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Butterfat Fatty Acids Differentially Regulate Growth and Differentiation in Jurkat T-Cells

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Abstract Synthetic Conjugated Linoleic Acid mixture (CLA; c9,t11; t10,c12-18:2) has been previously shown to inhibit growth, and enhance apoptosis and IL-2 mRNA synthesis in human lymphoblastic Jurkat T-cells. In this study, two different butterfat types were evaluated and compared for their effects on Jurkat cell viability, oxidative stress, pro-apoptotic activity, and cytokine synthesis: the conventionally produced butterfat (CBF), and organic butterfat (OBF) containing significantly higher amounts of c9,t11 (Rumenic Acid, RA), trans-vaccenic acid (VA; t11-18:1), α-linolenic acid (ALA), and lower levels of linoleic acid (LA). Results from cell treatment with both butterfat mixtures showed comparable oxidative stress (superoxide production, intracellular GSH depletion,and lipid peroxides yield), NADPH oxidase activation, cytotoxicity (LDH release), and IL-2 transcript level, whereas the effects of enhanced growth-inhibitory and pro-apoptotic activities were associated with OBF treatment. To then investigate each butterfat-induced effect caused by RA, VA, LA, and ALA, cells were exposed to synthetic FA concentrations similar to those from the different butterfats. Higher oxidative stress (superoxide production, intracellular GSH depletion) was induced by α-linolenic (ALA) and linoleic (LA) incubation ($P < 0.01$) and superoxide production was suppressed by specific PKCα inhibitor (Gö 6976) and linked to increased toxicity and IL-2 synthesis inhibition. By contrast, cell treatment with RA increased apoptosis and IL-2 synthesis. These results suggest that a supply of ALA and LA is responsible for BF-induced oxidative stress via PKCα-NADPH oxidase pathway, and that enhanced antiproliferative effects in OBF treated cells is essentially determined by RA-induced pro-apoptotic activity. J. Cell. Biochem. 96: 349–360, 2005. © 2005 Wiley-Liss, Inc.

Key words: conjugated linoleic acid; butterfat fatty acids; apoptosis; immunomodulation

Relatively high concentrations of cholesterol and saturated fatty acids in milk fat are known as factors in increasing the risk of heart disease. On the other hand, more attention has been given to several butterfat components such as CLA, trans-vaccenic acid (VA), and α-linolenic Acid (ALA) because of their potentially beneficial effects for human health.

Long-chain n-3 and n-6 polyunsaturated fatty acids (PUFA) have been reported to inhibit cell division, block the cell cycle, and induce cell death in malignant epithelial cells in vitro [Falconer et al., 1994; Nano et al., 2003], and during the past decade, several livestock feeding strategies have also been proposed that increase ALA levels content in cow milk to improve its nutritional value [Baer, 1996]. CLA is a collective term to indicate positional and geometric isomers of linoleic acid (LA) and consists of up to eight possible isomers [Ha et al., 1987], one being the *cis*- 9, *trans*- 11 isomer, which is the most abundant CLA isomer in the human diet. Because of its predominant presence in foods of ruminant origin, in fact, it has also been named the "rumenic acid" (RA) [Kramer et al., 1998]. VA is the major precursor of CLA in milk fat [Griinari et al., 2000] and its contribution in milk fat-induced anti-cancer response has been recently described [Ip et al., 1999; Corl et al., 2003]. Additionally, its significant bioconversion of dietary VA in humans by Δ^9-desaturase [Turpeinen et al., 2002], supports the hypothesis that increased VA content in dairy, and in other ruminant products, may increase RA availability in human tissue.

The animal's diet is the major determinant for modifying the fatty acid composition of milk fat, and there is a continued interest in the development of dairy products enriched with the

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fatty acids that are healthier for humans. Increased RA quantities in milk has been successfully achieved through animal diet manipulation [Bauman et al., 2001] and enhanced RA, VA, and ALA concentrations were found in milk fat produced according to organic husbandry [Jahreis et al., 1997; Bergamo et al., 2003] or in pasture-fed dairy cows [Hauswirth et al., 2004].

The importance of dietary CLA to human health was first recognized by Pariza and Hargraves [1985] who demonstrated that these fatty acids could inhibit cancer. Since then, many beneficial properties have been attributed to CLA including antiatherosclerotic, antiadipogenic, antidiabetogenic, and immunomodulating activity [Wahle et al., 2004]. However, only relatively little evidence has been published regarding the antineoplastic effect of RA-rich butterfat [Ip et al., 1999; O'Shea et al., 2000; Corl et al., 2003; Miller et al., 2003] and the biochemical events governing this activity have not yet been characterized.

In this study, butterfat (BF) produced according to organic measures (OBF) was used as an RA-rich source, and its ability to modify growth, apoptosis, and cytokine synthesis of Jurkat T-cells was evaluated. The results were then compared to the effects on the same cell activities observed for butterfat produced by conventional husbandry (CBF). Moreover, we investigated the degree to which RA, VA, LA, or ALA each contributed to the BF-induced biological effects, in addition to their underlying biochemical mechanisms.

MATERIALS AND METHODS

Reagents

Caspase-3 substrate, acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-p-NA), caspase inhibitor, acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO), and Gö 6976 were purchased from Calbiochem (San Diego, CA). RPMI 1640 and non-essential aminoacids (NEAA) were from Cambrex Bioproducts Europe (Verviers, Belgium). All other reagents were from Sigma (St. Louis, MO).

Fatty Acids Analysis and Butterfat Sample Preparation

Butter samples from organic ($N = 3$) or conventional management system ($N = 3$) were purchased at local supermarkets. Butter aliquots (0.5 g) were carefully weighed and fat was extracted by using the Folch procedure as modified by Chin et al. [1992]. Methyl esters were prepared by transmethylation of extracted lipids and fatty acid composition of the different butterfat was evaluated by gas-chromatographic analysis [Bergamo et al., 2003]. Samples prepared from the same butter type were pooled and stored at −20°C.

