Modulation of Arachidonic Acid Distribution by Conjugated Linoleic Acid Isomers and Linoleic Acid in MCF-7 and SW480 Cancer Cells

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ABSTRACT: The relationship between growth and alterations in arachidonic acid (AA) metabolism in human breast (MCF-7) and colon (SW480) cancer cells was studied. Four different fatty acid preparations were evaluated: a mixture of conjugated linoleic acid (CLA) isomers (*c*9,*t*11, *t*10,*c*12, *c*11,*t*13, and minor amounts of other isomers), the pure *c*9,*t*11-CLA isomer, the pure *t*10,*c*12-CLA isomer, and linoleic acid (LA) (all at a lipid concentration of 16 μg/mL). ^{14}C-AA uptake into the monoglyceride fraction of MCF-7 cells was significantly increased following 24 h incubation with the CLA mixture ($P < 0.05$) and *c*9,*t*11-CLA ($P < 0.02$). In contrast to the MCF-7 cells, ^{14}C-AA uptake into the triglyceride fraction of the SW480 cells was increased while uptake into the phospholipids was reduced following treatment with the CLA mixture ($P < 0.02$) and *c*9,*t*11-CLA ($P < 0.05$). Distribution of ^{14}C-AA among phospholipid classes was altered by CLA treatments in both cell lines. The *c*9,*t*11-CLA isomer decreased ($P < 0.05$) uptake of ^{14}C-AA into phosphatidylcholine while increasing ($P < 0.05$) uptake into phosphatidylethanolamine in both cell lines. Both the CLA mixture and the *t*10,*c*12-CLA isomer increased ($P < 0.01$) uptake of ^{14}C-AA into phosphatidylserine in the SW480 cells but had no effect on this phospholipid in the MCF-7 cells. Release of ^{14}C-AA derivatives was not altered by CLA treatments but was increased ($P < 0.05$) by LA in the SW480 cell line. The CLA mixture of isomers and *c*9,*t*11-CLA isomer inhibited ^{14}C-AA conversion to ^{14}C-prostaglandin E_2 (PGE_2) by 20–30% ($P < 0.05$) while increasing ^{14}C-$PGF_{2\alpha}$ by 17–44% relative to controls in both cell lines. LA significantly ($P < 0.05$) increased ^{14}C-PGD_2 by 13–19% in both cell lines and increased ^{14}C-PGE_2 by 20% in the SW480 cell line only. LA significantly ($P < 0.05$) increased 5-hydroperoxyeicosatetraenoate by 27% in the MCF-7 cell line. Lipid peroxidation, as determined by increased levels of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$), was observed following treatment with *c*9,*t*11-CLA isomer in both cell lines ($P < 0.02$) and with *t*10,*c*12-CLA isomer in the MCF-7 cell line only ($P < 0.05$). These data indicate that the growth-promoting effects of LA in the SW480 cell line may be associated with enhanced conversion of AA to PGE_2 but that the growth-suppressing effects of CLA isomers in both cell lines may be due to changes in AA distribution among cellular lipids and an altered prostaglandin profile.

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Compelling evidence indicates that conjugated linoleic acid (CLA), a derivative of linoleic acid found in milk and ruminant fats, is among the more potent naturally occurring anticarcinogens. *In vivo* model studies of experimental carcinogenesis have revealed that a synthetic mixture of CLA isomers containing 21% *c*11,*t*13, 29% *t*10,*c*12, 29.5% *c*9,*t*11, and 12.3% *c*8,*t*10 (1) possesses powerful inhibitory effects on mammary, colon, forestomach, and skin carcinogenesis in rodents (2–8). Other physiological benefits include a reduction in severity of atherosclerotic plaques, improvement of glucose tolerance in diabetic animals, body fat reduction, enhanced immune responses, and positive effects on bone formation, all of which have been well documented in numerous reviews (9–12). The specific CLA isomers that possess biological activity have not yet been clearly identified. Most of the mechanistic work to explain the potent anticancer effects of CLA has involved a commercial free fatty acid preparation containing up to 16 different CLA isomers (1). The availability of the pure isomers of *c*9,*t*11- and *t*10,*c*12-CLA has paved the way for determining the magnitude of biological responses to these isomers; these two are predominantly present in the synthetic CLA mixture and are produced by ruminants (13), and consequently they are found in the human diet. A study by Ip *et al.* (14) revealed that CLA-enriched butterfat, containing predominantly the *c*9,*t*11-CLA isomer, had a powerful protective effect against the risk of mammary cancer development in rodents.

