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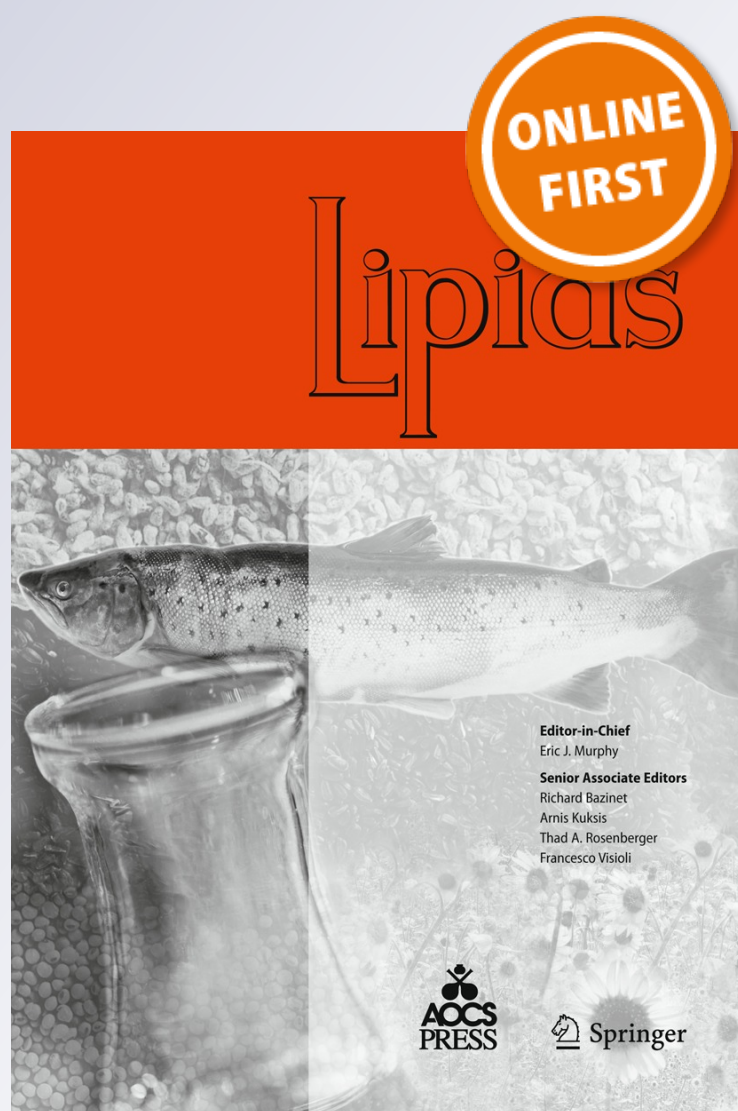
Manuela Oraldi, Marina Maggiora, Elena Paiuzzi, Rosa A. Canuto & Giuliana Muzio

Lipids

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ORIGINAL ARTICLE

CLA Reduces Inflammatory Mediators from A427 Human Lung Cancer Cells and A427 Conditioned Medium Promotes Differentiation of C2C12 Murine Muscle Cells

Manuela Oraldi · Marina Maggiora · Elena Paiuzzi · Rosa A. Canuto · Giuliana Muzio

Abstract Conjugated linoleic acid (CLA) is thought to have anti-proliferative and anti-inflammatory properties, but its effect on cancer cachexia is unknown. Two effects were here investigated: that of CLA on inflammatory mediator production in human lung cancer cells, and that of reduced mediators on the myogenic differentiation of murine muscle C2C12 cells. The latter cells were grown in medium conditioned by human lung cancer A427 cells, with or without CLA, to mimic only the effect of molecules released from the tumor "in vivo", excluding the effect of host-produced cachectic factors. The results obtained show that CLA was found to reduce the production of tumor necrosis factor-α, interleukin (IL)-1β and prostaglandin E2 (PGE2), but had no effect on IL-6 production. The mechanisms underlying the effect of CLA on cytokine or PGE2 release in A427 cells are probably mediated by activation of peroxisome proliferator-activated receptor (PPAR)α, which increased at 24 h CLA treatment. In turn, the reduced content of inflammatory mediators in medium conditioned by A427 cells, in the presence of CLA, allowed muscle cells to proliferate, again by inducing PPAR. The involvement of PPARα was demonstrated by treatment with the antagonist MK-886. The findings demonstrate the anti-inflammatory and myogenic action of CLA and point to its possible application as a novel dietary supplement and therapeutic agent in inflammatory disease states, such as cachexia.