Cell Culture

Jurkat cells were grown at 37°C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated foetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and non-essential amino acids (NEAA) (1%). Cells were kept in the exponential growth phase by passages at 2–3 days intervals. Cultures at $6–7 \times 10^5$ cell/ml density in complete RPMI-1640 medium were used in each experiment, unless otherwise stated. In this study, the amount of BF used was chosen on the basis of its RA content (from 15 to 60 μM) and dissolved in ethanol according to literature [O'Shea et al., 2000; Miller et al., 2003]. In particular, cells exposed to the same amount of ethanol (0.1%) were used as controls and no significant differences were observed compared to untreated culture (data not shown).

Cell Proliferation Assay

Jurkat T-cells were plated in flat bottom 96-well trays (1×10^4/well) and cultured for 72 h in the presence of butterfat (0.8 or 1.7 mg/ml) or different concentrations of synthetic RA, VA, ALA, and LA (15–130 μM).

In experiments with inhibitors, cells were exposed to 50 U/ml superoxide dismutase (SOD) or 10 μM alpha-Tocopherol (α-Toc). Eighteen hours prior to harvesting, cells were pulsed with 1 μCi/well [^{3}H]-thymidine. At the end of the incubation, cultures were harvested on filters using a semiautomatic cell harvester (Filtermate, Packard, Danvers, MA) and the presence of [^{3}H] thymidine in DNA was assessed by a microplate liquid scintillator (Top Count NXT™, Packard, Danvers, MA). Results were expressed as Proliferation Index (PI = cpm treated/cpm untreated cultures).

Measurement of Lipid Peroxides

Lipid hydroperoxides (LOOH) yield was spectrophotometrically determined following the

formation of a Fe(III) xylenol orange complex according to Gay and Gebicki [2003]. Jurkat T-cells were incubated (48 h) in culture medium supplemented with 1.7 mg/ml butterfat or with different amounts of synthetic FA at concentrations similar to those found in O- or CBF samples (30 or 60 μM RA, 130 or 90 μM VA, 130 or 90 μM LA, and 30 or 60 μM ALA, respectively). The absorbance was measured at 560 nm against blank (cell extract reduced with 1 mM triphenylphosphine) and converted in amount of LOOH ($\varepsilon = 52{,}100\ mM^{-1}\ cm^{-1}$). Following determination of protein content, using the Bradford Protein Assay Kit (Bio-Rad, Hercules Ca) [Bradford, 1976], LOOH concentration was finally expressed as mmoles LOOH/mg of protein.

Measurement of Butterfat and Fatty Acid-Induced Toxicity

Cytotoxic effects were measured by CytoTox 96® detection kit (Promega Bioscience, Inc., San Luis Obispo, CA) according to the manufacturer's instructions. In brief, cultures were exposed to 1.7 mg/ml BF for different times or incubated for 48 h in the presence of synthetic FA (60 μM RA, 130 μM VA, ALA, or 60 μM LA). Cytosolic lactate dehydrogenase (LDH) activity released into the medium was evaluated by colorimetric analysis.

Measurement of NADPH Oxidase Activity

NADPH oxidase complex activation was visualized by the *p*-nitro blue tetrazolium (NBT) reduction assay [Bruchhaus et al., 1998]. In brief, Jurkat T-cells at a density of 2×10^6 cells/ml were exposed (30 min, 37°C) to 1.7 mg/ml of different BF. At the end, cells were resuspended (30 min, 4°C) in 25 mM HEPES, pH 7.6, 40 mM NaCl, 20 mM DTT, 1 mm Phenyl-methyl-sulphonyl-fluoride, 10 μg/ml Leupeptin, 100 μg/ml Pepstatin A, and 0.5% Triton X-100. Protein extracts were fractionated by electrophoresis on a 12% non-denaturing polyacrylamide gel. The NADPH oxidase band was visualized as previously described [Bergamo et al., 2003].

Additionally, butterfat or synthetic fatty acids ability to activate NADPH oxidase was measured by the cytochrome-*c* reduction assay [Arroyo et al., 2002]. In brief, cell aliquots (2×10^6 cells/ml) were incubated up to 40 min at 37°C in the presence of BF or FA at different times; cytochrome c reduction was measured at 550 nm mixture by subtracting the absorbance value read in culture supplemented with SOD. Values were expressed as mmoles $O_2^{\cdot -}$/mg of total protein. In some experiments the superoxide anion ($O_2^{\cdot -}$) yield was evaluated in the presence/absence of 2.5 μM Gö 6976, an inhibitor of PKC-α; as positive control cells were treated with 0.12 μg/ml PMA, a specific PKC activator.

Measurement of Intracellular Reduced Glutathione (GSH)

Cytosolic GSH depletion was evaluated by the dithiobis (2-nitrobenzoic acid) (DTNB) GSSG reductase assay [Hyde et al., 1997]. Cultures were incubated for 48 h at 37°C with butterfat or with synthetic FA. Then, cell aliquots (4–5×10^6 cells) were washed twice with PBS and centrifuged (3 min, 10,000 rpm); cell pellet was then dissolved in 100 μl of trichloroacetic acid (TCA) solution (5% TCA, 0.1 mM HCl, 10 mM EDTA) and incubated for 10 min; suspensions were centrifuged (3 min, 10,000 rpm). Fifty microliters of supernatant aliquots was transferred in a 96-well plate; 140 μl of 0.3 mM NADPH and 100 μl of 1 U/ml GSH reductase, both in 125 mM sodium phosphate buffer pH 7.5 containing 6.3 mM EDTA, were added to each well. Twenty microliters of 6 mM DTNB was then added into each well; the absorbance change at 405 nm was monitored for 10 min and calculated as Mean V [Mean V = (OD at 10 min − OD at 0 min)/10] and converted into total GSH concentration by using a GSH standard curve. TCA cell precipitates were dissolved in 100 μl of 6 M Guanidine-HCl for protein determination. GSH concentration was finally expressed as nmoles GSH/min/mg total protein.

Measurement of Caspase-3 Activity

Butterfat or synthetic fatty acid-induced caspase-3 activity was spectrophotometrically determined as described by Bergamo et al. [2004]. In brief, cells were incubated with BF (up to 72 h) or FA (48 h) and caspase-3 activity was measured at 24 h intervals. Cultures exposed to 10 μM actinomycin D were used as positive control and caspase-3 activity was expressed as nmoles of *p*-nitroanilide (pNA)/h/mg of total protein.