The mechanisms by which CLA exerts its anticarcinogenic effects have not yet been fully elucidated. Induction of apoptosis by CLA *via* downregulation of a membrane protein bcl-2 has been reported to be the mode of cell death in cultured mammary tumor cells and in differentiated colonies of mammary epithelial organoids (15). The incorporation of CLA isomers into membrane phospholipids of breast, skin, and liver tissue has been reported but with variable levels of displacement from membranes of linoleic acid (LA) and arachidonic acid (AA) (7, 16–19). This suggests that CLA may influence the fatty acid

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Abbreviations: AA, arachidonic acid; CLA, conjugated linoleic acid; 5-HPETE, 5-hydroperoxyeicosatetraenoate; IP_3, inositol triphosphate; LA, linoleic acid; LTB_4, leukotriene B_4; MG, monoglyceride; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, prostaglandin; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PI, phosphatidylinositol; PKC, protein kinase C; PL, phospholipid; PLA_2, phospholipase A_2; PLC, phospholipase C; PS, phosphatidylserine; TG, triglyceride; TLC, thin-layer chromatography.

composition of cell membranes, resulting in alterations in eicosanoid production and other signal transduction pathways downstream of the cell membrane. Eicosanoids, comprising prostaglandins and leukotrienes, are a family of membrane-derived lipid mediators that have been an attractive target for cancer chemoprevention (20). Research has shown that CLA can affect the synthesis of eicosanoids, in particular prostaglandin E_2 (PGE_2) (16), a prostanoid that has been shown to promote growth and metastasis in many experimental tumors (21).

Previously we used mammary (MCF-7) and colon (SW480) tumor cell lines as *in vitro* models to investigate the mechanisms by which CLA may affect breast and colon cancer. The MCF-7 epithelial cell line retains several characteristics of differentiated mammary epithelium including the ability to process estradiol *via* cytoplasmic estrogen receptors (22). The SW480 cell line, which was established from a primary adenocarcinoma of the colon, is a dedifferentiated cell line that expresses elevated levels of the p53 protein and small amounts of carcinoembryonic antigen (23). We previously demonstrated that the mixture of CLA isomers induced a dose- and time-dependent cytotoxicity against both cell lines and provided evidence that this effect may be due in part to increased lipid peroxidation (24). The anticancer effect of CLA may be due in part to a redistribution of AA among cellular lipids, which might influence oxidative susceptibility of particular membrane phospholipids and/or alter eicosanoid synthesis during tumor growth. This study was undertaken to examine the modulatory effects of CLA isomers on cell viability in addition to AA uptake, distribution, release, and conversion to eicosanoid classes in breast (MCF-7) and colon (SW480) human cancer cell lines.

MATERIALS AND METHODS

Materials. ^{14}C-AA (specific activity, 55 mCi/mmol), Biotrak enzyme immunoassay kit for leukotriene B_4 (LTB_4), and radioreceptor kit for inositol triphosphate (IP_3) were purchased from Nycomed Amersham (Little Chalfort, Buckinghamshire, United Kingdom). The CLA mixture (21% *c*11,*t*13, 29% *t*10, *c*12, 29.5% *c*9,*t*11, and 12.3% *c*8,*t*10) (1) was obtained from Nu-Chek-Prep (Elysian, MN). Individual CLA isomers, *c*9, *t*11 and *t*10,*c*12, were purchased from Matreya (Pleasant Gap, PA). LA, authentic PGE_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), prostaglandin D_2 (PGD_2), 5-hydroperoxyeicosatetraenoate (5-HPETE), phospholipid (PL) standards, and Supelclean LC-18 SPE columns were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Silica Sep-Pak columns were obtained from Waters Corporation (Milford, MA). The Bioxytech immunoassay kit for 8-epi-$PGF_{2\alpha}$ was obtained from Bio-Stat (Stockport, United Kingdom). DC-Alufliien Kiesegel 60 thin-layer chromatography (TLC) plates were obtained from Lennox (Dublin, Ireland). The CellTitre®AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Southampton, United Kingdom). All other chemicals and solvents used were high-performance liquid chromatography grade.

Cell culture. Human breast (MCF-7) and colon (SW480) cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland). Both cell lines were maintained in Dulbecco's minimal essential medium supplemented with fetal bovine serum (5% by vol), 0.2 mM L-glutamine, 1 mM HEPES, and 1 unit/mL penicillin and streptomycin. The MCF-7 cells required an additional supplement of 10 mM sodium pyruvate. Cells were grown in Falcon T-25 cm^2 flasks and maintained as previously described (24).

Quantification of cell numbers. The comparative effects of four different fatty acid preparations on cell viability were evaluated: (i) the CLA mixture of isomers, (ii) the pure *c*9,*t*11-CLA isomer, (iii) the pure *t*10,*c*12-CLA isomer, and (iv) LA. MCF-7 and SW480 cells were seeded in 96-well plates at densities of 1×10^3/well and 5×10^2/well, respectively. Cells were cultured for 24 h to allow the cells to attach to the substratum. The medium was then replaced with medium supplemented with either the CLA mixture of isomers, the pure *c*9,*t*11-CLA, the pure *t*10,*c*12-CLA, or LA at two different lipid concentrations: 5 and 16 μg/mL corresponding to 17.8 and 57 μM, respectively. The CLA concentrations used have been reported to be within the physiological range of concentrations of the *c*9,*t*11-isomer in human PL (25), plasma, bile, and duodenal juice (26) and have been previously used in cell culture work (27). The fatty acids were dissolved in ethanol and, therefore, control wells were supplemented with equivalent volumes of ethanol (0.25 or 0.8% by vol). After 24 h and 4 d of incubation, viable cell numbers were quantified using the MTS assay kit (CellTitre®AQ_{ueous} Non-Radioactive Cell Proliferation Assay).