Abbreviations

CLA	Conjugated linoleic acid
COX	Cyclooxygenase
IL	Interleukin
LDH	Lactate dehydrogenase
PGE2	Prostaglandin E2
PPAR	Peroxisome proliferator-activated receptor
TNF	Tumour necrosis factor

Introduction

One of the commonest manifestations of advanced malignant disease is the development of cancer cachexia, which is responsible for about 20 % of cancer-related deaths. Associated abnormalities include anorexia, weight loss, muscle loss and atrophy, anemia, and alterations in carbohydrate, lipid and protein metabolism [1–3]. Muscle wasting is the most important feature of cancer cachexia, and the principal cause of morbidity and mortality in cancer patients [4]. Skeletal muscle is a highly adaptable tissue, which responds to physiological challenges by changing size and metabolism, undergoing changes in growth and development under the strict control of myogenic regulatory factors [5].

Although cachectic factor(s) have long been sought, and considerable scientific effort and economic resources have been devoted to their investigation, the picture is still

M. Oraldi · M. Maggiora · E. Paiuzzi · R. A. Canuto (✉) · G. Muzio
Dipartimento di Medicina ed Oncologia Sperimentale, Università di Torino, Corso Raffello 30, 10125 Turin, Italy
e-mail: rosangela.canuto@unito.it

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fragmentary. Experimental research and clinical studies have demonstrated that cytokines are involved in inducing cachexia [6, 7], although the results require careful interpretation. Episodic administration of tumor necrosis factor (TNF) has proved unsuccessful at inducing cachexia in experimental animals; repetitive TNF administration initially induces a cachectic effect, but tolerance to the cytokine soon develops and food intake and body weight return to normal. Escalating doses of TNF have been shown necessary to maintain the cachectic effect [3].

Treatment with anti-mouse interleukin (IL)-6 antibody, or with nonpeptide IL-6 receptor antagonist, successfully reversed the key parameters of cachexia in colon-adeno-carcinoma-bearing mice [8, 9]. Conversely, studies on incubated rat skeletal muscle have clearly shown that IL-6 has no direct effect on muscle proteolysis [10]. IL-1β production in human and animal cancer cell lines is well documented, and patients with IL-1β-producing tumors generally have a poor prognosis [11]. This cytokine is also reported to be responsible for enhanced protein degradation in rats, in which repeated administration of IL-1β induced anorexia and weight loss [11].

Since cancer cachexia is regarded as a chronic inflammatory condition, in which pharmacological and nutritional interventions aimed at increasing muscle mass and weight are of limited benefit [12], the current strategies for managing this condition focus on the use of agents with anti-inflammatory and nutritional properties. Conjugated linoleic acid (CLA) comprises a group of positional and geometric isomers of linoleic acid, found naturally in meat and dairy products from ruminant animals. It has shown promise both in reducing inflammation and as an anti-carcinogenic agent [13, 14]. CLA is formed in the rumen during a process of bacterial biohydrogenation of linoleic acid. Although 28 different isomers of CLA exist, the c9,t11 form accounts for more than 90 % of the CLA intake in the human diet [13]. The health benefits deriving from CLA have been attributed to the two main isomer forms, c9,t11 and t10,c12; these are the predominant biologically active forms and exhibit numerous physiological properties [15, 16]. The health-related effects of CLA have been confirmed in numerous animal and human-intervention studies; they include anti-carcinogenic, anti-atherogenic, anti-diabetogenic, and immunomodulatory actions [17–19]. In vitro studies have shown CLA to inhibit proliferation of several human tumor cell lines, in a dose- and time-dependent manner [17, 20, 21].

It has been suggested that the anti-carcinogenic properties of CLA stem from its anti-inflammatory properties, as the acid negatively regulates the expression of pro-inflammatory genes such as TNF-α, IL-1β, and IL-6 [22]. Due to its anti-proliferative and potentially anti-inflammatory properties, CLA might be a novel dietary supplement for patients suffering from muscle wasting in chronic inflammatory-related disease, particularly cancer cachexia.

CLA is not universally considered to be of use as a supplement in cachexia patients [23]. The addition of c9,t11-CLA-rich oil to the diet did not ameliorate wasting in mice with cancer cachexia [24]. However, other studies have shown that CLA can preserve muscle mass in tumor-bearing cachectic animals, decreasing skeletal muscle wasting and reducing the catabolic effects of TNF-α, and have suggested that it may have overall protective properties in cachexia [25, 26]. In particular, one study reported CLA to possess specific tumor-related effects on myogenic and inflammatory gene expression, in an in vitro human co-culture model comprising a pancreatic tumor cell line and human primary muscle cells [27].

The present study investigated the effects of CLA on the proliferation and production of inflammatory mediators in human lung cancer A427 cells. It also examined the effects of the secretion products of cancer cells, both treated and untreated with CLA, on the myogenic differentiation of murine muscle C2C12 cells.

Materials and Methods

A427 Cell Culture Conditions

Human lung A427 cells (ATCC, MD, USA) were cultured (20,000 cells/cm^2) in DMEM/F12 medium supplemented with 2 mM glutamine, 1 % antibiotic/antimycotic solution and 10 % FBS (medium A).