Measurement of Cytokine mRNA Levels

IL-2 and IFN-γ mRNA gene expression in Jurkat T-cell was assessed by semi-quantitative

reverse transcription-polymerase chain reaction (RT-PCR) [Luongo et al., 2003]. Briefly, cells at 8×10^5/ml density were cultured in 75 cm^2 flasks in complete medium supplemented with different BF or single FA for 48 h and incubated, for additional 24 h, in the presence/absence of 1 μg/L PMA, 0.5 μM ionomycin, and 10 μg/L anti-human CD3 monoclonal antibody (OKT3; Pharmingen, S.Diego, CA). At the end of the incubation, total RNA was recovered [Chomczynski and Sacchi, 1987]. Reverse transcription of 1 μg RNA was primed using oligo-$(dT)_{12-18}$ and different aliquots of cDNA were then used for PCR. The temperature profile of the amplification consisted of 25–35 cycles of 1 min denaturation at 95°C, 1 min annealing at 58°C (β-actin) or 60°C (IL-2 and IFN-γ), and 1 min extension at 72°C. Sequences of primers and sizes of the corresponding PCR products have been already published by Jung et al. [1995]. Negative control was performed by omitting RNA from the cDNA synthesis and specific PCR amplification. PCR products were analyzed on a 1.5% agarose gel stained with VISTRA Green (Amersham International plc, Buckinghamshire, UK). Fluorescence scanning and quantitative analysis of detected bands were carried out on STORM 860 system by IMAGEQUANT software (Molecular Dynamics, Inc., Sunnyvale, CA) and the results were expressed as cytokine/β-actin mRNA ratio.

Statistical Analysis

Results were expressed as means ± SD and differences between treatments were identified by Student's *t*-test and considered significant at $P < 0.05$. Trend curves, regression analysis, and correlation test were done by using the program Graph Pad Prism 3.00 (Graph Pad Software, San Diego, CA) significance was determined according to the test of Sokal and Rohlf [1994].

RESULTS

OBF and CBF Mixtures Differ for Their Fatty Acid Composition

Fatty acid composition of butter samples produced according to different management systems is shown in Table I. RA, ALA, and VA concentration in OBF is significantly higher (10.2 ± 0.5, 9.6 ± 1.0, and 21.1 ± 0.6 mg/g of fat, respectively) compared to the levels in CBF (5.2 ± 0.3, 5.7 ± 0.7, and 16.6 ± 0.4 mg/g of fat, respectively) ($P < 0.01$). By contrast, a significantly lower LA concentration was measured in OBF with respect to the CBF mixture (16.1 ± 0.5 and 22.1 ± 0.2 mg/g fat, respectively) ($P < 0.05$).

TABLE I. Fatty Acid Composition of O and CBF Samples

Fatty acid		OBF	CBF
$C_{14:0}$		117.0 ± 0.1	118.1 ± 0.5
$C_{16:0}$		318.8 ± 2.2	323.5 ± 1.2
$C_{16:1}$		21.3 ± 0.3	20.7 ± 0.7
$C_{18:0}$		109.9 ± 1.1	111.4 ± 0.8
t_{11} $C_{18:1}$	Vaccenic	21.1 ± 0.6*	16.6 ± 0.4*
$C_{18:1}$		218.9 ± 0.1	216.2 ± 0.1
$C_{18:2}$	Linoleic	16.1 ± 0.5**	22.1 ± 0.2**
$C_{20:0}$		1.6 ± 0.7	1.5 ± 0.4
c_9 t_{11} $C_{18:2}$	Rumenic	10.2 ± 0.5*	5.2 ± 0.3*
$c_{9,12,15}$ $C_{18:3}$	α-Linolenic	9.6 ± 1.0**	5.7 ± 0.7**

Fatty acid composition was evaluated in different brands (N = 3) of organic (OBF) or conventional butter (CBF). FA concentration was expressed as mg/100 g of fat. Average values ± SD from triplicate analysis are shown. Superscripts indicated significant differences between fatty acid concentration.
*$P < 0.01$.
**$P < 0.05$.

Enhanced Antiproliferative Effect Associates to OBF Treatment

To establish the amount of butterfat necessary to produce effects on the proliferation of Jurkat cells, $[^3H]$-thymidine incorporation was measured upon culture incubation (56 h) with 0.8 or 1.7 mg/ml BF. Exposing the cells to 0.8 mg/ml BF did not modify cell proliferation, while treatment with 1.7 mg/ml OBF or CBF significantly decreased cell growth compared to control (70% or 30% respectively, $P < 0.01$). Moreover, the inhibitory effect induced by OBF on Jurkat T-cell proliferation was significantly higher than that of CBF-treated culture ($P = 0.005$) (Fig. 1A). Cell incubation with all tested synthetic FA resulted in a dose-dependent growth decrease, with VA as the only exception. Remarkable anti-proliferative effects actually resulted from incubation with 90 and 130 μM LA (60% and 90%, respectively), and from 60 μM ALA treatment (75%), whereas a minor, but still significant, inhibition was obtained with 60 and 120 μM RA (18% and 33%, respectively) (Fig. 1B).

Noticeable Superoxide Anion Production Is Triggered by ALA and LA Supply Via PKC/NADPH Oxidase Pathway

Based on the fact that milk fat-induced cell growth inhibition was associated with increased peroxidation [O'Shea et al., 2000],

