) for 24 h at 37°C. Cells were then harvested and viewed with an Olympus FV500 laser scanning confocal microscope. Droplets of cholesterol are marked by black arrows.

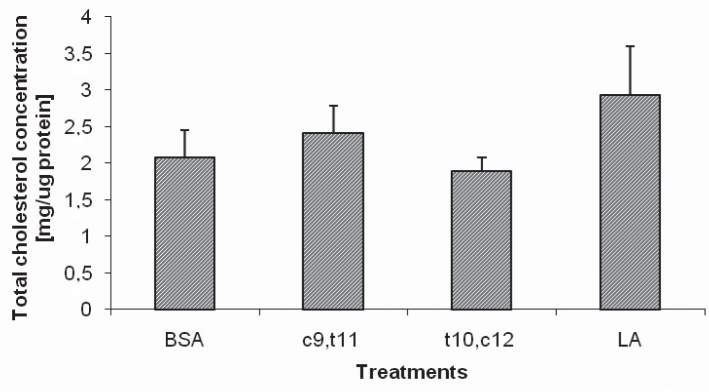

Figure 6. Effect of CLA on cholesterol concentration in foam cells
Cells were grown in medium containing BSA (a vehicle control), 30 μM *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, or linoleic acid for 2 days. Foam cells were obtained as described in Materials and Methods. Cholesterol concentration was determined spectrophotometrically. The data are expressed as mean(mg/mg protein) ± S.D. from ten separate experiments. Values with different letters are significantly different ($P < 0.05$).

cose uptake (Brown *et al.,* 2003). According to Brown and coworkers *trans*-10, *cis*-12 CLA leads to delipidation by down-regulation of the ativity of PPARγ receptor and several of its target genes. This phenomena may lead to insulin resistance, hyperglycemia, and hyperlipidemia. We suppose that a similar process may concern macrophages, where we noticed reduction of cholesterol and triacylglycerol content (Figs. 3 and 5). Also Ringseis *et al.* (2008) has shown that in mouse macrophages CLAs affect cholesterol efflux and reduce cholesterol accumulation. In the study of Ringseis *et al.* (20008) both *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA lowered cholesterol accumulation. This process consisted in stimulation of cholesterol efflux, and elevation of expression of genes CD36, ABCA1, LXRalpha, NPC-1, and NPC-2 (Ringseis *et al.,* 2008). Cholesterol metabolism and lipid concentration in macrophages by are regulated PPAR receptors. Activators of PPAR are capable of reducing cholesterol accumulation through the activation of genes involved in cholesterol homeostasis (Ringseis *et al.,* 2008) as well as in ox-LDL uptake (Nagy *et al.,* 1989; Tontonoz *et al.,* 1998; Kliewer *et al.,* 1999). Mice that were fed a diet containing 0.5% CLA had an elevated expression of both PPAR γ and PPAR α in the aortas (Toomey *et al.,* 2003). This coincided with the reduction of CD36 expression and elevation of apoptosis in the aorta (Toomey *et al.,* 2003). This upregulation of CD36 by PPAR can be substantially enhanced by various ligands: linoleic acid (Kliewer *et al.,* 1999; Nagy *et al.,* 1989), ox-LDL, 15d-PGJ2 ond other (Xie *et al.,* 2009). We hypothesize that *trans*-10,*cis*-12 C 18:2 may antagonize activation of PPAR α and in this way reduce CD36 expression and ox-LDL transport to foam cells. When the inhibitor of PPAR α GW 6471 was added to macrophages the triacylglycerol content increased (Figs. 1 and 2).

In summary, we show that *trans*-10,*cis*-12 CLA induces macrophage delipidation *in vitro* by down regulation of PPAR, however, further investigations are required to see whether the same effect occurs *in vivo*.

Acknowledgements

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