Treatment of A427 Cells with CLA

CLA was prepared in horse serum (HS) at a concentration of 5 mM. A mixture of the two principal CLA isomers, c9,t11 and t10,c12 (Sigma, MO, USA) was used. Twenty-four hours after cell seeding, medium A was removed and replaced with DMEM/F12 medium supplemented with 2 mM glutamine, 1 % antibiotic/antimycotic solution, 2 % HS (medium B), and CLA (final concentration 5 or 50 μM). In control cells, a quantity of HS equivalent to the highest dose administered with CLA was added to the cell culture, in addition to the 2 % HS present in the medium B.

Twenty-four hours after CLA or HS addition, the culture media were collected, pooled, and centrifuged at 2,800g for 10 min (centrifuge J6B Beckman Culture, CA, USA) at room temperature. The resulting medium was used as conditioned medium in which to culture C2C12 cells; it was also used to determine pro-cachectic factor production and lactate dehydrogenase (LDH) release. Cells were washed with PBS, trypsinized and centrifuged at 900g for

10 min (centrifuge J6B Beckman Culture, CA, USA) for the assays listed below.

Treatment of A427 Cells with CLA and MK886

A427 cells were divided into four groups: (1) control cells; (2) CLA group: cells treated with 5 µM CLA; (3) MK886 group: cells treated with 5 µM MK886 dissolved in DMSO; (4) CLA plus MK886 group: cells treated with both substances. After 24 h, in all groups cells were trypsinized, centrifuged at 900g (centrifuge J6B Beckman Culture, CA, USA) for 10 min, and cell proliferation was evaluated; the culture medium was used to evaluate the production of TNF-α and prostaglandin E2 (PGE2), and to culture C2C12 cells.

C2C12 Cell Culture Conditions

Murine muscle C2C12 cells (ATCC, MD, USA) were seeded at 6,000 cells/cm^2 in medium A and maintained for 4 days. After this time, to induce differentiation into myotubes, medium A was removed and replaced with medium B, in which the C2C12 cells were maintained for a further 3 days. Cells were then subdivided into five groups: group I (Tdiff): cells grown in medium B; group II (control): cells grown in medium conditioned by A427 cells; group III (5 µM CLA): cells grown in medium conditioned by A427 cells treated with 5 µM CLA; group IV (MK886): cells grown in medium conditioned by A427 cells treated with 5 µM MK886; group V (MK886 + CLA): cells grown in medium conditioned by A427 cells treated with 5 µM CLA plus 5 µM MK886.

Cells and culture media of these groups were analyzed after 1 or after 4 days. After medium removal, cells were maintained frozen until use. The culture medium was centrifuged at 2,800g (centrifuge J6B Beckman Culture, CA, USA) for 10 min at 4 °C and used to determine LDH release.

Cell Growth and Viability

The number of A427 cells in the monolayer and that in the culture medium were evaluated using a Burker chamber, and expressed as cells/cm^2. For both A427 and C2C12 cells, viability was evaluated by determining LDH release, as described elsewhere [28].

Immunofluorescence Staining

To evaluate production of myosin and myofibrillar structures, C2C12 cells were washed with PBS, fixed with acetone and methanol (1:1) at 20 °C for 20 min and kept at 4 °C until use. Cells were then permeabilized with 0.1 % Triton X-100 in PBS and incubated with the monoclonal anti-skeletal (fast) myosin antibody (1:400 in PBS-Triton 0.1 %) (Sigma, MO, USA) overnight at 4 °C. After washing in PBS-Triton 0.1 % and then in PBS alone, cells were incubated with the appropriate Cy3-conjugated secondary antibody (1:1000) (GE Healthcare, UK) for 1 h at room temperature.

To visualize nuclei, cells were stained with Hoechst dye for 20 min. After incubation, cells were thoroughly washed with bidistilled H$_2$O and mounted with glycerol/PBS (1:1). Fluorescence-labeled cells were viewed using a fluorescence reverted microscope, and photographed using a ×32 lens (Zeiss, Germany). For image processing, Zeiss Axio-Vision software was used.

