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CLA reduces breast cancer cell growth and invasion through ER α and PI3K/Akt pathways

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Abstract

We previously reported that conjugated linoleic acid (CLA), a naturally occurring fatty acid, inhibits the growth of ER α (+) MCF-7 and ER α (-) MDA-MB-231 human breast cancer cells by negative modulation of the ERK/MAPK pathway and apoptosis induction. Here we show that in these cell lines CLA also down-regulates the PI3K/Akt cascade. In MCF-7 cells CLA also triggers ER α /PP2A complex formation reducing the phosphorylation state and transcriptional activity of ER α whereas in MDA-MB-231 cells CLA does not induce PP2A activation.

Moreover, CLA induces the expression of proteins involved in cell adhesion and inhibits cell migration and MMP-2 activity. These findings suggest that CLA may induce the down-regulation of ER α signalling and the reduction of cell invasion through the modulation of balancing between phosphatases and kinases.

Keywords

- CLA;
 - MCF-7;
 - MDA-MB-231;
 - ER α ;
 - PI3K/Akt;
 - β -Catenin/E-cadherin
-

1. Introduction

Conjugated linoleic acid (CLA) is the common element of a group of C18 fatty acids with two double bonds naturally found in food such as milk fat and meat from ruminant animals [1]. It has long been recognized that CLA exerts a protective effects against carcinogenesis in different tissues, particularly in the mammary gland [2].

In animal models, dietary CLA seems to inhibit the initiation, promotion and progression of carcinogenesis in several organs, such as the prostate, colon and breast [3] and [4]. Furthermore, CLA has been shown to inhibit the proliferation of bladder, colon, and breast cancer cells in vitro [5], [6], [7] and [8].

At present, despite the considerable amount of work done to demonstrate the importance of CLA as an anticancer agent, the mechanisms mediating its effects are not fully understood.

It has been suggested that the growth inhibitory effect of CLA on cancer cell lines could be related to the alteration of arachidonic acid metabolism, the inhibition of 5-hydroxyeicosatetraenoic acid production and the induction of lipid peroxidation [9], [10] and [11]. Some studies indicate that CLA isomers inhibited tumor FA uptake, intratumor cAMP content, 13-HODE release and [³H]-thymidine incorporation into tumor DNA. Similar responses were observed in ER(+) MCF-7 breast cancer xenografts in nude rats during perfusion in situ [12].

In addition, it or its metabolites have been found to alter the expression of key proteins regulating the cell cycle, such as cyclins and cdk inhibitors, on the gastric adenocarcinoma cell line and in rat mammary epithelium [13], and in colon cancer cells they negatively modulated ErbB3 signalling leading to the inhibition of Akt activation [14].

The PI3K/Akt plays a pivotal role in the initiation and progression of several human malignancies, including breast cancer [15]. PI3K is one of the key enzymes which regulates phosphoinositide metabolism and its activation results in phosphatidylinositol,3,4,5-triphosphate (PIP₃)-mediated phosphorylation of Akt. The Akt family is known to be a major downstream mediator of the PI3K pathway, which has been implicated in a variety of cellular processes, such as cell cycle regulation via p21 and p27 retention and an increased stabilization of cyclin D1 [16]. Also, Akt inhibits proapoptotic proteins and activates nuclear factor kappa-B transcriptional activity leading to the activation of pro-survival signalling [16]. In particular, Akt is recognized as a positive regulator of ER α -mediated signalling and as a negative regulator of glycogen synthase kinase 3 β (GSK3 β) [17].

The regulation of PI3K/Akt activation can occur through several mechanisms, among them the activation of its negative regulators such as PP2A (protein phosphatase 2A) and PTEN (phosphatase and the tensin homolog deleted on chromosome ten) [18]. At the same time PP2A activation may also dephosphorylate and activate GSK3 β [19] which is known to be important in a variety of signalling pathways that control cell proliferation, motility and apoptosis [20]. Inactivation of GSK3 β through the PI3K/Akt pathway is mainly due to protein phosphorylation at serine 9 [20], the level which tightly controlled the cytoplasmic pool of β -catenin and γ -catenin, multifunctional proteins involved in cell to cell adhesion and signal transduction. The membrane pool of both β and γ catenins mediates the formation of the cadherin-dependent cell adhesion complex that links cadherins to the actin cytoskeleton, while its cytosolic pool acts in the canonical wnt signalling pathway [21]. In the absence of wnt signals, free cytosolic β and γ