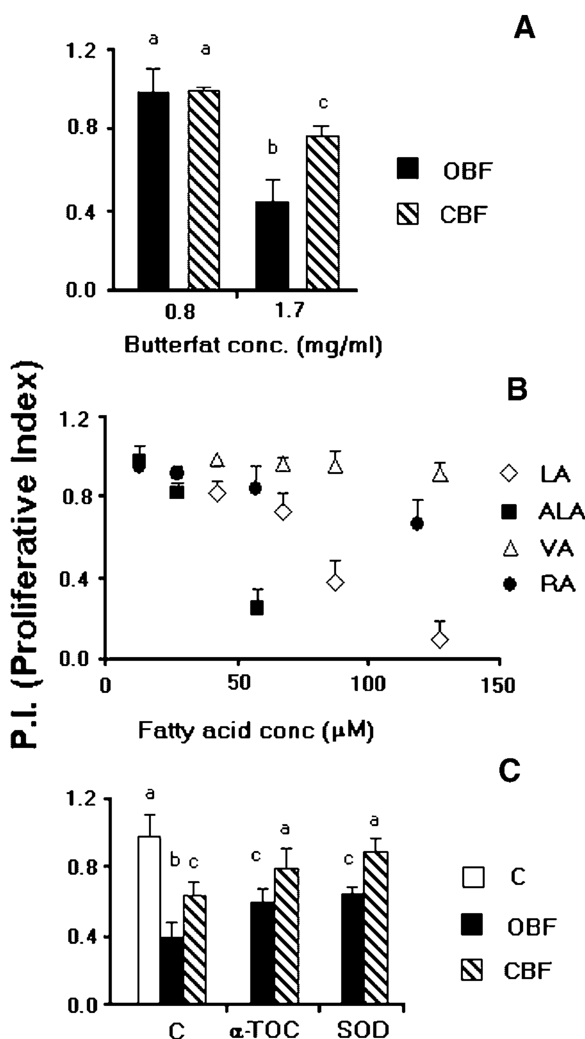

Fig. 1. Effect of OBF, CBF, and synthetic fatty acids on Jurkat T-cell proliferation. Antiproliferative effect of BF mixtures or synthetic FA were evaluated by [^{3}H]-thymidine incorporation assay. Jurkat T-cells proliferation was measured after 72 h incubation with different butterfat amounts (0.8 or 1.7 mg/ml) (**Panel A**) or with increasing RA, LA, ALA, or VA concentrations (**Panel B**). Cell proliferation of cells incubated in the presence of 1.7 mg/ml butterfat and different antioxidants (10 μM α-Toc or 50 U/ml SOD) are shown in **Panel C**. Cells treated with the same amount of ethanol were used as control. Results are mean values ± SD (error bars) from two separate experiments (n = 10) and values were reported as Proliferation Index (PI = cpm control/cpm treatment). Different letters denote values, which are significantly different from control ($P < 0.05$).

we then examined whether Reactive Oxygen Species (ROS) production or lipid peroxidation was involved in the effect of butterfat-induced antiproliferation. Cell proliferation was measured in cultures with 50 U/ml SOD or 10 μM α-Toc, which are known inhibitors of ROS production/lipid peroxidation induced by RA [Luongo et al., 2003], or by PUFA supplementation [Nano et al., 2003], respectively. Interestingly, the butterfat-induced antiproliferative effect was decreased by both antioxidants compared to control ($P < 0.05$) (Fig. 1C). In particular, cell growth in culture exposed to OBF or CBF in the presence of SOD was 25% or 11% lower, while with α-Toc, it was 50% or 20% lower, respectively.

The biochemical pathways mediating the prooxidant effect of butterfat treatment was investigated next. Butterfat ability to induce NADPH oxidase activation was directly shown in the zymogram (Fig. 2 insert) and the time-dependent increase of reduced ferrocytochrome-*c* (Fig. 2A). A rapid $O_2^{\cdot-}$ production occurred within few minutes of incubation reaching a maximum (4 nmoles/mg prot) after 30 min, whereas low amounts of superoxide anion (0.08 ± 0.014 nmoles/mg prot) were measured in controls. Next, the ability of synthetic FA to activate the NADPH oxidase complex was evaluated. The experiments were performed by using the highest concentrations previously detected for their natural counterparts in CBF (LA) or OBF (RA, VA, and ALA). We found that differential ROS production was triggered by synthetic FA. Cell treatment with RA ($P < 0.01$) elicited lower ROS concentration, whereas $O_2^{\cdot-}$ yield resulted from LA or ALA treatments at levels comparable with the positive control PMA. Cell exposure to VA gave $O_2^{\cdot-}$ concentration levels equivalent to that of untreated controls. In addition, involvement of PKC in FA-induced ROS production was supported by the suppressive effect of Gö 6976 (Fig. 2B). The very same concentrations of synthetic FA were used in subsequent experiments.

ALA and LA Supply Greatly Modifies Redox Cell Status

Cellular redox status is affected not only by enhanced ROS production, but also by antioxidant reduction. As GSH is the predominant low-molecular-weight thiol and plays a major role in cellular defences against oxidative stress [Meister, 1994], the effect of different treatment toward GSH depletion was examined. Both butterfat mixtures significantly reduced intracellular GSH concentration ($P < 0.05$) (Fig. 3A). Interestingly, maximal GSH depletion following synthetic FA treatments resulted upon cell incubation with ALA or LA (70% or 68%, respectively) ($P < 0.01$), whereas only minor or