Lipid extraction and fractionation. Cells were seeded in T-25 cm^2 flasks at a density of 2×10^5/flask and grown to 90% confluency. The medium was then replaced with medium containing ^{14}C-AA at 0.2 μCi along with either the CLA mixture of isomers, the pure *c*9,*t*11-CLA, the pure *t*10,*c*12-CLA, or LA, all at a lipid concentration of 16 μg/mL (57 μM). The CLA mixture of isomers at a lipid concentration of 16 μg/mL yielded a *c*9,*t*11-CLA and *t*10,*c*12-CLA concentration of approximately 4.7 μg/mL (17 μM) each. Control flasks were supplemented with an equivalent volume of ethanol (0.8% by vol). After 24 h incubation, cells were harvested using phosphate-buffered saline (PBS) containing trypsin (0.25% by vol). Total lipids were extracted from the cell pellet as described (28), dried under nitrogen, redissolved in chloroform, and applied to a silica Sep-Pak column to separate the triglyceride (TG), monoglyceride (MG), and PL fractions as described (29). An aliquot of each fraction was counted in a Beckman LS6500 scintillation counter before being dried under nitrogen. The PL fraction was separated using normal-phase TLC. Samples were co-migrated with authentic standards of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE). Chloroform/methanol/acetic acid (65:45:4, by vol) was used to separate the PL (30). Iodine vapors were used to identify the position of the PL compared with standards, and these bands were removed from TLC plates and placed in vials for counting by liquid scintillation.

Phospholipase C (PLC) activity. IP_3 was used as an index of PLC activity. Cells were treated with the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described above. After 24 h incubation the cells were harvested as described above, and IP_3 was extracted from cells using a perchloric acid extraction method previously described by Palmer *et al.* (31). A radioreceptor assay kit (Biotrak D-*myo*-inositol 1,4,5-triphosphate assay system) was used to quantify IP_3 levels according to the manufacturer's instructions. This assay is based on competition between a [^{3}H]-IP_3 tracer and unlabeled IP_3 in the samples for binding to a bovine adrenal cortex protein.

Release of ^{14}C-AA derivatives. Cells were seeded in T-25 cm^2 flasks at a density of 2×10^5/flask and grown to 80% confluency. Medium was replaced with medium containing ^{14}C-AA (0.2 μCi) and incubated for 24 h. After removal of media, cells were washed three times with PBS before addition of medium containing the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described earlier. After 24 h, medium containing the released ^{14}C-AA derivatives was removed and an aliquot was counted by liquid scintillation.

Primary PG and 8-epi-$PGF_{2\alpha}$. Cells were seeded and treated with ^{14}C-AA at 0.2 μCi along with the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described previously. After 24 h incubation the medium was removed from the flasks, and eicosanoids were extracted twice with ethyl acetate from medium acidified to pH 3.0 with 0.1 N HCl as described (17). Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate, and applied onto normal-phase TLC plates. Ethyl acetate/iso-octane/glacial acetic acid/water (55:25:10:50, by vol) was used to separate PG (16). Samples were comigrated with authentic standards of PGE_2, $PGF_{2\alpha}$, and PGD_2. Iodine vapors were used to identify the position of each PG compared with the standards. Bands of PGE_2, $PGF_{2\alpha}$, and PGD_2 were removed from TLC plates and placed in vials for counting by liquid scintillation. For the 8-epi-$PGF_{2\alpha}$ assay, culture medium was collected after 24 h incubation with the fatty acid treatments described earlier and 8-epi-$PGF_{2\alpha}$ was extracted as described (32). Briefly, ethanol was added to the medium to a final concentration of 15% (vol/vol) and acidified to pH 3.0 with formic acid (98% by vol). The sample was applied to Supelclean LC-18 SPE columns and washed with water (adjusted to pH 3.0 with formic acid), 15% (vol/vol) ethanol in water (pH 3.0), and hexane. Ethyl acetate containing 1% (vol/vol) methanol was used to elute 8-epi-$PGF_{2\alpha}$. The eluate was dried under nitrogen and resuspended in assay buffer (Bioxytech), and a competitive horseradish peroxidase enzyme-linked immunoassay kit (Bioxytech 8-isoprostane assay system) was used to quantify 8-epi-$PGF_{2\alpha}$ levels according to the manufacturer's instructions.

LTB_4 and 5-HPETE. Cells were seeded and treated with the four different fatty acid treatments (all at 16 μg/mL) or ethanol as described earlier. For the 5-HPETE assay, cells were lysed using Triton-X 100 (0.1% vol/vol). The assay was initiated by the addition of 50 μL of AA (70 mM prepared in 50 mM Tris-HCl buffer, pH 4) to 50 μL of cell lysate in an ice-cold 96-well plate and incubated at 37°C for 10 min. The reaction was terminated by the addition of 100 μL of the FOX reagent: sulfuric acid (25 mM), xylenol orange (100 μM), iron(II) sulfate (100 μM), methanol/water (9:1 vol/vol) (33). Absorbance was measured at 620 nm using an Anthos 2010 plate reader. For the LTB_4 assay, eicosanoids were extracted from the medium as described earlier and dried under nitrogen. An enzyme immunoassay kit (Biotrak LTB_4 enzyme immunoassay system) was used to quantify LTB_4 levels according to the manufacturer's instructions. This assay is based on the competition between unlabeled LTB_4 and a fixed quantity of peroxidase-labeled LTB_4 for binding sites on a LTB_4-specific antibody.