Western Blot Analysis

A427 cells were suspended in a lysis buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 0.015 mg/ml leupeptin, 0.1 % NP-40 substitute, and 1 mM Na-orthovanadate. Suspensions were kept on ice for 30 min. C2C12 cells were rubberized and suspended in a RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 % NP-40 substitute, 0.5 % Na-deoxycholate, 0.1 % SDS, 1 mM PMSF, 1 mM Na-orthovanadate and 0.015 mg/ml leupeptin. Suspensions of both cell types were kept on ice for 10 min and sonicated. Forty microgram of cell proteins were separated by SDS–polyacrylamide gel electrophoresis, then electrotransferred to a polyvinylidene difluoride (PVDF) membrane, which was then blocked overnight with TBS containing 5 % non-fat dry milk. The membrane was then rinsed and treated with polyclonal anti-PPARα, anti-PPARβ, or anti-PPARγ antibodies (Santa-Cruz Biotechnology Inc., CA, USA), and monoclonal anti-NF-kBp65, anti-cyclooxygenase (COX)-2, anti-skeletal (fast) myosin, anti-β-actin or anti-α-tubulin antibodies (Sigma, MO, USA). Protein bands were visualized using a chemiluminescent detection system (Immun-StarTM HRP Luminol/Enhancer, Bio-Rad Laboratories Inc., CA, USA).

Real-Time PCR

A427 cells were processed to determine PPAR expression by real-time PCR. Total RNA was extracted from the cells using the NucleoSpin RNA II Kit (Macherey–Nagel GmbH & Co. KG, Germany). Real-time PCR was run with single-stranded cDNA prepared from total RNA (1 µg) using a High-Capacity cDNA Archive kit (Applied Bio Systems, CA, USA). The forward (FW) and reverse (RV) primers were designed using the Beacon Designer® software package (Bio-Rad Laboratories Inc., CA, USA). Twenty-five microliters of a PCR mixture, containing cDNA

template equivalent 40 ng of total RNA, 5 pmol each of forward and reverse primers, and $2\times$ IQ SYBR Green SuperMix (Bio-Rad Laboratories Inc., CA, USA), were amplified in an iCycler PCR instrument (Bio-Rad Laboratories Inc., CA, USA) with an initial 10 min melt at 95 °C, followed by 35–40 cycles at 95 °C for 40 s, annealing temperature for each primer set for 40 s, and 72 °C for 40 s. Each sample was tested in duplicate, and threshold cycle (Ct) values were averaged. The variation in expression was defined as the relative expression in cells treated with CLA versus that in control cells, calculated as $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct_{CLA} - Ct_{GAPDH}$ and $\Delta\Delta Ct = \Delta Ct_{CLA} - \Delta Ct_{control}$.

Chemical Analysis

TNF-α, IL-1β and IL-6 were evaluated by the Enzyme Linked Immuno Sorbent Assay (Bender Medsystems, Austria), while PGE2 was evaluated with an Enzyme Immunoassay Kit (Oxford Biomedical Research, MI, USA).

Protein Determination

Protein concentrations in A427 and C2C12 cell lysates were measured using the Protein Assay Kit 2, following the manufacturer's instructions (Bio-Rad Laboratories Inc., CA, USA).

Statistical Analysis

All data are expressed as mean $\pm$ SD differences between group means were assessed by analysis of variance followed by a post hoc Newman-Keuls test or by the Student's t test.

Results

Effect of CLA on Human Lung Cancer A427 Cells

After 24 h, only the highest concentration of CLA (50 μM) significantly reduced the number of cells, whereas the lowest concentration (5 μM) left cell numbers similar to control values (Fig. 1). Neither concentration of CLA induced death, by necrosis or by apoptosis, as demonstrated by LDH release in the medium and by cytofluorimetric analysis (data not shown). For subsequent determinations, only the 5 μM CLA concentration was used, in order to avoid as far as possible any interference from decreased cell proliferation.

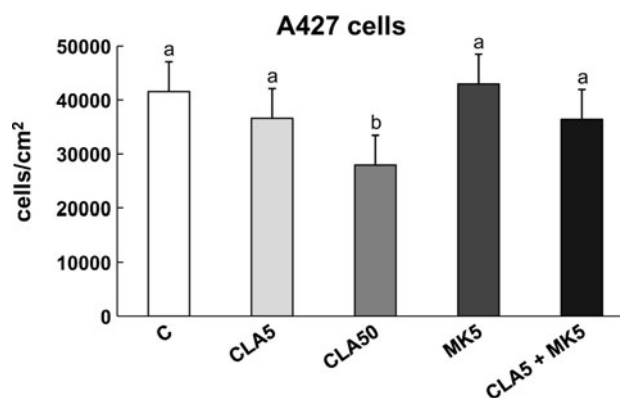Evaluation of cytokines in the culture medium (Fig. 2) shows that CLA caused a significant decrease of TNF-α

Fig. 1 Numbers of human lung cancer A427 cells, untreated or treated with MK886, CLA or CLA + MK886 for 24 h. Data are mean $\pm$ SD from four experiments. Means with *different letters* are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls analysis. *C* control cells, *CLA5* cells treated with 5 μM CLA, *CLA50* cells treated with 50 μM CLA, *MK5* cells treated with 5 μM MK886, *CLA5 + MK5* cells treated with 5 μM CLA + 5 μM MK886

and IL-1β versus control cells at 24 h, with no significant decrease of IL-6. Figure 2 also shows that the release of PGE2 in the culture medium was lower in cells treated with CLA than in control cells.