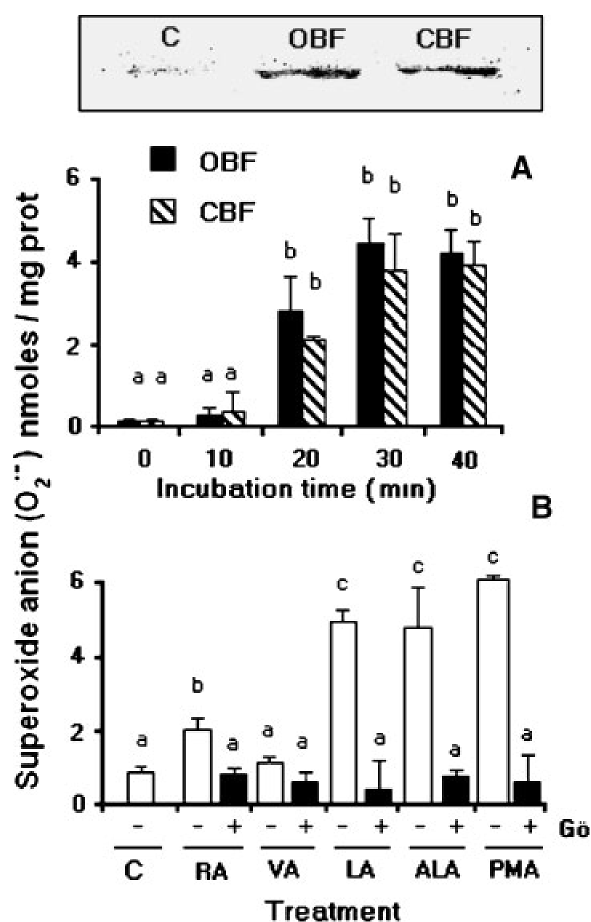

Fig. 2. NADPH oxidase activation in Jurkat T-cells treated with different BF or synthetic FA. NADPH oxidase activation was evaluated upon cells exposure (30 min) to 1.7 mg/ml of different BF by NBT assay carried out on cell proteins fractionated by native gel electrophoresis (insert). The extent of NADPH oxidase activation was measured by ferricytochrome-*c* reduction assay in Jurkat T-cells incubated with 1.7 mg/ml of OBF (black bars) or CBF (lined bars) for the indicated periods of time. As control, cells cultured in the presence of 0.1% ethanol were collected at the same time and used as control (C) (white bars) (**Panel A**). Incubation (30 min) with 60 μM RA, 130 μM VA, 130 μM LA, or 60 μM ALA was carried out. Cultures in the presence of ethanol were used as control (C), cells treated with 50 μM PMA were used as positive control (PMA). PKCα involvement in NADPH oxidase activation was evaluated by Gö 6976 preincubation (**Panel B**). Results are mean values ± SD (error bars) from three separate experiments (n = 9). Average values NADPH oxidation activity was expressed as nmoles $O_2^{\cdot-}$/mg protein. Different letters denote values, which are significantly different from control ($P < 0.05$).

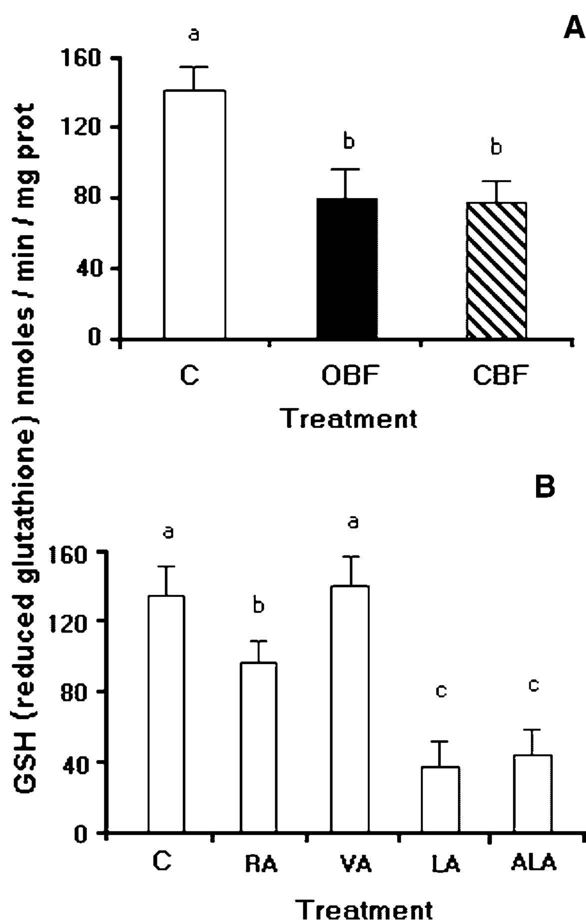

Fig. 3. GSH measurement in culture exposed to different BF or synthetic FA. GSH was measured in Jurkat T-cells exposed for 48 h with butterfat (**Panel A**) or 60 μM RA, 130 μM VA, 130 μM LA, or 60 μM ALA (**Panel B**). As control, cells were cultured in the presence of 0.1% ethanol. Intracellular GSH concentration was expressed as GSH μM/min/mg protein. Results are mean values ± SD (error bars) from two separate experiments (n = 6). Different letters denote values, which are significantly different from control ($P < 0.05$).

negligible modifications were detected in culture treated with RA or VA, respectively (Fig. 3B). The negative correlation between ROS production and intracellular GSH concentration ($r = 0.87$; $P < 0.01$) further supported the strong relationship between the production of oxidant molecules and cell redox modification.

Prooxidant Effect of ALA and LA Associates to Butterfat Cytotoxic Effect

LDH release was evaluated to assess the cytotoxic effect of both butterfat or fatty acid treatments. A time-dependent cytotoxic effect resulted from both OBF and CBF incubations ($P < 0.05$); LDH release reached a plateau after 48h, however no significant difference among the two different treatments was observed (Fig. 4A). Again, comparable amounts of lipid hydroperoxides (LOOH) were produced by both mixtures ($P < 0.05$) (Fig. 5A). The analysis of

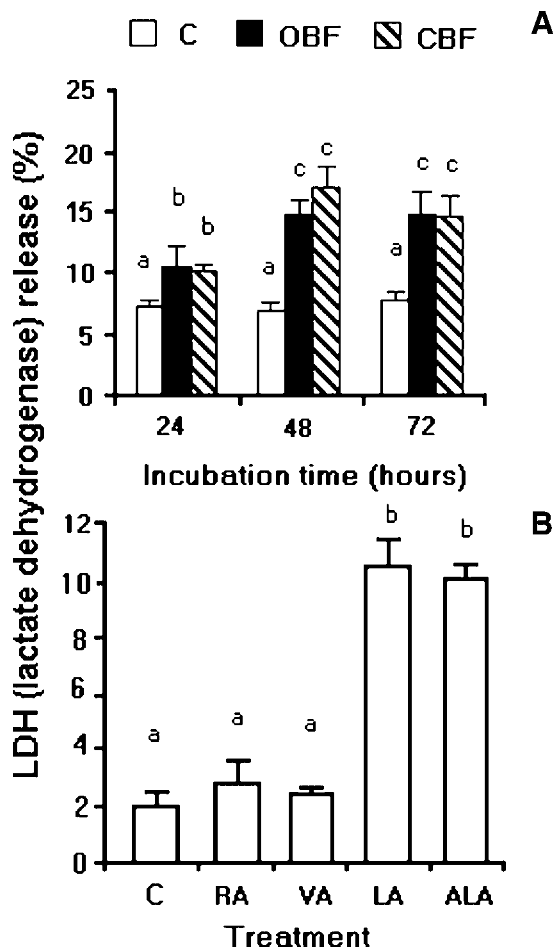

Fig. 4. Cytotoxic effect of culture treatment with different BF or synthetic FA. The cytotoxic effect of O- or CBF was evaluated by measuring the LDH leakage from Jurkat T-cells. Cells (0.5×10^6/ml) were incubated with O- or CBF (black or lined bars, respectively) for the indicated periods of time. Cells cultured in the presence of 0.1% ethanol were used as control (white bars) (**Panel A**). LDH release was measured in cells incubated (48 h) with 60 μM RA, 130 μM VA, 130 μM LA, or 60 μM ALA. Cultures treated with 0.1% ethanol were used as controls (C) (**Panel B**). Results are mean values $\pm$ SD (error bars) from four separate experiments (n = 8). Different letters denote values, which are significantly different from control ($P < 0.05$).