Statistical analysis. Three independent experiments were performed in triplicate. Student's *t* test was used to determine significant differences between treatments.

RESULTS

Effect of CLA isomers on cell viability. MCF-7 and SW480 cells were incubated for 24 h and 4 d with the CLA mixture of isomers, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and LA at two different lipid concentrations (5 and 16 μg/mL corresponding to 17.8 and 57 μM, respectively). None of the fatty acids at either 5 or 16 μg/mL significantly altered cell viability after 24 h. The CLA mixture of isomers (16 μg/mL) caused a reduction in cell viability after 4 d in both cell lines, with a greater reduction noted in MCF-7 cells (58%) (Fig. 1A) compared with SW480 cells (52%) (Fig. 1B). The *c*9,*t*11-CLA isomer caused a similar reduction (~50%) in cell viability to the CLA mixture of isomers following 4 d of incubation at both 5 and 16 μg/mL. In both cell lines, the *t*10,*c*12-CLA isomer at 5 and 16 μg/mL reduced viability by 38–39 and 50–60%, respectively, following 4 d of incubation. Incubation of SW480 cells with LA (16 μg/mL) for 4 d increased cell viability by 23%, but the lower concentration of 5 μg/mL had no effect at either time point (Fig. 1B). LA (5 and 16 μg/mL) had no effect on the viability of MCF-7 cells following 4 d of incubation in this study.

Effect of CLA isomers on incorporation of ^{14}C-AA into cellular lipid fractions. One of the mechanisms involved in growth suppression is an alteration in the AA cascade of events leading to eicosanoid production (17). In order to examine if cellular AA distribution was altered by CLA, we investigated the effect of CLA isomers on incorporation of ^{14}C-AA into cellular lipid fractions. ^{14}C-AA was preferentially incorporated into the PL fraction in untreated and CLA-treated MCF-7 cells and SW480 cells (Table 1). Levels of uptake into PL, TG, and MG were 60, 33, and 7%, respectively, in control MCF-7 cells (Table 1). ^{14}C-AA uptake into the MG fractions was increased in MCF-7 cells treated with the CLA mixture ($P < 0.05$) (7.2%) and the pure *c*9,*t*11-CLA isomer ($P < 0.02$) (16.6%). None of the fatty acid treatments had any effect on uptake of ^{14}C-AA into the TG and PL fractions of the MCF-7 cell line.

Levels of uptake into PL, TG, and MG were 76, 21, and 3%,

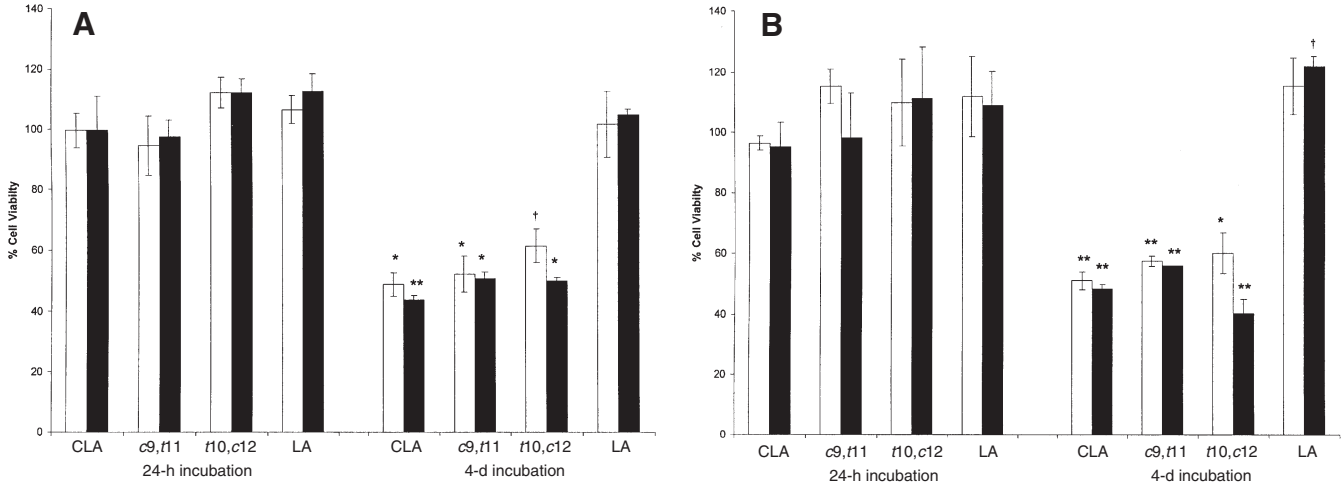

FIG. 1. Cell viability for (A) MCF-7 (human breast cancer) and (B) SW480 (human colon cancer) cells incubated with 5 (open bar) or 16 μg/mL (solid bar) conjugated linoleic acid (CLA) mixture, *c*9,*t*11-CLA, *t*10,*c*12-CLA, linoleic acid (LA), or ethanol control for 24 h and 4 d. Data represent cell viability expressed as a percentage of the control, which was taken to be 100% (**$P < 0.001$, *$P < 0.02$, and $^{\dagger}P < 0.05$). Error bars represent standard deviation.