catenins undergo GSK3 β -induced phosphorylation and degradation through the ubiquitin pathway. Activation of wnt receptors inhibits β and γ catenins phosphorylation and leads to the cytosolic accumulation of the protein that can move into the nucleus, thus regulating the expression of the target genes involved in cell proliferation and migration [22]. Intriguingly, despite the similar structures and functions of β and γ catenins, including their binding to APC, no γ -catenin mutations have been reported in human cancer [23]. In fact, wild-type γ -catenin was reported to exert tumor suppressive effects upon its overexpression in some cancer cells [24]. In addition to β and γ catenin system, cell migration is regulated by several signalling systems integrated in the concept of focalized adhesion, including the main protagonists PI3K and extracellular signal-regulated protein kinase (ERK)1/2, which are the best characterized MAPK [25]. The ERK pathway is involved in cell proliferation and survival in breast cancer cells and its activation is known to inhibit apoptosis and induce cell proliferation in several human malignancies.

We previously established that a physiological concentration of CLA (60 μ M) lowers human breast cancer cell proliferation through mechanisms that can be related to ERK1/2 inhibition in ER α (+) MCF-7 cells [26] and to apoptosis induction in ER α (-) MDA-MB-231 cells [27]. In addition, we also evidenced that CLA represents a PPAR γ agonist and that PPAR γ activation in MCF-7 cells is accompanied by the inhibition of cell growth and β -catenin/E-cadherin induction [28].

Since PI3K- and ER α -mediated signal transduction cascades are interconnected with ERK and PPAR γ pathways in a variety of cells, in the present study we examined CLA effects on the activation and regulation of several key proteins of the PI3K/Akt signalling pathway. We additionally investigated whether CLA might exert direct antiestrogenic activity on or otherwise interfere with estrogen signalling in ER α (+) breast cancer cells.

2. Material and methods

2.1. Materials and reagents

CLA and mouse monoclonal anti- β -actin antibody were purchased from Sigma Chemical Co. (MO, USA). Rabbit polyclonal antibody anti-GSK3 β and -phospho-GSK3 β were from Cell Signalling Technology. Rabbit polyclonal antibody anti-ER α and -phospho-Akt1/2/3, mouse monoclonal antibody anti-E-cadherin, - β -catenin, -ICAM-1, -PTEN, -Akt and -PI3K-p85 α , goat polyclonal antibody anti-PP2A and -p-ER α , secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Secondary Cy3-conjugated antibody was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). TransAM ER kit was obtained from Vinci-Biochem (Firenze, Italy).

2.2. Cell culture

MDA-MB-231 and MCF-7 breast cancer cell lines were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured at 37 °C in a humidified incubator with 5% CO₂ and 95% air in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 μ g/mL streptomycin and 25 μ g/mL amphotericin B. For treatments, cells were seeded at a density of 3×10^4 cells/cm² and cultured for 24 h to allow them to adhere to the substratum. The medium was then replaced with serum-

free DMEM supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 25 µg/mL amphotericin B, 2 mM glutamine, 1% ITS (insulin, transferrin, sodium selenite), 1% vitamin solution, 0.4% serum bovine albumin (fatty acid free) and CLA 60 µM. CLA (cis- and trans-9,11 and 10,12 isomers in approximately a 50:50 ratio) was dissolved in FBS (0.01% v/v final concentration in the medium). Control groups received the same amount of FBS.

2.3. Protein extraction

2.3.1. Total extracts

Cells were seeded in 75 cm² plates and properly treated. Collected cells were suspended in lysis buffer containing 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.05% aprotinin, 0.1% IGEPAL and then incubated for 30 min at 4 °C. The suspension was centrifuged for 25 min at 12,000 rpm; the supernatant from this centrifugation was saved as the total extracts.

2.3.2. Nuclear extracts

Cells were seeded in 75 cm² plates and properly treated. Collected cells were suspended in lysis buffer containing 10 mM Hepes–NaOH (pH 7.8), 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1 mM PMSF and then incubated for 15 min at 4 °C. After addition of 10% (v/v) Nonidet P-40, the cell suspension was mixed, incubated for 30 min at 4 °C, and centrifuged for 15 min at 3000 rpm. The pellet was resuspended in lysis buffer containing 50 mM Hepes–NaOH (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% (v/v) glycerol. The suspension was mixed for 30 min at 4 °C and centrifuged for 15 min at 3000 rpm; the supernatant from this centrifugation was saved as the nuclear extract.