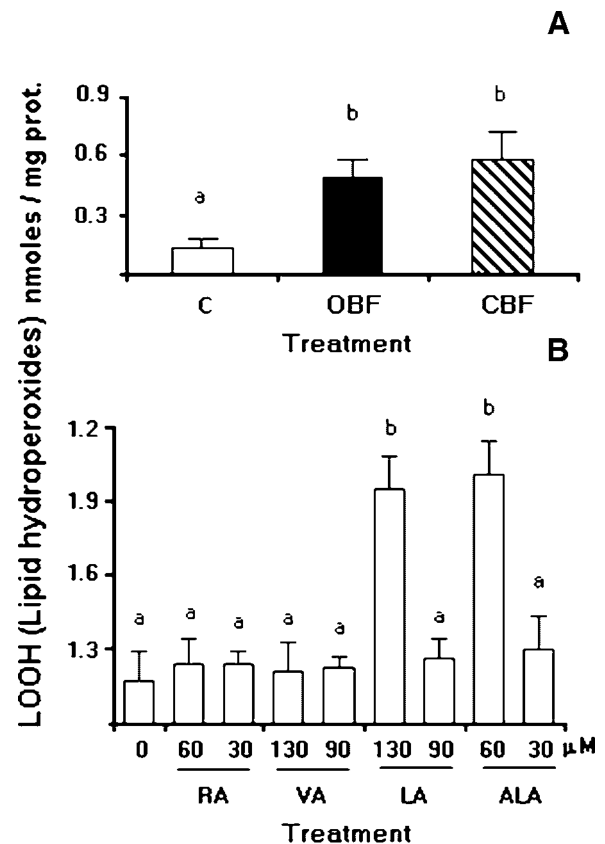

Fig. 5. Lipid peroxidation extent in Jurkat T-cells treated with different BF or synthetic FA. The effect of O- or CBF treatment on the extent of lipid hydroperoxides in Jurkat T-cells was evaluated by measuring LOOH content. Cells (0.5×10^6/ml) were incubated (48 h) with O- or CBF (black or lined bars, respectively). Cells cultured in the presence of 0.1% ethanol were used as control (C) (white bars) (**Panel A**). LOOH levels were measured in cells exposed to 30–60 μM CLA, 90–130 μM VA, 90–130 μM LA, or 30–60 μM ALA. Cultures treated with 0.1% ethanol were used as controls (C) (**Panel B**). Results are mean values $\pm$ SD (error bars) from three separate experiments (n = 6). Different letters denote values, which are significantly different from control ($P < 0.05$).

single FA showed significantly higher LDH and LOOH amounts upon 48-h Jurkat cells incubation with LA or ALA compared to control ($P < 0.05$), whereas no significant variation occurred in cells exposed to RA or VA (Fig. 4B, Fig. 5B). Interestingly, LA and ALA failed to produce an increase in lipid peroxidation when tested at lower concentrations, the same we previously detected in CBF and OBF. As expected, the positive correlation between LOOH and LDH amount ($r = 0.68$ respectively; $P < 0.05$) suggested that there is a close relationship between lipid peroxidation and cytotoxicity. Low LOOH concentration was measured in control culture (0.2 ± 0.1 nmoles/mg protein), and the contribution of BF-delivered peroxide can be excluded since we were unable to detect any LOOH amounts in a cell-free system (data not shown).

RA-induced Apoptosis Contributes to Antiproliferative Effect Elicited by OBF

Caspase-3 activity was measured at different times up to 72 h in order to evaluate the apoptosis contribution to butterfat-mediated antiproliferative effect. Cell treatment with

both butterfats caused a time-dependent apoptosis reaching a plateau after 48 h, and yielded significantly higher caspase-3 activity in cells exposed to OBF compared to CBF cultures ($P < 0.05$) (Fig. 6A). Moreover, a significant induction of caspase-3 activation was found in RA-treated cells, as compared to other FA treatments or to control ($P < 0.05$) (Fig. 6B), suggesting that RA-induced apoptosis contributes substantially to the greater OBF-induced antiproliferative effect. Significantly lower caspase activity in the culture exposed to BF or synthetic FA was measured (2.8 ± 1.2 nmoles pNA/mg prot/h; $P < 0.05$) (Fig. 6A).