Effect of Medium Conditioned by A427 Cells on Murine Muscle C2C12 Cells

Medium conditioned for 24 h by A427 cells, untreated or treated with CLA, did not affect the viability of C2C12 cells, as evidenced by LDH determination in the culture medium (data not shown). Figure 3 shows the differentiation degree of C2C12 cells, grown for 1 or for 4 days in conditioned medium; number and size of myotubes were evidenced by immunofluorescence staining. At both 1 and 4 days, cells grown in medium conditioned by A427 cells without CLA showed little or no differentiation, compared to cells induced to differentiate in the appropriate medium (Tdiff). Conversely, cells grown for 4 days in medium conditioned by A427 cells treated with 5 μM CLA showed differentiation increased in a time-dependent manner, reaching almost the same degree of differentiation as cells grown in Tdiff. These results were confirmed by evaluating myosin protein expression, which significantly increased in C2C12 cells grown for 4 days, in medium conditioned by A427 cells with 5 μM CLA, compared to control cells (Fig. 3).

PPAR Involvement

CLA is a PPAR ligand; these receptors were thus examined in A427 cells, to investigate their possible involvement in the CLA-induced decrease of cytokine and PGE2 production. Figure 4 shows the PPAR trend, evaluated as mRNA

(Fig. 4a) and protein expression (Fig. 4b). Both measures of PPARα significantly increased versus control cells, 24 h after treating A427 cells with CLA; conversely, PPARγ was unchanged, and PPARβ showed a small but not significant increase. As Fig. 4c shows, the protein expression of NF-kB and COX-2 decreased in CLA-treated cells versus controls cells after 24 h treatment.

Since PPARs are involved in reducing the inflammatory process, their influence in regulating myosin production in C2C12 cells was also examined. Figure 5 shows the PPAR trend evaluated as mRNA (Fig. 5a, b) and as protein (Fig. 5c): PPARα increased in C2C12 cells grown for 1 day in the culture medium of A427 cells treated with

CLA, whereas no variation was found at 4 days. PPARγ remained unchanged. As regards PPARβ, a small but not significant increase occurred after 1 day, and a significant increase after 4 days.

To investigate the relation between PPARα and cytokine and PGE2 production in A427 cells, a concentration of PPARα-antagonist MK886 that does not affect cell proliferation was administered to the cells (Fig. 1). The presence of MK886 prevented the reduction in TNF-α and PGE2 production in A427 cells (Fig. 6), and the increase of number and size of myotubes at both 1 and 4 days in C2C12 cells, as evidenced by immunofluorescence staining (Fig. 7).
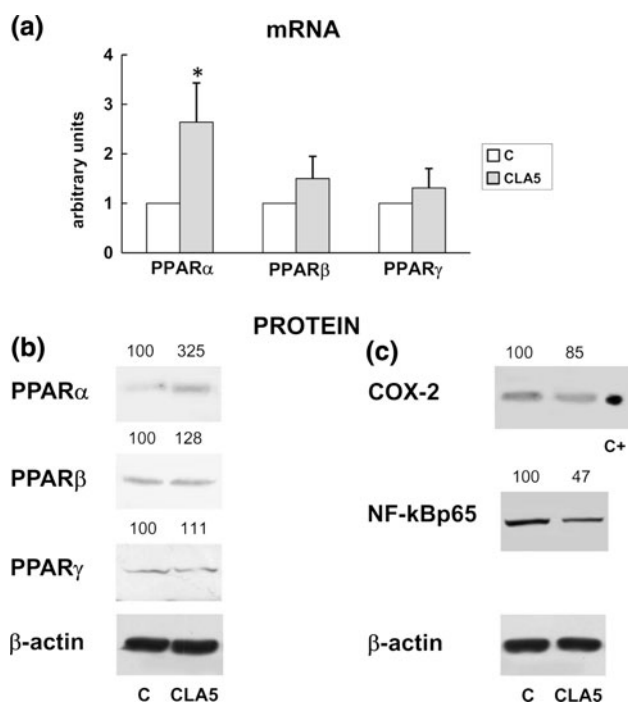
(a) mRNA

(b) PROTEIN

(c)

Fig. 4 PPAR, COX-2 and NF-kBp65 expression in human lung cancer A427 cells, untreated or treated with CLA for 24 h. PPARs were analyzed by real-time PCR (**a**) and Western blot (**b**), COX-2 and NF-kBp65 were analysed by Western blot (**c**). Data are mean ± SD from three experiments. For real-time PCR (1 µg of cDNA), the values of cells treated with CLA are referred to control value, taken as 1 (*white bar*). For Western blot (40 µg of cell proteins for each lane), the densitometry value given for each protein is normalized to the corresponding β-actin value and expressed as a percentage, setting the control value (*C*) arbitrarily at 100. Student's *t* test: *$p < 0.05$, control cells versus cells treated with 5 µM CLA. *C* control cells, *CLA5* cells treated with 5 µM CLA, *C+* positive control