2.3.3. Membrane-associated fraction

Cells were seeded in 75 cm² plates and properly treated. Collected cell were suspended in hypotonic buffer containing 10 mM Tris (pH 7.4), 0.2 mM MgCl₂, 2 µg/mL pepstatin A, 2 µg/mL leupeptin and 100 µg/mL PMSF for 10 min on ice and then homogenized with a Dounce homogenizer. The homogenates were centrifuged for 37 min at 20,750 rpm; the resulting precipitate, to which an equal volume of the SDS-PAGE sample buffer was added, was analysed as the membrane-associated fraction.

2.4. Western blot analysis

Protein contents in the supernatants were measured using a commercially available assay (Protein Assay Kit 2, Biorad) with bovine serum albumin as a standard.

Equal amounts of proteins were mixed with solubilization buffer containing 250 mM Tris (pH 8.8), 4% SDS, 16% glycerol, 8% 2-mercaptoethanol and 0.1% bromophenol blue, and then fractionated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose for 2 h in a Biorad electroblotting device. Nitrocellulose matrices were blocked with 5% milk in TBST (1 M Tris buffer saline, pH 7.4, 5 M NaCl, 0.1% Tween-20) for 1 h at room temperature. For immunodetection, matrices

were incubated overnight at 4 °C with primary antibody. The matrices were then detected by incubation for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the ECL system. Band intensities were quantified by densitometry and the expression of proteins was reported as a proportion of β -actin or Akt protein expression to monitor any discrepancies in gel loading. Fold change versus control values was calculated by normalizing densitometric values obtained from the various proteins with those obtained for β -actin or Akt (VersaDoc Imaging System 3000, Biorad).

2.5. Invasion assay

Cells were seeded on 6-well culture plates, and properly treated. After treatment, cell invasion was evaluated with Boyden chambers equipped with 8 μ m porosity polyvinylpyrrolidone-free polycarbonate filters that were coated with 50 μ g/mL of Matrigel solution. With a Zeiss microscope (Oberkochen, Germany) equipped with bright field optics (40 \times) invasiveness was quantified by counting crystal violet-stained cells that invaded Matrigel. For each filter, the number of cells in ten randomly chosen fields was counted, and the counts were averaged (means \pm S.D.). Results are expressed as the number of migrated cells per high-power field.

2.6. Gelatinolytic zymography

Cells were seeded on 6-well culture plates, allowed to adhere for 24 h and then treated. After treatment, conditioned media were harvested and centrifuged to remove cellular debris. Conditioned media from the cell cultures were analysed for gelatin degradation activity by SDS-PAGE under non-reducing conditions. Samples for analysis were diluted in 1:1 non-reducing buffer (12.5% 0.5 M Tris-HCl (pH 6.8), 10% glycerol, 4% SDS, and 0.05% bromophenol blue) and then fractionated by electrophoresis on sodium dodecyl sulfate polyacrylamide gel electrophoresis co-polymerized with 0.2% gelatin A. After separation, the gels were washed twice in 2.5% Triton-X 100 for 15 min and incubated in buffer (Tris-HCl 50 mM, CaCl₂ 10 mM, NaCl 50 mM, pH 7.6) at 37 °C over night. The gelatinolytic activities of Matrix Metalloproteinase 2 (gelatinase-A, 72 kDa gelatinase, 72 kDa type IV collagenase, MMP-2) were visualized by staining the gel with 0.1% Coomassie brilliant blue and destained with 45% methanol, 10% (v/v) acetic acid until clear bands suggestive of gelatin digestion were present. The gels were washed out with distilled water and scanned for densitometry (VersaDoc Imaging System 3000, Biorad).

2.7. Immunoprecipitation

Cells were seeded in 75 cm² plates and properly treated. Collected cell were suspended in immunoprecipitation buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 1 mM EDTA, 0.7 μ g/mL pepstatin A, 5 μ g/mL leupeptin, 1 μ g/mL aprotinin and 1 μ M PMSF for 15 min on ice. ER α /PP2A complex formation was immunoprecipitated with anti-ER α antibody and detected with anti-PP2A antibody. The complex was absorbed from solution through the addition of an immobilized antibody binding protein such as Protein A-Sepharose beads. Upon centrifugation, the complex is brought down in the pellet; the subsequent liberation of the antigen was achieved by boiling the sample in the presence of SDS.

2.8. ER transcription factor assay

ER α transactivation was checked by Transam ER kit (Active Motif), a highly sensitive ELISA-based assay, according to the manufacturer's directions. 5 μ g of nuclear extracts of CLA-treated or untreated MCF-7 cells were added into a 92-well plate to which oligonucleotide containing a consensus-binding site was immobilized. ER α binds specifically to the oligonucleotide (5'-GGTCACAGTGACC-3'). The revelation was done by incubation with a primary antibody that recognized an accessible epitope on ER α protein upon DNA binding, followed by incubation with an HRP-conjugated secondary antibody. After incubation with standard developing solution, the samples were quantified by an ELISA plate reader (450 nm). Positive and negative controls were run in parallel.