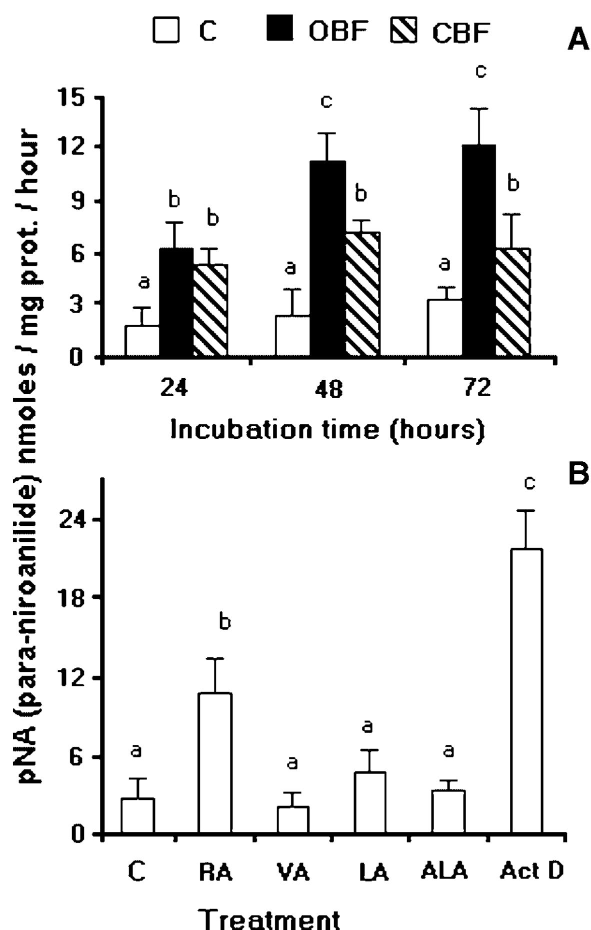

Fig. 6. Pro-apoptotic activity of different BF or synthetic FA. Caspase-3 activity in cultures treated with BF or synthetic FA was measured to evaluate their pro-apoptotic effects. Jurkat T-cells were incubated with OBF or CBF (black or lined bars, respectively) for the indicated periods of time (**Panel A**); cells cultured in the presence of 0.1% ethanol were used as control (C) (white bars). Cells were treated with 60 μM RA, 130 μM VA, 130 μM LA, or 60 μM ALA. Cells cultured in the presence of 0.1% ethanol were used as negative control (C), cells treated with 50 μM actinomycin D were used as positive control (Act D) (**Panel B**). Results are the mean values ± SD (error bars) from three separate experiments ($n = 6$). Different letters denote values, which are significantly different from control ($P < 0.05$).

Cell Redox Status Modulates IL-2 Expression

To analyze the influence of the different treatments on cytokine transcription, IL-2 and IFN-γ mRNA transcript levels were determined in Jurkat T-cells. An increased IL-2 mRNA amount was found in O- or CBF-treated cells (85% and 64% higher, respectively), whereas the levels of IFN-γ mRNA was similar to that produced in control cultures (Fig. 7A,B). Cell incubation with ALA or LA markedly inhibited IL-2 production (70% and 50%, respectively) and, by contrast, enhanced IL-2 production (36%) resulted from cell exposure to RA. Culture incubation with VA gave a IL-2 mRNA levels comparable to that of untreated cultures (Fig. 8A,B).

DISCUSSION

In the present study, the Jurkat T-cell line has been used as model for investigating butterfat-induced oxidative signaling that leads to apoptosis and necrosis. This model was chosen for the fact that cell membrane markers strongly resemble those found in normal T-lymphocytes [Konikova et al., 1992]. The involvement of an oxidative mechanism in the CLA-induced reduction of cell proliferation has been previously described [Luongo et al., 2003]. For further extending the analysis, the effects of two butterfat types, differing in FA composition, was determined in this study.

Results obtained indicate that RA-rich butterfat (OBF) induces greater growth-inhibitory effects in Jurkat T-cells than does the conventional butterfat (CBF). A major finding of this study is that both apoptotic and cytotoxic cell death mechanisms contribute to the remarkable antiproliferative effect elicited by OBF. In particular, the data reported suggest that prooxidant mechanism is responsible for ALA- and LA-mediated cytotoxicity, whereas RA yield may be responsible for the BF-induced pro-apoptotic effect.

It is widely accepted that ROS represent important signaling molecules involved in the regulation of a variety of cellular functions including proliferation, differentiation or apoptosis, as well as modulation of immune responses [Dröge, 2002]. NADPH oxidase is probably the most important ROS source in both

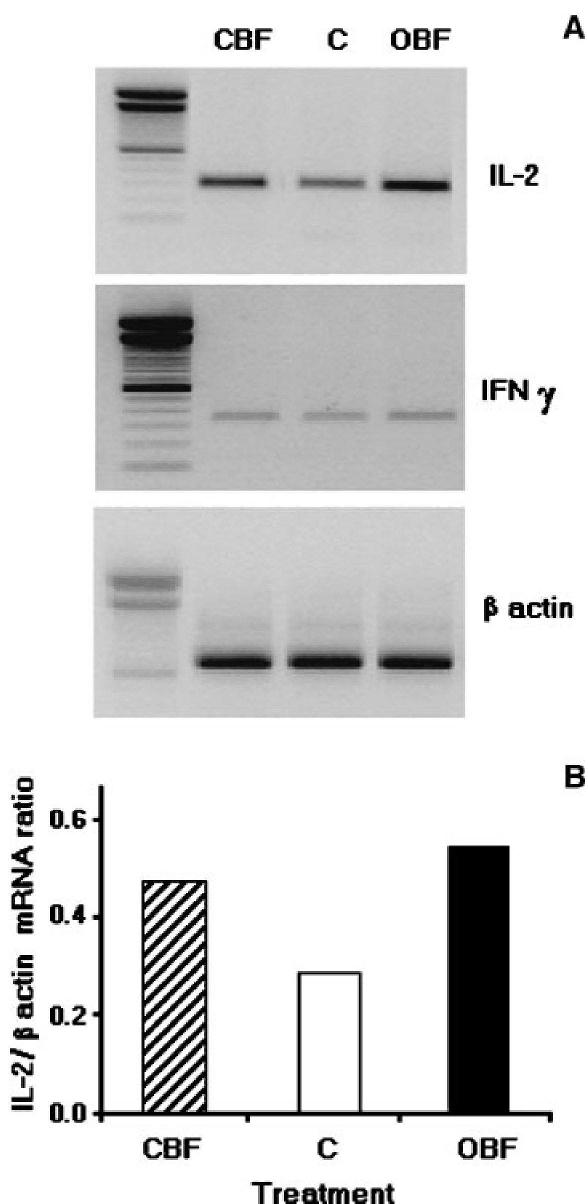

Fig. 7. Effect of different BF on cytokine expression. IL-2 and IFN-γ mRNA productions of Jurkat cells treated with O- or CBF for 48 h before stimulation with agonists, were assessed by a semi-quantitative RT-PCR. (**Panel A**). As control, cells were cultured in the presence of 0.1% ethanol (C). Densitometric analysis of cytokine mRNA; after electrophoresis, a densitometric scanning was carried out and results are expressed as cytokine/β-actin mRNA ratio (**Panel B**). Results are from two independent experiments with similar results.

