Extracellular signal-regulated kinase 1/2 and protein phosphatase 2A are involved in the antiproliferative activity of conjugated linoleic acid in MCF-7 cells

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Conjugated linoleic acid (CLA) has protective properties in breast cancer. Here, we studied the mechanisms underlying the effects of CLA on MCF-7 breast cancer cell proliferation, especially in correlation with the involvement of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and protein phosphatase 2A (PP2A). CLA inhibits MCF-7 cell growth in a concentration- and time-dependent manner, without triggering apoptosis. In assessing expression levels of proteins that play obligatory roles in the ERK cascade, we evidenced that CLA down-regulated Raf-1 and decreased levels of phospho-ERK1/2, as well as c-myc expression. Increase in PP2A expression rates were additionally observed after CLA treatment of MCF-7 cells. The above effects, as well as CLA-induced inhibition of cell growth, were reversed by okadaic acid, a specific inhibitor of PP2A. Thus, PP2A likely participates in deactivation of ERK1/2, and its up-regulation may represent a novel mechanism for CLA-induced inhibition of cell proliferation.

Conjugated linoleic acid: MCF-7 cell line: Extracellular signal-regulated kinase 1/2: protein phosphatase 2A: Proliferation

Mammary cancer is the most commonly diagnosed and represents one of the major leading cause of cancer mortality in women living in the Western world (Boelsma et al. 2001). Among identifiable risk factors associated with mammary cancer occurrence are dietary fatty acids, some of which are implicated in cancer promotion and others which can suppress tumour development (Escrich et al. 2001; Stoll, 2002).

Conjugated linoleic acid (CLA) is a dietary fatty acid predominant in ruminant food products and it has received considerable attention because of its anticarcinogenic and antimutagenic properties (Belury, 2002; Ip et al. 2002). CLA is the generic term of a group of positional and geometric isomers of the n-6 essential fatty acid linoleic acid, which generally stimulates tumour growth (Reyes et al. 2004). In animal models CLA inhibits tumour development (Ip et al. 1985; Belury et al. 1996), while in cancer cellular models, including mammary cancer cells, CLA is an effective inhibitor of tumour growth (Shultz et al. 1992; Ochoa et al. 2004). However, the cellular mechanisms by which CLA elicits these antitumor effects are not well explained at present.

The mitogen-activated protein kinase (MAPK) plays a pivotal role in breast cancer progression (Santen et al. 2002) and can represent one of the major targets for breast cancer therapy. Of this cascade, an important signal transduction pathway is the Ras/Raf-1/ERK cascade, whose activation results in the phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2), of intense interest because of its role in the regulation of proliferation and differentiation (Williams et al. 1993). Activated ERK1/2 proteins in turn phosphorylate and activate a variety of substrates including transcription factors, protein kinases and phosphotyrosine protein phosphatases, leading to positive or negative regulation of signalling cascades (Chang et al. 2003). Elevated ERK1/2 activity has been noted in a proportion of clinical breast cancers v. benign disease (Adeyinka et al. 2002) and a relationship between elevated MAPK activity and shorter disease-free survival in primary breast cancer has been reported (Seboll-Leopold & Herrera, 2004).

Recent evidence indicates a growth-suppressive function of protein phosphatase 2A (PP2A), an important negative regulator of the ERK-signalling pathway (Muzio et al. 2003). PP2A forms stable complexes with protein kinase signalling molecules, playing a regulatory role in signal transduction mediated by reversible protein phosphorylation (Janssens & Goris, 2001); although the role of PP2A in the regulation of cell growth is not clear (Gotz et al. 1998). PP2A acts as a tumour suppressor (Schonthal, 2001) and is negatively influenced by oncogenic signals. In breast carcinoma cell lines its presence is related to oestrogen receptor status, and in oestrogen receptor-positive MCF-7 cells its presence is high (Gopalakrishna et al. 1999).

Recent findings suggest that CLA may be an interesting non-cytokine nutrient modulator of ERK signalling in several

Abbreviations: CLA, conjugated linoleic acid; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase 1/2; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PP2A, protein phosphatase 2A.

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cancer cells (Park et al. 2000; Brown et al. 2004) and that hyperactivity of ERK1/2 may play a central role in breast cancer progression (Zivadinovic & Watson, 2005). The goals of the present study are to evaluate the effects of CLA on the proliferation of human breast cancer cells in culture and to characterize the role of the ERK1/2 pathway and PP2A in breast cancer cell response to CLA.