2.9. Statistical analysis

Differences between the means were analysed for significance using the one-way ANOVA test with Bonferroni post hoc multiple comparisons, used to assess the differences between independent groups. All values are expressed as means \pm S.D., and differences were considered significant at $P < 0.05$.

3. Results and discussion

The anti-carcinogenic properties of CLA were established by several investigators against different types of experimental cancers [29], including breast cancer [30]. The molecular mechanisms that facilitate these effects are not currently well known, even if there is growing evidence that a wide array of cellular signalling components comprising phosphoinositide-3-kinase/serine/threonine protein kinase B (PI3K/Akt), the mitogen-activated protein (MAP) kinase family and the estrogen receptor (ER) signalling pathways are implicated in CLA anti-carcinogenic effects [6], [7] and [8].

The purpose of this study was to determine the effect of CLA on Akt signalling and its downstream targets on estrogen receptor α positive (MCF-7) and on estrogen receptor α negative (MDA-MB-231) human breast cancer cells.

Although PI3K has many potential downstream targets, we focused our attention on Akt because of several reports showing the importance of this kinase for its functional role in aggressive, therapy-resistant malignancies [31]. As it is frequently constitutively active in cancer cells, several drugs are being investigated for their ability to inhibit Akt signalling.

To address this question, we evaluated the ability of CLA to modulate the expression of p85 α , a regulatory subunit of PI3K protein. All the experiments were conducted by treating the cells for a time period of 72 h with 60 μ M CLA, an experimental condition that allowed the observation of the most significant effects on cell growth [26] and [27]. This concentration of CLA is in the physiological range: in fact, CLA concentration in human plasma is from 8 to 80 μ M, whereas it is only present in trace amounts in the phospholipids of cell membranes [32].

As reported in Fig. 1, CLA induces the reduction of p85 levels in both cell lines. This effect leads to the inhibition of Akt, the downstream target of PI3K, as the active form of Akt (phospho-Akt)

is substantially decreased after CLA treatment ([Fig. 1](#)). Akt signalling plays a crucial role in the initiation and progression of breast cancer and also regulates several downstream targets that are responsible for cell proliferation and survival. Many reports show that inactivation of Akt by dephosphorylation plays a key role in tumor suppression [\[17\]](#) and [\[18\]](#).

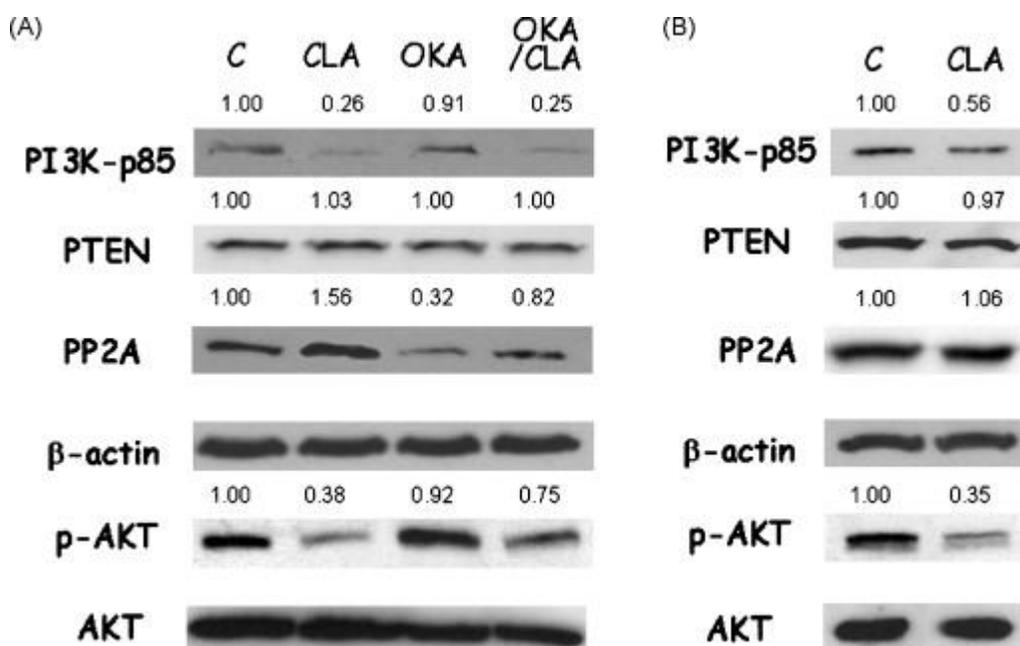


Fig. 1.