respectively, in control SW480 cells (Table 1). In contrast with MCF-7 cells, uptake of ^{14}C-AA into PL was significantly lowered ($P < 0.02$) (~25%) in the SW480 cells treated with the CLA mixture and *c*9,*t*11-CLA, while both the CLA mixture and *c*9,*t*11-CLA increased AA uptake into TG (25–30%) ($P < 0.05$). These data suggest that ^{14}C-AA uptake into TG occurred at the expense of PL in the SW480 cell line. None of the fatty acid treatments had any effect on uptake of ^{14}C-AA into the MG lipid fraction of the SW480 cells. The *t*10,*c*12-CLA isomer and LA (both at 16 μg/mL) had no effect on ^{14}C-AA incorporation into any of the lipid fractions in either cell line.

Effect of CLA isomers on ^{14}C-AA distribution among PL fractions. Having shown that ^{14}C-AA was preferentially incorporated into the PL fraction of CLA-treated cells, we examined the effect of CLA isomers on ^{14}C-AA distribution among individual PL. PC and PE were the predominant PL classes in which ^{14}C-AA was taken up by control cells. Levels of uptake into PC, PI, PS, and PE were 45, 8, 6, and 41%, respectively, in the MCF-7 control cells and 34, 3, 3, and 60%, respectively, in the SW480 control cells (Tables 2 and 3). Of all the treatments, only incubation with the pure *c*9,*t*11-CLA isomer altered the distribution of ^{14}C-AA among PL classes in the MCF-7 cells (Table 2). The *c*9,*t*11-CLA treatment at 16 μg/mL significantly ($P < 0.05$) reduced uptake of ^{14}C-AA into PC (32%) and increased uptake into PE (41%). The CLA mixture at 16 μg/mL (which yielded a *c*9,*t*11-CLA isomer concentration of 4.7 μg/mL) had no effect. The *t*10,*c*12-CLA isomer at 16 μg/mL had no effect on the incorporation of ^{14}C-AA into any of the PL fractions in the MCF-7 cells.

Incubation of the SW480 cell line with the *c*9,*t*11 CLA isomer (16 μg/mL) decreased uptake of ^{14}C-AA into PC by 24% ($P < 0.01$) and increased uptake into PE by approximately 20% ($P < 0.01$) (Table 3). In contrast with MCF-7 cells, both the CLA mixture at 16 μg/mL (which yielded a *t*10,*c*12-CLA isomer concentration of 4.7 μg/mL) and the *t*10,*c*12 isomer (16 μg/mL) increased uptake into PS by 12–15% ($P < 0.05$) in the SW480 cell line. LA treatment had no effect on ^{14}C-AA distribution among PL fractions in either cell line. None of the CLA isomers or LA had any effect on the uptake of ^{14}C-AA in PI.

Effect of CLA isomers on AA release. AA can be released by two major pathways, the first through the action of phospholipase A_2 (PLA_2), which catalyzes the hydrolysis of *sn*-2 fatty acyl bond of membrane PL to liberate free AA (34), and the second by sequential cleavage of PI by PLC and diacylglyceride lipase (35). IP_3 levels were used as an index of PLC

TABLE 1
Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into Lipid Fractions[a]

	MCF-7			SW480		
Fatty acid	MG	TG	PL	MG	TG	PL
Control	7.0 ± 1.3	33.5 ± 5.2	59.5 ± 6.2	2.9 ± 0.9	21.0 ± 0.6	76.1 ± 1.5
CLA mixture	14.8 ± 1.6[b]	28.4 ± 1.2	56.8 ± 2.8	3.8 ± 1.0	47.2 ± 3.2[a]	48.9 ± 2.2[a]
*c*9,*t*11-CLA	23.6 ± 1.0[a]	26.4 ± 4.1	49.9 ± 5.1	4.7 ± 1.3	45.7 ± 6.1[b]	49.6 ± 7.2[a]
*t*10,*c*12-CLA	6.2 ± 0.8	34.4 ± 4.5	59.5 ± 5.1	4.0 ± 1.9	22.1 ± 4.2	73.4 ± 5.7
LA	10.3 ± 2.0	31.2 ± 3.3	58.4 ± 5.2	3.8 ± 2.0	26.0 ± 9.1	70.1 ± 7.6

[a]Data represent the mean (± SD) percentage of total cellular lipids. Superscript roman letters indicate values that are significantly different compared to controls ([a] denotes $P < 0.02$ and [b] denotes $P < 0.05$). MCF-7, human breast cancer cells; SW480, human colon cancer cells; CLA, conjugated linoleic acid; AA, arachidonic acid; MG, monoglyceride; TG, triglyceride; PL, phospholipid; LA, linoleic acid.