Discussion

The study provides some insight into anti-inflammatory and myogenic effects of the fatty acid CLA, and provides a glimpse of its possible applications as a therapeutic agent in inflammatory states underlying cachexia in cancer patients. An in vitro model in which murine muscle C2C12 cells were grown in a medium conditioned by A427 human lung cancer cells, exposed or not to CLA, was used to mimic the effect of molecules released from the tumor in vivo; this excluded any possible interference of the effects of cachexia-mediator factors produced by the host. One difficulty of in vivo research is that of differentiating between effects due to pro-cachectic mediator factors produced by the host, and those produced by cancer cells. A mixture of c9,t11 and t10,c12 CLA isomers was used, because they are the two major CLA forms present in the diet and showing biological activity, even if c9,t11 CLA constitutes up to 80 % of total CLA.

Exposing A427 cells to CLA reduced production of TNF-α, IL-1β and PGE2, the reduction of PGE2 being due

to the decrease of COX-2. COX-2 is the pro-inflammatory COX isoform, and is the rate-limiting enzyme in the synthesis of prostaglandin PGE2 from arachidonic acid, one of many fatty acids in the phospholipids of cell membranes [29].

In murine muscle C2C12 cells, medium removed from A427 cells not treated with CLA did not enable differentiation to occur, as evidenced by the minimal number and size of myotubes (immunofluorescence staining) and low level of myosin expression (Western blot). Conversely, medium conditioned by the presence of CLA, which had a lower content of pro-inflammatory mediators than control medium, allowed time-dependent differentiation to occur, evidencing the importance of these factors on muscle cell modifications. In our study, CLA mixture administered to A427 cells enabled C2C12 cell differentiation, despite report that only the c9,t11 isomer stimulates C2C12 cell differentiation [30]. It appears probable that, in the case of administration of a CLA mixture, the effect of the c9,t11 isomer prevails.

There is considerable evidence from animal studies that TNF-α plays a role in muscle loss in cancer cachexia, although its role in the human condition is more questionable [31, 32]. Be that as it may, in our model the reduction of PGE2, coupled with the reduction of cytokines, regardless of cytokine type, was associated with muscle cell differentiation. Mechanisms thought to underlie the effect of CLA on the decrease of cytokines or of PGE2 in A427 cells were mediated by activation of PPARα, which increased after 24 h of CLA treatment, whereas the other PPARs did not show any statistically significant variations.

The PPAR family comprises three ligand-activated transcription factors: PPARα (NR1C1), PPARβ (NR1C2), and PPARγ (NR1C3). All three PPARs play important roles in regulating metabolic pathways, including those of lipids and glucose. They are also involved in cell differentiation, proliferation, and apoptosis pathways, and great interest has been shown in their involvement in inflammatory processes. In particular, PPARα and PPARγ inhibit the activation of inflammatory gene expression, and may negatively interfere with pro-inflammatory signaling pathways in vascular and inflammatory cells. The role played by PPARβ in regulating inflammation and immunity is only now emerging [33].