Effect of CLA on PI3K/Akt, PTEN and PP2A. MCF-7 (A) and MDA-MB-231 (B) cells were pretreated or not for 2 h with 0.1 nM okadaic acid before adding 60 μ M CLA for 72 h and total cell lysates were probed with an anti-PI3K-p85, anti-PTEN, anti-PP2A and anti-p-Akt antibodies. Protein contents were normalized with anti- β -actin (for PI3K-p85, PTEN and PP2A) or anti-Akt (for p-Akt) antibodies and analysed by densitometry. The blots shown are from a representative experiment repeated three times with similar results.

[Figure options](#)

Thus, to better clarify the effects of the fatty acid on the PI3K/Akt cascade, we analysed the expression level of PTEN and PP2A, both involved in the regulation of this pathway. The phosphatase PTEN negatively regulates the growth-promoting effects of PI3K by removing the phosphates from PIP3, while PP2A, a major cellular serine/threonine phosphatase, directly removes phosphate groups from active Akt [\[33\]](#).

Unexpectedly, in both cell lines no change was observed in PTEN protein levels after CLA treatment ([Fig. 1](#)), suggesting that this phosphatase is not involved in CLA-induced PI3K/Akt dephosphorylation. As regards PP2A, in MCF-7 cells CLA triggers a strong increment in the phosphatase expression, an expression that is not modified in MDA-MB-231 cells ([Fig. 1A](#)).

The involvement of PP2A in the decrease of the phospho-Akt level was confirmed by the fact that pre-treatment of MCF-7 cells with okadaic acid (OKA), a well known inhibitor of PP2A, completely reverted CLA effects ([Fig. 1A](#)). These results demonstrate that CLA reduces

PI3K/Akt activation via PP2A induction. This is in agreement with our previous study, in which CLA reduced ERK1/2 activation and consequently inhibited MCF-7 cell growth through PP2A involvement [26].

GSK3 β is one of the major downstream targets of Akt, involved in the phosphorylation and inactivation or degradation of a broad range of substrates, such as glycogen synthetase, cyclin D1, c-Myc, and β -catenin [20]. Here we evidenced that CLA-induced PP2A also exerts a regulatory role on GSK3 β , since in MCF-7 cells high levels of PP2A correlate with low levels of phospho-GSK3 β (inactive form), and consequently with a high rate of β -catenin degradation. Also in this case, the direct involvement of PP2A was demonstrated by the fact that pre-treatment with OKA abrogated CLA effect (Fig. 2A). These results show that CLA induces the dephosphorylation of GSK3 β , thereby maintaining GSK3 β in its active form through PP2A induction and phospho-Akt inhibition.

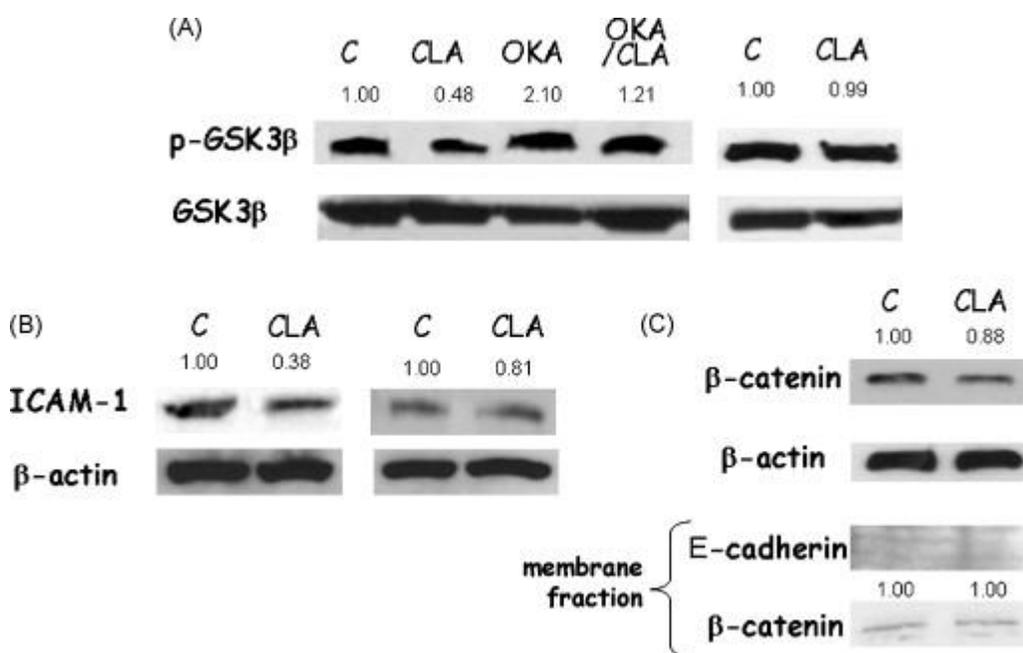


Fig. 2.