Materials and methods

Antibodies and chemicals

MCF-7 breast cancer cell lines were a kind gift of Professor Sebastiano Ando, Department of Pharmaco-Biology, Faculty of Pharmacy, University of Calabria, Cosenza, Italy; the MCF-7 epithelial cell line retains several characteristics of differentiated mammary epithelium including the ability to process oestradiol via cytoplasmic oestrogen receptors. CLA, chemicals and culture media were purchased from Sigma Chemical Co. (St Louis, MO, USA). Cell culture plasticware was from Corning-Celbio (Pero, Milan, Italy). Mouse monoclonal antibody to β-actin was from Sigma Chemical Co.; rabbit polyclonal antibody specific for Raf-1 (sc 133), c-myc (C-19) (sc-788), ERK1 K-23 (sc 94) and phospho-ERK1/2 (sc 7383), goat polyclonal antibody specific for PP2A (sc 6110), goat anti-rabbit (sc-2004), goat anti-mouse (sc 2005) and mouse anti-goat (sc 2354) secondary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An enhanced chemiluminescence detection system was obtained from Amersham Pharmacia Biotech (Uppsalra, Sweden).

Cell culture

MCF-7 breast cancer cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U penicillin/ml, 100 µg streptomycin/ml and 25 µg amphotericin B/ml. Cells were cultured at 37°C in a humidified incubator with 5% CO₂ and 95% air, and regularly examined using an inverted microscope.

For treatments, cells were seeded at a density of 3 x 10⁴ cells/cm², allowed to adhere overnight and then treated with serum-free DMEM, supplemented with 100 U penicillin/ml and 100 µg streptomycin/ml, 25 µg amphotericin B/ml, 2 mM-glutamine, 1% insulin–transferrin–sodium selenite, 1% vitamin solution, 0.4% albumin, containing various concentrations of CLA.

Proliferation assay

Cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the conversion of the yellow tetrazolium dye MTT to purple formazan crystals by metabolically active cells. Cells were seeded in ninety-six-well culture plates; 1 d later the medium was changed with DMEM containing the drugs and eight wells were assigned to each experimental treatment. After treatment, 30 µl MTT dye solution (5 mg/ml in PBS) were added to each well, and the plate was incubated for 3 h at 37°C. Dimethyl sulfoxide (150 µl) was added for 20 min and the absorbance at 540 nm was recorded using an ELISA plate reader.

Lactate dehydrogenase activity

Cells were seeded in twenty-four-well culture plates; 1 d later cultures were treated with DMEM containing CLA. The supernatant (1 ml) was then collected for the measurement of lactate dehydrogenase (LDH) activity. The LDH activity was determined by a spectrophotometric assay based on the oxidation of NADH and the rate of decrease in absorbance at 340 nm.

Whole cell extract preparation

Cells were seeded in 75 cm² plates and then properly treated. Whole cell lysates were obtained by direct dissolution of cells using an ice-cold lysis buffer containing 20 mM-Tris-HCl (pH 7.4), 150 mM-NaCl, 5 mM-EDTA, 0.1 mM-phenylmethylsulphonyl fluoride, 0.05% aprotnin and 0.1% Igepal. The lysates were analysed by Western blotting for Raf-1, phospho-ERK1/2, ERK1, PP2A and c-myc.

Protein determination

Protein contents of whole cell lysates were measured in triplicate using a commercially available assay (Bio-Rad, Hercules, CA, USA).

Western blotting

Cell extracts were mixed with solubilization buffer 250 mM Tris (pH 8.8), 4% SDS, 16% glycerol, 8% 2-mercaptoethanol and 0.1% bromophenol blue (60 µg/well) and resolved by 10% SDS–PAGE. Proteins were transblotted on to nitrocellulose for 2 h in a Bio-Rad electroblotting device. Nitrocellulose matrices were blocked with 5% milk in buffer (1 M-Tris buffer saline (pH 7.4), 5 mM- NaCl, 0.1% Tween-20) for 1 h at room temperature. For immunodetection, blocked matrices were incubated overnight at 4°C with primary antibody. The matrices were detected by incubation for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using the enhanced chemiluminescence system. Blots were washed, reprobed with anti-β-actin or anti-ERK1 antibody, and developed in an identical manner for assessing protein levels to ensure equal protein loading.

Densitometric scanning

Band intensities were quantified by densitometry, and expression proteins were reported as a proportion of β-actin or ERK1 protein expression to control for any discrepancies in gel loading. Fold change versus control values has been calculated by normalizing densitometric values obtained from the various proteins with those obtained for β-actin or for ERK1 (VersaDoc Imaging System 3000; Bio-Rad).