PPARα agonist-induced activation has been shown to inhibit a number of inflammatory pathways, including TNF-α, inducible nitric oxide synthase, and COX-2, and that it also inhibits infiltration of adhesion molecules and cells into the tissues [34]. In this study, inhibition of TNF-α, IL-1β and PGE2 by PPARα may have been mediated by the decrease of p65 NF-kB, a hypothesis that would agree with reports showing that increased PPARα expression may inactivate TNF-α and COX-2, by inhibiting NF-kB.
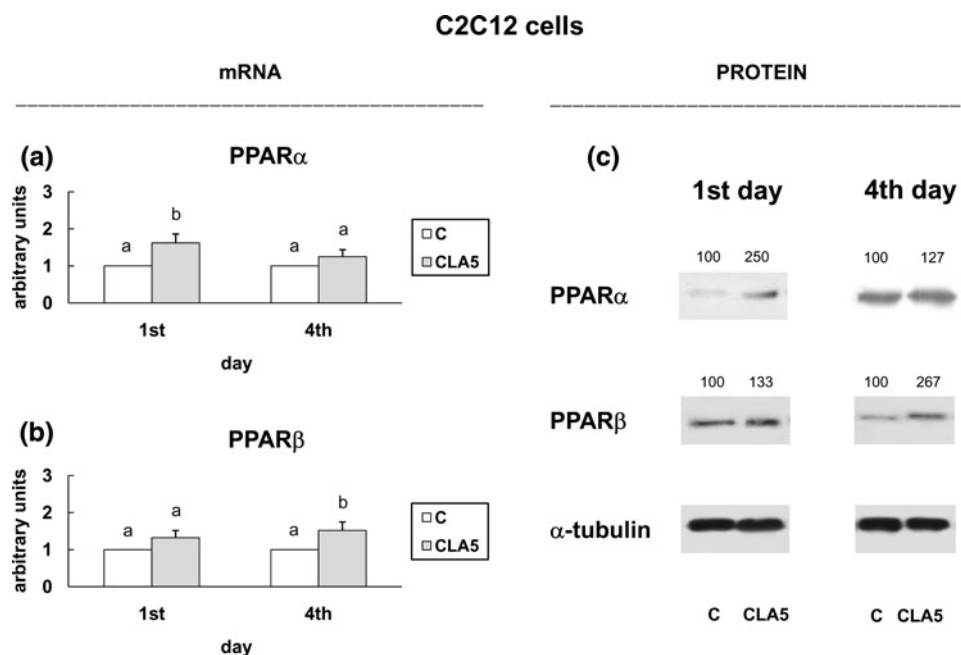
Fig. 5 PPAR expression evidenced by real-time PCR (**a**, **b**) and Western blot (**c**) in murine muscle C2C12 cells after 1 and 4 days of differentiation. Differentiation was in medium conditioned by human lung cancer A427 cells, untreated or treated with CLA for 24 h. Data are mean $\pm$ SD from three experiments. For real-time PCR (1 μg of cDNA), the values of cells treated with CLA are referred to the control value, taken as 1 (*white bar*). For Western blot (40 μg of cell proteins for each lane), the densitometry value given for each protein is normalized to the corresponding β-actin value and expressed as a percentage, setting the control value (*C*) arbitrarily at 100. For each PPAR, means with *different letters* are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. *C* cells grown in conditioned medium removed from A427 cells untreated with CLA, *CLA5* cells grown in conditioned medium removed from A427 cells treated with 5 μM CLA
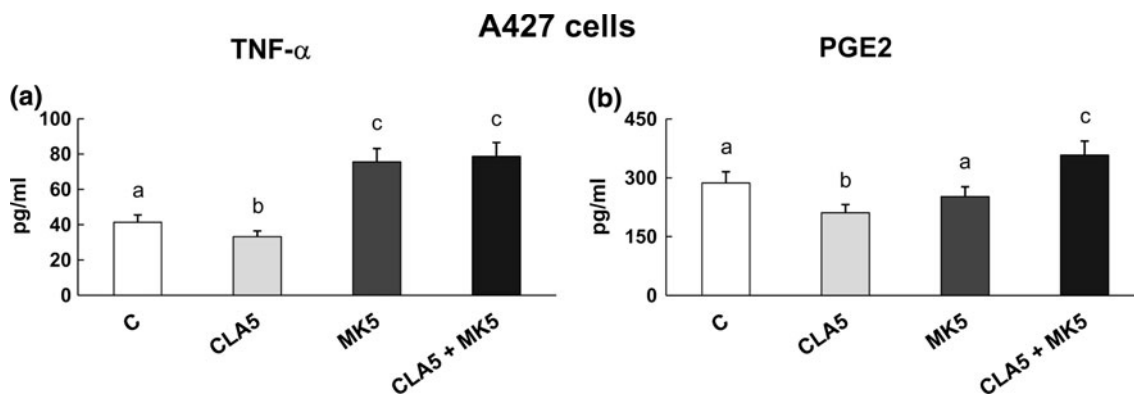

Fig. 6 Concentrations of TNF-α (**a**) and PGE2 (**b**) in medium of human lung cancer A427 cells, untreated or treated with CLA and MK886 for 24 h. Data are means $\pm$ SD from three experiments. For each panel, means with *different letters* are significantly different from one another ($p < 0.05$) as determined by analysis of variance followed by post hoc Newman–Keuls test. *C* control cells, *CLA5* cells treated with 5 μM CLA, *MK5* cells treated with 5 μM MK886, *CLA5 + MK5*, cells treated with 5 μM CLA + 5 μM MK886