Effect of CLA on expression of p-GSK3 β , ICAM-1, β -catenin and E-cadherin. In A, MCF-7 (left) and MDA-MB-231 (right) cells were treated as described above; after treatment total cell lysates were probed with anti-p-GSK3 β antibody and normalized with an anti-GSK3 β antibody and analysed by densitometry. In B, MCF-7 (left) and MDA-MB-231 (right) cells were incubated with 60 μ M CLA for 72 h and were processed for western blot analysis with anti-ICAM-1 antibody, normalized with anti- β -actin and analysed by densitometry. In C, total and membrane-associated extracts of MDA-MB-231 cells, treated as described in B, were probed with anti- β -catenin and anti-E-cadherin antibodies and normalized with anti- β -actin or Ponceau Red dye (not shown) and analysed by densitometry. The blots shown are from a representative experiment repeated three times with similar results.

[Figure options](#)

PP2A has also been shown to regulate β -catenin-dependent transcriptional events [34]. β -Catenin is a protein with dual functions: it can be either complex with E-cadherin to promote cell–cell adhesion or enter into the nucleus as a transcriptional activator. The association between the loss or down-regulation of β -catenin/E-cadherin proteins and the development or progression of sporadic breast cancer has been extensively documented [35].

Our recent data show that in MCF-7 and Caco-2 cells CLA is able to stimulate the formation of E-cadherin/ β -catenin complex [28] and [36]. Consistent with these results, CLA caused a down-regulation of ICAM-1 (Fig. 2B), a cell surface glycoprotein associated with invasion and metastasis in a variety of human malignancies [37]. The magnitude of this effect was greater in MCF-7 cells than in MDA-MB-231 cells, in which neither the expression (Fig. 2C) nor the intracellular distribution of E-cadherin and β -catenin was modified by CLA (data not shown).

In most epithelial cancers, the loss of E-cadherin/ β -catenin complex is not only associated with a loss of cell–cell adhesion, but also can promote invasion and metastasis [38]. Thus, we ascertained whether CLA induction of E-cadherin/ β -catenin complex is accompanied by reduction of breast cancer cell migration and invasion. The Boyden chamber assay showed that in both cell lines CLA treatment results in a significant reduction of Matrigel invasion, to a greater extent in MCF-7 cells than in MDA-MB-231 cells (Fig. 3A). The zymogram revealed that CLA significantly reduces the activity of matrix metalloproteinase (MMP) 2 (also known as gelatinase-A and 72-kDa type IV collagenase) involved in the breakdown of the basement membrane by extracellular matrix (ECM)-degradation in MDA-MB-231 cells while no effect was detected in MCF-7 cells (Fig. 3B).