Statistical analysis

Statistical significance of the differences was assessed by ANOVA followed by Bonferroni’s procedure. All values are expressed as means and standard deviations, and differences were considered significant at P<0.05.
Results and discussion

Effects on cell growth and viability

Figure 1 summarizes the time–response effects of a mixture of CLA isomers (cis- and trans-9,11 and 10,12 isomers in approximately a 50:50 ratio) on the growth of MCF-7 human breast cancer cells.

The cells were exposed to different concentrations of CLA, ranging from 0.1 μM to 60 μM. The growth inhibitory activity of CLA can be observed at higher concentrations, with a significant decrease in cell viability at 72 h. The CLA concentration determining the maximum inhibitory activity was 60 μM (30% at 48 h, 65% at 72 h, decrease in cell number with respect to untreated control cells).

The three incubation times gave acceptable data, but 72 h assays allowed significant growth-inhibiting concentrations. Additionally, 60 μM was the concentration of CLA that significantly inhibited cell growth at 48 and 72 h. Thus, 60 μM CLA administered for 72 h was the condition generally used for subsequent assays.

We further evaluated the cytotoxic activity exerted by 60 μM-CLA by estimating the LDH release in the medium. The LDH activity of MCF-7 cells treated for 24 and 48 h was similar to that detected in control cells (not shown) and the increase was significant only after treatment of cells for 72 h (Fig. 2). The present result suggests that a remarkable growth inhibitory activity of CLA can be accompanied by a cytotoxic side-effect.

Treatment with 60 μM-CLA for 72 h was without effect on the expression levels of proteins involved in programmed cell death signalling, such as members of the bcl-2 family Bak and BclXl, and caspases 3, 8, 9 (data not shown).

The lack of apoptosis induction in this experimental condition does not agree with other data reported for CLA in breast cancer cells, indicating a positive up-regulation of pro-apoptotic and a negative down-regulation of anti-apoptotic protein expression (Ip et al. 1999; Majumder et al. 2002; Maggiora et al. 2004).

Effects on extracellular signal-regulated kinase 1/2 activation pathway

In order to better understand the molecular mechanisms affected by CLA in its growth inhibitory activity exerted on MCF-7 cells, we investigated whether CLA could directly interfere with the ERK pathway, a large network of signalling molecules regulating cell growth and differentiation (Williams et al. 1993; Liu et al. 2004). Inhibitors of the ERK1/2 cascade are often being explored as anticancer agents (Boldt et al. 2002) so we had supposed a correlation between the inhibition of cell growth exerted by CLA and the modulation of ERK1/2.

Firstly, the effect of CLA was assessed on the expression rate of Raf-1 protein, an upstream component of the extracellular signal-regulated kinase (ERK) pathway that functions as a MAPK kinase kinase, determining the amplitude of ERK activity (Zhang et al. 2002). We observed that a prolonged cell exposure (72 h) to 60 μM-CLA caused a reduction of Raf-1 protein rates (Fig. 3(A)). The level of ERK1/2 activation in MCF-7 cells exposed to the same treatment was further evaluated. A pronounced decrease in the phosphorylated form of ERK1/2 was seen (Fig. 3(B)), in accordance with the effects exerted by CLA on cell proliferation.

We completed our studies by evaluating the expression levels of c-myc, the transcription factor which co-operates with the ERK pathway to regulate cell proliferation (Jaattela, 2004; Mawson et al. 2005). Reduced levels of c-myc were observed (Fig. 3(C)), consistently with the dephosphorylation/inactivation of ERK1/2 and with the lack of apoptosis. Our data can well agree with reports showing that inhibition of the MAPK pathway reduces c-myc protein levels and cell growth (Mawson et al. 2005) and are additionally supported by the knowledge that the c-myc oncogene product plays key functions in cell proliferation, differentiation and apoptosis (Jaattela, 2004).
Involvement of protein phosphatase 2A

Phosphorylations leading to activation and subsequent inactivation of proteins are fundamental regulatory mechanisms for control of cell growth and differentiation (Cohen, 2000). ERK are known to play a key role in cell proliferation, and protein phosphatases are generally identified as inhibitors of cell proliferation by participating in the dephosphorylation of ERK. Several lines of evidence indicate that PP2A is an important negative regulator of the ERK signalling pathway (Park et al. 2000) and that it may even function as a tumour suppressor (Garcia et al. 2003; Muzio et al. 2003). Based on these considerations, we had hypothesized a contribution of PP2A to cellular growth inhibition exerted by CLA. MCF-7 cells were treated at the optimal growth inhibitory concentration of CLA (60 μM for 72 h), which resulted in substantial dephosphorylation of ERK1/2. As we expected, CLA-treated cells exhibited increased PP2A protein content (Fig. 4(A)), which accompanied the reduced levels of phospho-ERK1/2 (Fig. 4(B)).