TABLE 2
Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into MCF-7 Phospholipid Fractions[a]

Fatty acids	PC	PI	PS	PE
Control	44.4 ± 9.7	8.0 ± 2.8	6.2 ± 4.04	1.4 ± 8.2
CLA mixture	33.5 ± 3.9	6.7 ± 3.7	11.4 ± 5.6	48.4 ± 3.3
*c*9,*t*11-CLA	11.6 ± 2.7[a]	4.3 ± 0.6	1.8 ± 0.5	82.2 ± 3.5[b]
*t*10,*c*12-CLA	27.5 ± 6.9	5.5 ± 1.7	10.8 ± 6.5	56.3 ± 3.0
LA	33.5 ± 1.6	6.8 ± 3.0	3.7 ± 3.1	55.9 ± 7.7

[a]Data represent the mean percentage incorporation of total cellular phospholipids. Superscript roman letters indicate values that are significantly different compared to controls ([a] denotes $P < 0.05$ and [b] denotes $P < 0.02$). PC, phosphatidycholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; for other abbreviations see Table 1.

activity in this study. The CLA mixture of isomers, the pure *c*9,*t*11- and *t*12,*c*10-CLA isomers, and LA did not affect IP_3 in either cell line (data not shown). Total ^{14}C-AA derivatives were increased by 28% ($P < 0.05$) in SW480 cells treated with LA only, whereas none of the CLA isomers had any effect on the total level of ^{14}C-AA derivatives released by cells (Fig. 2).

Effect of CLA isomers on eicosanoid synthesis. The effects of various fatty acid treatments on enzymatic conversion of AA to primary eicosanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, LTB_4, and 5-HPETE) and on its nonenzymatic, free radical-catalyzed conversion to 8-epi-$PGF_{2\alpha}$, were examined. Following incubation of MCF-7 and SW480 cells in the presence of the CLA mixture and the pure *c*9,*t*11-CLA isomer at 16 µg/mL, it was found that ^{14}C-AA conversion to ^{14}C-PGE_2 was decreased by 20–30% ($P < 0.05$) whereas conversion to ^{14}C-$PGF_{2\alpha}$ was increased by 17–44% relative to control (Figs. 3A,B). CLA treatments had a negligible effect on ^{14}C-PGD_2. The *t*10,*c*12-CLA isomer had no effect on the three PG examined in either cell line. LA significantly ($P < 0.05$) increased ^{14}C-PGD_2 by 13–19% in both cell lines and increased ($P < 0.05$) ^{14}C-PGE_2 by 20% in the SW480 cell line only. Incubation of cells with either the CLA mixture of isomers or the pure *c*9,*t*11- or *t*10,*c*12-CLA isomers did not alter 5-HPETE or LTB_4 levels in the cells (data not shown), suggesting that CLA may mediate its effect *via* the cyclooxygenase component of the AA cascade. LA significantly increased 5-HPETE levels by 27% ($P < 0.05$) in the MCF-7 cell line (data not shown) but had no effect in the SW480 cells compared with untreated controls. The *c*9,*t*11-CLA isomer significantly increased ($P < 0.02$)

TABLE 3
Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into SW480 Phospholipid Fractions[a]

Fatty acids	PC	PI	PS	PE
Control	32.5 ± 8.0	3.5 ± 0.3	2.5 ± 0.7	61.5 ± 8.5
CLA mixture	25.9 ± 1.7	6.7 ± 1.9	14.5 ± 1.2[a]	52.9 ± 0.8
*c*9,*t*11-CLA	8.3 ± 0.2[b]	3.7 ± 0.1	5.5 ± 2.2	82.5 ± 2.1[b]
*t*10,*c*12-CLA	25.8 ± 8.2	10.6 ± 4.4	9.1 ± 3.6[b]	54.4 ± 7.5
LA	36.7 ± 9.6	3.7 ± 1.1	3.2 ± 0.4	56.4 ± 8.5

[a]Data represent the mean percentage incorporation of total cellular phospholipids. Superscript roman letters indicate values that are significantly different compared to controls ([a] denotes $P < 0.01$ and [b] denotes $P < 0.05$). For abbreviations see Tables 1 and 2.