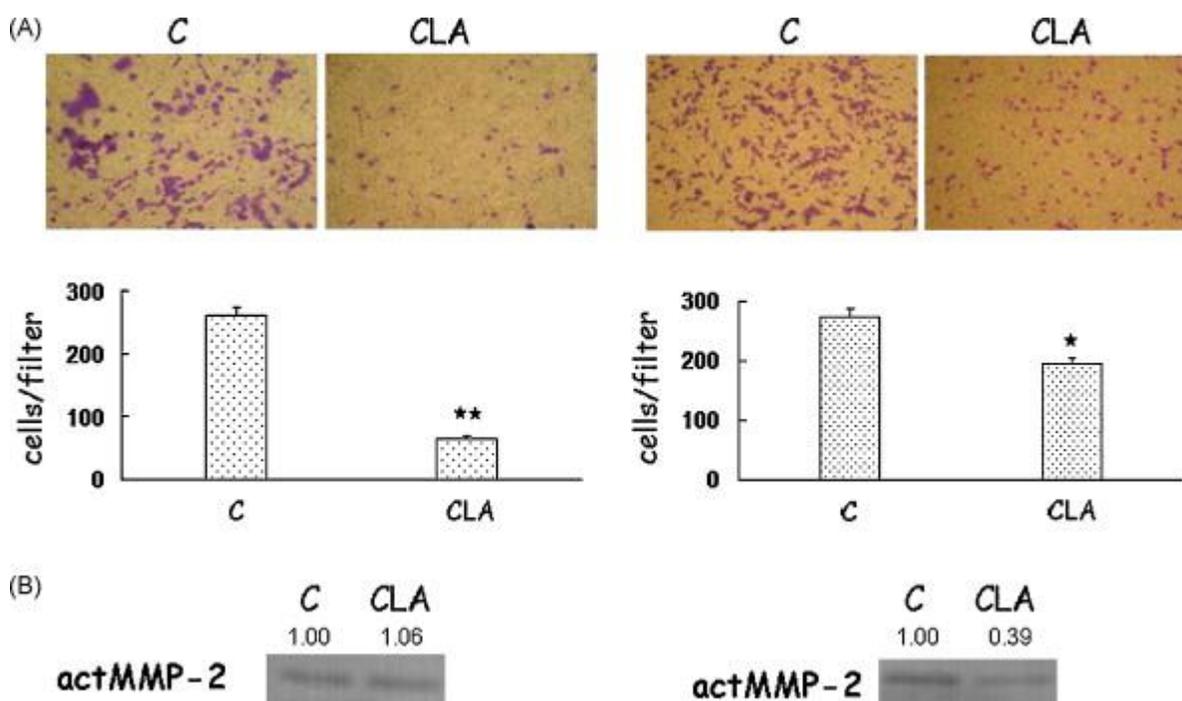


Fig. 3.

Effect of CLA on cell migration and invasion. MCF-7 (left) and MDA-MB-231 (right) were incubated with 60 μ M CLA for 72 h. In A, Matrigel invasion was evaluated with a Boyden chamber assay. Each filter was examined with a Zeiss microscope and the number

of cells was counted. Results are expressed as the number of migrated cells (means) per high-power field. The values represent the mean of three independent experiments each performed in triplicate (bars, S.D.). Statistical significance compared with untreated control: * $P < 0.05$, ** $P < 0.001$, by one-way ANOVA test with the Bonferroni. In B, the gelatinolytic activities of MMP-2 were visualized by scanning and illustrated active (act) MMP-2. The blots shown are from a representative experiment repeated three times with similar results.

[Figure options](#)

These results suggest that in CLA-treated MCF-7 cells the modulation of E-cadherin/ β -catenin system could be associated with the reduction of cell migration, whereas in MDA-MB-231 no correlation was detected.

The fact that CLA exerted strong growth inhibitory effects on ER α (+) MCF-7 cells but had relatively little activity on ER α (-) MDA-MB-231 cells [26] and [27] suggested the possibility of cross-talk between the compound and the ER α pathway. The evaluation of CLA effect on the expression of ER α indicated that the fatty acid did not influence total ER α level in MCF-7 cells and confirmed the absence of the receptor in MDA-MB-231 cells (Fig. 4A).

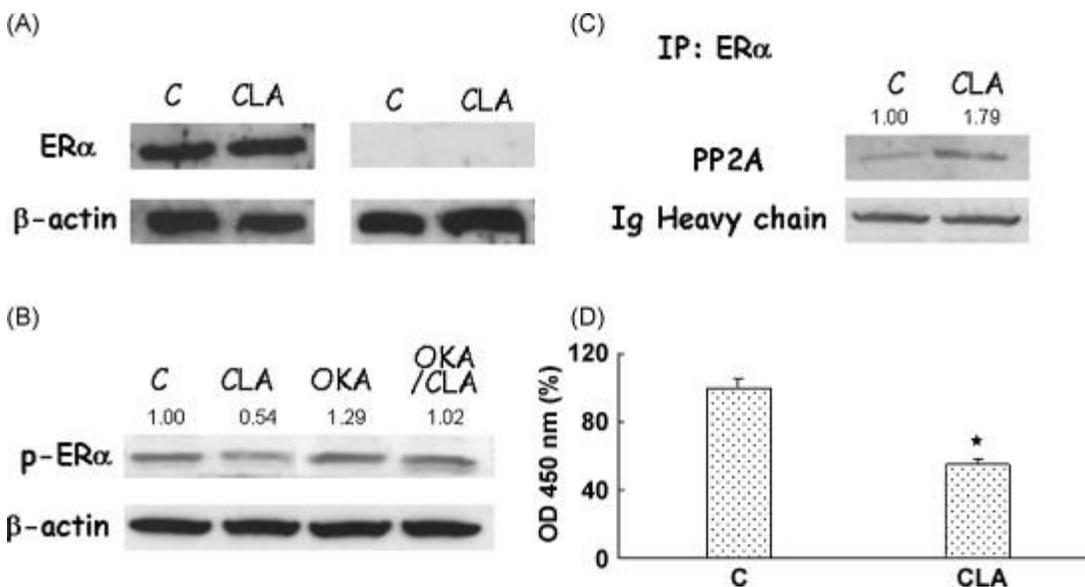


Fig. 4.