PPARα translocates from the cytosol to the nucleus, where it binds with NF-kB to inhibit gene activation [35–37]. The promoter regions of TNF-α, IL-1β and COX-2 genes contain numerous transcription-factor binding sites, including AP-1 and NF-kB. Several lines of evidence suggest that PPARα exerts its anti-inflammatory effects through negative regulation of NF-kB. In human aortic smooth-muscle cells, PPARα has been shown to inhibit the NF-kB transactivation function, by directly interacting with the Rel homology domain of the p65 subunit, which mediates DNA binding and dimerization activity [38]. PPARα ligands have also been found to upregulate expression of the NF-kB inhibitor protein IkBα, in both human aortic smooth-muscle cells and microglial cells, leading to decreased DNA binding of NF-kB and gene expression [39, 40].
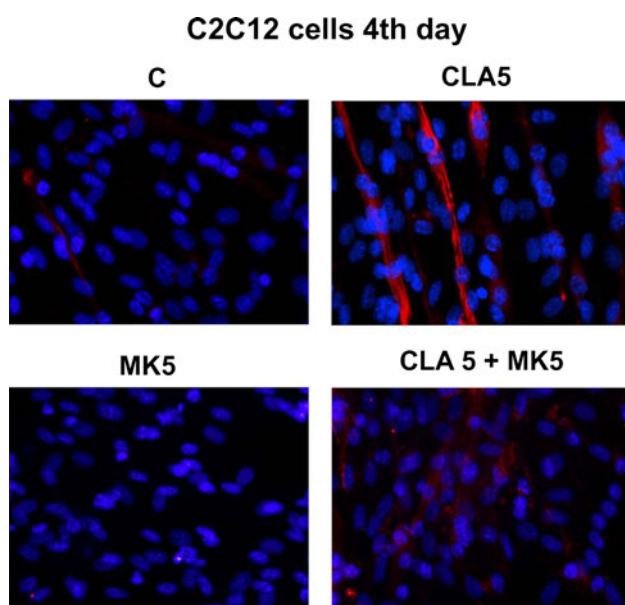
C2C12 cells 4th day

Fig. 7 Myosin content evidenced by immunofluorescence staining in murine muscle C2C12 cells after 4 days of differentiation. Differentiation was in medium conditioned by human lung cancer A427cells, untreated or treated with MK886, CLA, or CLA + MK886 for 24 h. *C* cells grown in conditioned medium removed from A427 cells untreated with CLA, *CLA5* cells grown in conditioned medium removed from A427 cells treated with 5 μM CLA, *MK5* cells grown in conditioned medium removed from A427 cells treated with 5 μM MK886, *CLA5 + MK5* cells grown in conditioned medium removed from A427 cells treated with 5 μM CLA + 5 μM MK886

The involvement of PPARα in reduction of inflammatory factors induced by CLA was confirmed by using the PPARα antagonist MK886, which enabled TNF-α and PGE2 release to return to near control values in A427 cells, while in C2C12 cells it permitted the anti-miogenic effect of cytokines to occur. In our experimental model, PPARα was involved not only in the effect of CLA on A427 cells, but probably also in the effect of cytokines and PGE2 on C2C12 cell differentiation. In the latter cells, medium conditioned by A427 treated with CLA, and with a lower content of pro-inflammatory factors than the medium of untreated cells, caused an increase in PPARα, especially at 1 day, and in PPARβ at 4 days. The increased PPAR expression might be the cause of the increased myosin content and increased number and size of myotubes, that occurred in C2C12 cells grown in medium with reduced inflammatory mediator content. The increase in PPAR caused by the reduction of cytokines and PGE2 could also be explained by the inhibition of NF-kB: TNF-α induces muscle protein degradation by activating NF-kB, dependently of ROS production or otherwise [31, 41].

The physical interaction between PPAR and the p65 subunit of NF-kB interferes with the activity of both transcription factors [38, 40]. Because interaction between PPAR and NF-kB leads to modulation of the inflammatory response, a reciprocal effect of NF-kB on the PPAR transactivation function is obviously to be expected. In vitro assays have shown that translocation of NF-kB into the nucleus represses PPAR transactivation of a PPRE-driven promoter [42]. Increased PPAR expression may explain the positive effect of reduced inflammatory mediator content on increasing myosin content, number and size of myotubes.

PPARβ activation has been shown not only to increase the number of oxidative myofibres and capillaries in skeletal muscle, but also to promote myonuclear accretion [43], which is a characteristic response to exercise [44–46]. Increased myonuclear density has been found in transgenic muscle-specific over-expressing animals, as well as in wild-type mice treated for only 2 days with a PPARβ agonist. As mouse aging is associated with reduced PPARβ expression, and with decreased numbers of oxidative myofibres and myonuclei, which could be partially reversed by pharmacological PPARβ activation, a potential use of PPARβ agonists for the treatment of age-related muscle atrophy has been suggested [43].

In conclusion, CLA was here found to reduce release into the culture medium of inflammatory factors, produced by lung cancer cells, through activation of PPAR. In turn, the reduced content of these factors enabled differentiation of muscle cells, again through induction of PPAR. The study provides insight into the anti-inflammatory and myogenic actions of CLA, and points to its possible application as a therapeutic agent in inflammatory disease states, such as cachexia.

Conflict of interest All authors have read and agreed to the editorial policies, and declare that there are no financial or other conflicts of interest that might be construed to influence the results or interpretation of their manuscript.

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