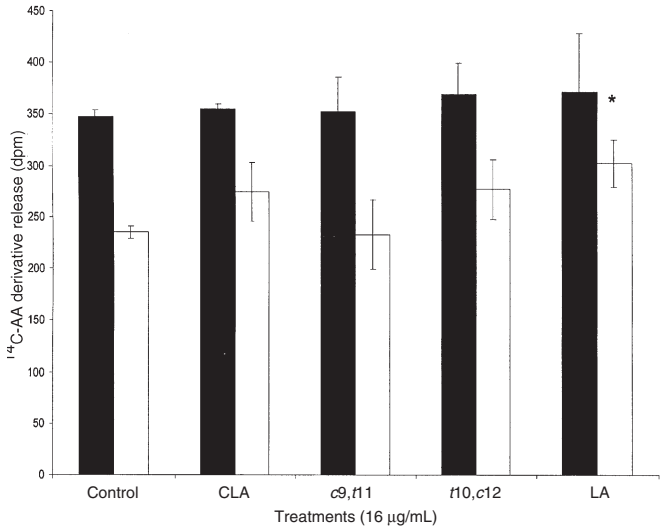

FIG. 2. The effect of treatments on total ^{14}C-arachidonic acid (AA) release in MCF-7 (solid bars) and SW480 (open bars) cells. Cultures were treated with ^{14}C-AA at 0.2 µCi for 24 h after which medium was replaced to contain either CLA mixture, LA, *c*9,*t*11-CLA, *t*10,*c*12-CLA (16 µg/mL), or ethanol (control) and then incubated for 24 h. Medium containing the released ^{14}C-AA was removed, and an aliquot was counted by liquid scintillation. Results were expressed as mean ^{14}C-AA released (dpm). Error bars represent standard deviation. *Values that are significantly different ($P < 0.05$) compared to controls. For abbreviations see Figure 1.

8-epi-$PGF_{2\alpha}$ in MCF-7 and SW480 cells by 38 and 48%, respectively (Figs. 3A,B). The *t*10,*c*12-CLA isomer increased ($P < 0.05$) levels of 8-epi-$PGF_{2\alpha}$ by 30% in the MCF-7 cell lines but had no effect in the SW480 cells (Fig. 3B). However, treatment of both cell lines with the mixture of CLA isomers and LA treatments had no relative effect in either cell line.

DISCUSSION

This study shows that the MCF-7 and SW480 cell lines were sensitive to growth-inhibitory effects of not only the CLA mixture but also both the *t*10,*c*12-CLA and the *c*9,*t*11-CLA isomers following 4 d of incubation with physiological levels of CLA (5–16 µg/mL) (27). The CLA mixture of isomers at 16 µg/mL (yielding a *c*9,*t*11-CLA and *t*10,*c*12-CLA concentration of approximately 4.8 µg/mL each) was equally effective in inhibiting growth of both cell lines as the pure *c*9,*t*11-CLA and *t*10,*c*12-CLA isomer added at 16 µg/mL. This suggests that a plateau effect was reached or that one or more of the other isomers present in the mixture may be capable of altering cell viability. It is imperative, however, that more basic research be undertaken to determine the specific biological effects of other isomers present in the mixture, particularly *c*11,*t*13, which has recently been detected in natural products (36) and in liver microsomes (37). The growth-stimulatory effect of LA previously reported (27,38) was also seen in this study in the SW480 cell line treated with LA, but no effect was seen in the MCF-7 cells at the concentrations used.

This study provides an insight into the early responses of breast and colon cancer cell lines before growth is altered. Inter-

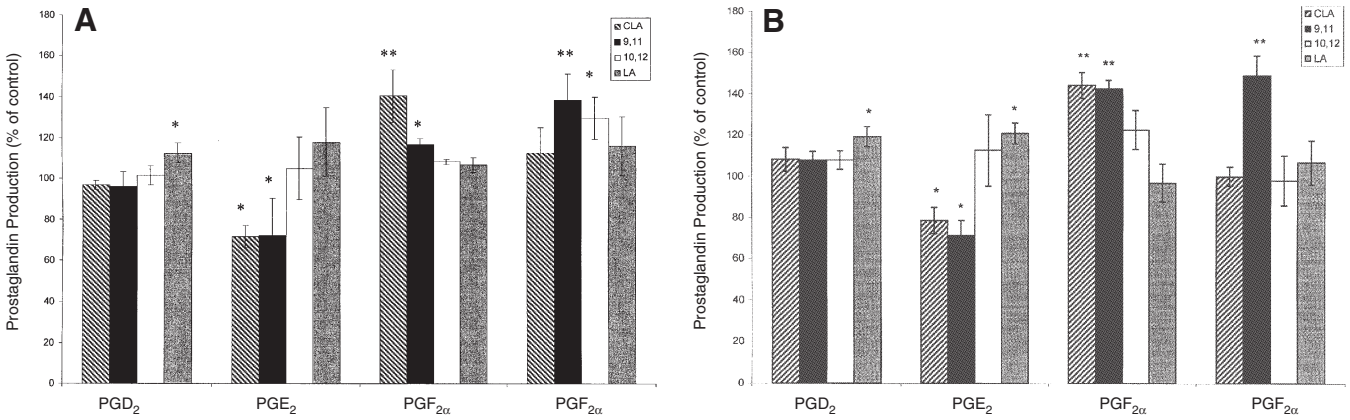

FIG. 3. Effect of treatments on primary prostaglandins (PG) and 8-epi-$PGF_{2\alpha}$ synthesis in (A) MCF-7 and (B) SW480 cells. Cultures were treated with 0.2 μCi/mL ^{14}C-AA along with either the CLA mixture, *c*9,*t*11-CLA, *t*10,*c*12-CLA, and LA (16 μg/mL) or ethanol control for 24 h. Eicosanoids were extracted from medium, and PG were separated using thin-layer chromatography and counted by liquid scintillation. Data represent the mean ^{14}C-PG synthesis expressed as a percentage of the control, which was taken to be 100%. 8-Epi-$PGF_{2\alpha}$ levels were quantified using an enzyme immunoassay kit from Bioxytech (Bio-Stat, Stockport, United Kingdom). Error bars represent standard deviation. Asterisks indicate values that are significantly different compared to controls (**$P < 0.02$; *$P < 0.05$). For abbreviations see Figures 1 and 2.