Effect of CLA on ER α expression and activation. In A, MCF-7 (left) and MDA-MB-231 (right) were incubated with 60 μ M CLA for 72 h and were processed for western blot analysis with anti-ER α antibody, normalized with anti- β -actin and analysed by densitometry. In B, MCF-7 cells were pretreated or not for 2 h with 0.1 nM okadaic acid before adding 60 μ M CLA for 72 h and the cell lysates were probed with an anti-p-ER α antibody, normalized with anti- β -actin and analysed by densitometry. In C, ER α -PP2A complex formation was detected in MCF-7 cells after treatment with 60 μ M CLA for 72 h. 300 μ g of total proteins were immunoprecipitated with anti-ER α antibody. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-PP2A antibody. The immunoglobulin heavy chain is also shown as a control for loading. The

relative fold change in the protein band to its own control band was quantitated by densitometry. The blots shown are from a representative experiment repeated three times with similar results. In D, nuclear lysates of MCF-7 cells after treatment with 60 μ M CLA were probed with primary antibody specific for the active form of bound ER α , and then with HRP-conjugated secondary antibody. The values represent the mean of three independent experiments each performed in triplicate (bars, S.D.). Statistical significance compared with untreated control: * $P < 0.001$, by one-way ANOVA test with the Bonferroni.

[Figure options](#)

Since there is a growing body of evidence that the phosphorylation status of many transcription factors, including nuclear hormone receptors, is involved in modulating their activation, we sought to determine if CLA may affect the activation state of ER α through the evaluation of the serine phosphorylation state of the receptor. As shown in [Fig. 4B](#), in ER α (+) MCF-7 cells CLA treatment strongly reduces the phosphorylation level of the receptor.

ER α exists in complexes with both kinases and phosphatases that exert opposing effects on ER α phosphorylation status. In this regard, the best understood phosphorylation-related event involves MAPK [\[39\]](#), and the dephosphorylation could involve phosphatases such as PP2A. As expected, the involvement of PP2A in the reduction of the ER α phosphorylation state induced by CLA was confirmed by the observation that MCF-7 cell pre-treatment with PP2A inhibitor okadaic acid prevents CLA-induced ER α dephosphorylation ([Fig. 4B](#)).

Since it has been reported that ER α directly binds to the catalytic subunit of PP2A and this results in the dephosphorylation and inactivation of the receptor [\[40\]](#), we focused specifically on the interaction of ER α with PP2A. According to literature, immunoprecipitation assays revealed that CLA was able to induce the formation of the ER α /PP2A complex, thus inhibiting ER α phosphorylation and activation ([Fig. 4C](#)).

The phosphorylation status of ER α is closely linked with estrogen response element (ERE) binding and with the subsequent transcriptional activation of target genes involved in cell growth [\[41\]](#). Transactivation assays confirmed the data obtained from immunoprecipitation experiments as nuclear extracts from MCF-7 cells treated with CLA showed decreased binding activity to the canonical ERE ([Fig. 4D](#)).

Our findings provide evidence that CLA possesses antiestrogenic properties that can account for the strongest antiproliferative activity detected on ER α (+) MCF-7 as compared to ER α (-) MDA-MB-231 cells. The CLA growth inhibitory effect is in part due to the alteration of balancing between the activation of phosphatases and the inhibition of kinases.

Here we evidenced that the degree of ER α phosphorylation could be regulated by two pathways: the inhibition of PI3K/Akt cascade or the induction of PP2A activity. In MCF-7 cells the stimulation of PP2A expression by CLA can at least partly account for the reduction of ER α transcriptional activity and contributes to the inhibition of Akt. In MDA-MB-231 cells the down-regulation of Akt is not related to PP2A induction but presumably involves other mechanisms.

Collectively, our results suggest that CLA functions as an antiproliferative agent by inhibiting the pro-survival signalling pathways Akt, ERK and ER α . The inhibition of these signalling pathways can contribute to tightly regulate the interplay between the formation and loosening of adhesive contacts of tumor cells with the extracellular matrix, thus reducing breast cancer cell invasion and spreading.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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