To test whether dephosphorylation of ERK1/2 effectively depends on higher PP2A activity, we used the specific PP2A inhibitor okadaic acid to block PP2A activity. We measured PP2A expression levels and ERK1/2 phosphorylation in MCF-7 cells incubated with okadaic acid alone at a concentration that would inactivate PP2A (0·1 nM), in comparison

![Fig. 3. Reduction in Raf-1 (A), phospho-ERK1/2 (pERK) (B) and c-myc (C) expression in MCF-7 cells. Cells were treated with 60 μM-conjugated linoleic acid (CLA) for 72 h before harvesting for measurement of protein levels by Western blotting with anti-Raf-1, anti-phospho-ERK1/2 or anti-c-myc antibody. β-Actin and extracellular signal-regulated kinase 1 (ERK1) served as a loading control. The relative fold change in protein band to its own control band was quantified by densitometric scanning and is expressed in arbitray units. For details of procedures, see p. 23. Values are means of three independent experiments with standard deviations depicted by vertical bars. Blots shown are from a representative experiment.](https://www.cambridge.org/core/doi/10.1079/BJN20051619)

![Fig. 4. Okadaic acid (OKA) pretreatment reverses protein phosphatase 2A (PP2A) protein expression (A) and phospho-ERK1/2 (pERK) (B) reduction induced by conjugated linoleic acid (CLA). Cells were cultured for 2 h in medium containing 0·1 nM-OKA and treated with 60 μM-CLA for 72 h before harvesting for measurement of protein levels by Western blotting with anti-PP2A or anti-phospho-ERK1/2 antibody. β-Actin and extracellular signal-regulated kinase 1 (ERK1) served as a loading control. The relative fold change in protein band to its own control band was quantified by densitometric scanning and is expressed in arbitrary units. For details of procedures, see p. 23. Values are means of three independent experiments with standard deviations depicted by vertical bars. Blots shown are from a representative experiment.](https://www.cambridge.org/core/doi/10.1079/BJN20051619)
pendent experiments, each performed in quadruplicate, with assay. For details of procedures, see p. 23. Values are means of three inde-

determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

animal models where the most relevant findings have been

2004). These antitumour effects have been mainly observed in

arcinogenic properties (Whigham et al. 2004). Okadaic acid the inhibitory effects of CLA on breast cancer proliferation.

okadaic acid before CLA led to a reversion of inhibition of cell growth, MCF-7 cells were pretreated with okadaic acid before treatment with CLA, and cell viability was detected after 72 h. Treatment with okadaic acid alone did not influence the growth of MCF-7 cells, while pretreatment with okadaic acid before CLA led to a reversion of CLA effects on cell growth (Fig. 5). PP2A can indeed be suggested to participate in dephosphorylation of ERK1/2 caused by CLA, as demonstrated by efficient reversal of ERK1/2 inactivation with okadaic acid. In the presence of okadaic acid the inhibitory effects of CLA on breast cancer cell growth were reduced too, suggesting a pivotal role for ERK1/2 and PP2A in the regulation of breast cancer cell proliferation.

Conclusions

Diet, particularly dietary fat, has been shown to be a major risk factor in mammary cancer, which represents a relevant healthcare problem. Therefore, the identification of nutrients which could suppress the initiation and development of breast cancer would provide more information for new preventive or adjuvant nutritional strategies. In this sense CLA has received a great deal of attention due to its reported anticarcinogenic properties (Whigham et al. 2000; Maggiora et al. 2004). These antitumour effects have been mainly observed in animal models where the most relevant findings have been marked antitumorigenic activity (Ip et al. 1985; Belury et al. 1996); CLA also elicited inhibition of proliferation and induction of apoptosis in cancer cells in culture (Shultz et al. 1992; Maggiora et al. 2004; Ochoa et al. 2004).

Although the literature relating to the effects of CLA on mammary cancer is extensive, little information is available concerning the molecular and cellular mechanisms of its anti-proliferative effects.

In a previous study from our laboratory we demonstrated that CLA inhibits the growth of the oestrogen unresponsive MDA-MB-231 human breast cancer cell line by triggering apoptosis (Miglietta et al. 2006), and that both the mitochondrial pathway and ERK1/2 repression are involved in apoptosis induction.

Taken together, our findings can provide evidence that major CLA isomers exert antiproliferative activity in MCF-7 breast cancer cells through the repression of the ERK signaling pathway, which can represent the result of PP2A activation. Hence, the results herein reported may be useful for future studies aimed at identifying novel dietary factors important as preventive or therapeutic tools for cancer.

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