estingly, the CLA mixture of isomers containing 4.7 μg/mL *c*9,*t*11-CLA was less effective than the pure *c*9,*t*11-CLA isomer (16 μg/mL) at redistributing AA among lipid fractions in the MCF-7 cell line and had no effect in altering AA content of individual PL of these cells. Our data demonstrate that the *c*9,*t*11-CLA isomer decreased AA uptake into PC while increasing uptake into PE in both cell lines. The decrease in uptake into PC is very significant as this is the PL preferentially hydrolyzed by PLA_2 to provide AA for eicosanoid synthesis (39). None of the other treatments had any effect on PS in the MCF-7 cell line, but in the SW480 cell line the CLA mixture and the *t*10,*c*12-CLA isomer both increased uptake of AA into PS. Although PS is a biosynthetic precursor of PE, it is in itself an important membrane lipid as it is an activator of membrane-associated protein kinase C (PKC), an enzyme that phosphorylates serine and threonine residues of an extremely diverse group of proteins regulating cell proliferation, activating cellular function, differentiation, and even apoptosis (40). It has been postulated that CLA may modulate PKC (41). However, activation of this enzyme is also dependent on diacylglycerol, a product of PLC activity, and Ca^{2+} released from intracellular stores by IP_3. None of the treatments investigated altered the levels of IP_3 in the cells or uptake of AA into PI, suggesting that growth modulatory effects of various treatments in this study were not associated with PLC-mediated signal transduction. Other reports also indicate that physiological concentrations of CLA did not mediate changes in either PLC or PKC activity in MCF-7 cells (42) or in normal rat mammary epithelial cell organoids (43).

Interestingly, none of the CLA treatments influenced AA release from cells, yet both the CLA mixture and the *c*9,*t*11-CLA isomer decreased ^{14}C-PGE_2 synthesis and increased ^{14}C-$PGF_{2\alpha}$ in both cell lines, suggesting that a modulation of cyclooxygenase and/or downstream isomerase or reductase gene expression may be responsible. By contrast, LA stimulated PGD_2 production in both cell lines while stimulating PGE_2 production in the SW480 cell line. These changes in PG synthesis may have been responsible for the differential effects of LA and CLA treatments on growth. Levels of LTB_4 and 5-HPETE were not altered by any of the CLA treatments, suggesting that the anticancer effect of CLA may be mediated independently of the lipoxygenase component of the AA cascade as has been already proposed (44, 45).

A differential effect between physiological levels (0.5–5 μg/mL) of *c*9,*t*11 CLA and LA on growth of MCF-7 cells after 4 d has been reported (42). Growth inhibition by the CLA isomer was not mediated through PLC, PKC, or PGE_2-dependent signal transduction pathways, suggesting that another inhibitory mechanism may be involved. Because our study did show that PGE_2 synthesis was reduced by higher but near-physiological concentrations of CLA, it is apparent that there may be a threshold requirement for CLA and LA to affect cellular PGE_2 synthesis. A similar inhibitory effect of CLA on PGE_2 synthesis was observed in keratinocytes (17) and mouse epidermis (46). More recently CLA has been shown to inhibit prostaglandin H synthase activity in ram seminal vesicle microsomes (47).

Basu and coworkers (48) reported that CLA induced lipid peroxidation in humans by using urinary 8-iso-$PGF_{2\alpha}$ excretion as a biomarker of nonenzymatic lipid peroxidation. We showed that incubation of both cell lines with the *c*9,*t*11-CLA isomer led to significantly increased 8-epi-$PGF_{2\alpha}$ in both cell lines, while incubation with the *t*10,*c*12-CLA led to increases in 8-epi-$PGF_{2\alpha}$ levels in the MCF-7 cell line only. These isomers may be promoting nonenzymatic oxidation of AA at the expense of the formation of enzymatically derived eicosanoids. The mixture of CLA isomers (at 16 μg/mL) had no effect on 8-epi-$PGF_{2\alpha}$ levels, suggesting that a concentration of *c*9,*t*11-CLA higher than 4.7 μg/mL is needed to induce nonenzymatic oxidation of AA. A number of studies have now shown that the production of reactive oxygen species

serves to trigger an apoptotic signal transduction pathway (reviewed in Ref. 49). Further studies to investigate the effects of CLA isomers on the expression of cyclooxygenase isoforms and other signal transduction pathways are warranted to explain the potential inhibitory role of CLA on *in vitro* growth. Intervention studies have shown that increasing CLA intake led to increases in the CLA content in human milk (50), plasma (51), and adipose tissue (52). Although it is attractive to speculate that CLA may be useful in nutritional prevention of cancer in humans, evidence of beneficial effects in cancer patients receiving CLA as a dietary supplement is required. To this end, appropriate molecular and biochemical markers of both CLA nutritional status and of tumorigenesis are currently being sought.

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