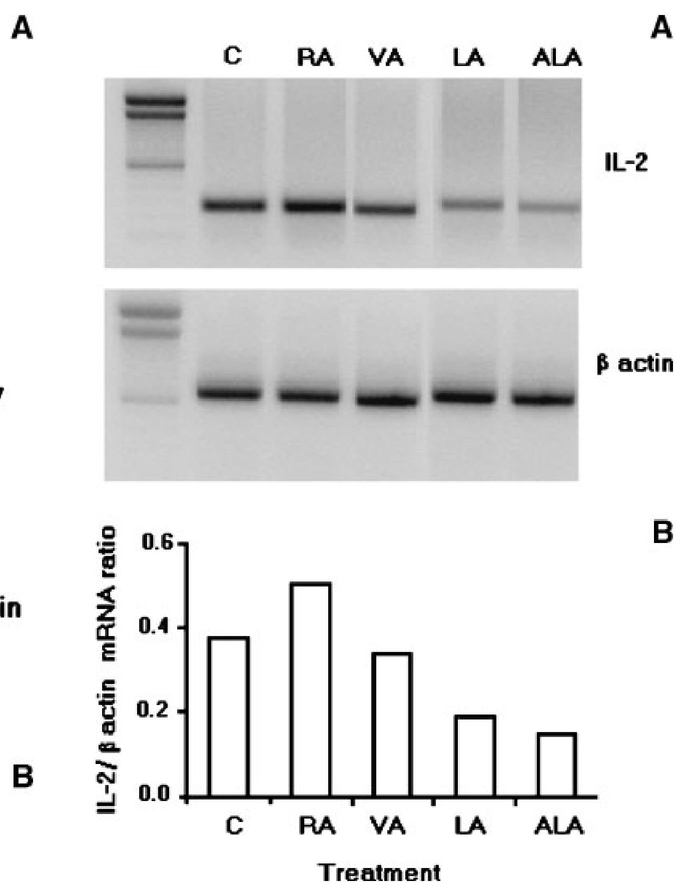

Fig. 8. Effect of synthetic FA on cytokine expression. IL-2 mRNA productions of Jurkat cells treated with 60 μM RA, 130 μM VA, 130 μM LA, or 60 μM ALA for 48 h were assessed by a semi-quantitative RT-PCR. (**Panel A**). As control, cells were cultured in the presence of 0.1% ethanol (C). Densitometric analysis of cytokine mRNA; after electrophoresis, a densitometric scanning was carried out and results are expressed as IL-2/β-actin mRNA ratio (**Panel B**). Results are from two independent experiments with similar results.

neutrophils [Lee and Koretzky, 1998] and in lymphocytes [Devadas et al., 2002]. NADPH oxidase activation by FA has been demonstrated for the first time by Cui and Douglas [1997] and butterfat ability to activate NADPH oxidase has been shown in this work for the first time. In particular, by analyzing single fatty acid components, our data suggest that ALA and LA greatly contribute to ROS generation, via PKC/NADPH oxidase pathway, in accordance with literature [Padma and Das, 1996].

PKC is a group of serine/threonine kinases involved in cell-signaling pathways regulating fundamental cellular functions including proliferation, death, differentiation, tumorigenesis, and stress responses [Buckner, 2001; Jeong et al., 2001]. Several n-3, n-6 PUFA [Nishikawa et al., 1988; Huang et al., 1997] have been demonstrated to modulate PKC activity and superoxide production in vitro and α-Toc was previously shown to impair the NADPH oxidase by decreasing PKC activity [Cachia et al., 1998]. Therefore, the α-Toc-mediated inhibition of anti-proliferative effect

following butterfat treatment, together with data showing the suppressive effect of Gö 6976, indicates PKCα involvement in the prooxidant activity of butterfat.

As expected, GSH levels paralleled the ROS amount generated by the different cell treatments. GSH is the prominent intracellular non-protein sulphydril compound necessary for maintaining cellular redox homeostasis toward reduction; cell redox homeostasis, besides its regulatory effects on cell growth, has been reported to play an important role in modulating the immune system [Dröge, 2002]. A decrease in the GSH pool, markedly inhibiting T-cell activity was indeed evidenced by Morel and Barouki [1999]. This agrees with our data showing an association between a moderate GSH alteration and enhanced IL-2 transcription in RA treated cells, and an ALA- or LA-induced association between a severe GSH depletion and reduced IL-2 transcription. Very interestingly, the activity of RA seems to dominate over the other fatty acid components in experiments with BF. The immunomodulatory ability of synthetic CLA has been recently published [Luongo et al., 2003], and the present study goes further to address the issue, which evidences that RA contribution to butterfat-induced enhancement of IL-2 synthesis is mediated by cell redox modulation in Jurkat T-cells.

The tumoricidal action of PUFA has been related to their ability to increase ROS-mediated cytotoxicity [Das, 1991] or to induce apoptosis [Colquon and Schumacher, 2001]. The different activities were largely dependent on concentration, exposure time, and cellular type used [Rudolph et al., 2001]. Exposure of a mammary cancer cell line to milk fat, containing 30–60 μM RA, was previously shown to induce lipid peroxidation and decrease cell proliferation [O'Shea et al., 2000]. Our finding of noticeable anti-proliferative effect exhibited by ALA and LA treatment, concurs with literature [Lima et al., 2002], suggesting that ALA- and LA-induced toxicity is mainly responsible for butterfat-induced antiproliferative effects. Moreover, we found that ALA and LA are mostly responsible for prooxidant-induced antiproliferative effect, which is further supported by data indicating the close relationship between oxidative stress with growth inhibitory effect [Hammer et al., 1997] and ^{3}H-thymidine incorporation [Muzio et al., 1999]. However, in our study OBF-mediated growth inhibition is not merely the consequence of induced cytotoxicity.

Apoptosis is a key biological process in cells and tissues, in whose pathway several cysteinyl proteases (caspases) are activated. Among them, caspase-3 has been identified as one of the key executioner of apoptosis in mammalian cells [Cohen, 1997]. In this work, butterfat ability to induce apoptosis in cancer cell lines has been evidenced for the first time. In particular, our data highlight RA ability to induce caspase-3 activation, supporting the hypothesis that apoptosis induction in butterfat-treated cells is essentially elicited by the presence of this fatty acid. This is in agreement with the work of Ip et al. [1996] where biological properties of CLA appeared to be independent of the presence of other dietary FA, and with data indicating the enhanced antitumor activity of high CLA milk fat [Ip et al., 1999; O'Shea et al., 2000].

In conclusion, our experiments suggest that ROS production in butterfat treated cells is greatly induced by ALA and LA supply via PKC/NADPH oxidase pathway, whereas the RA-induced pro-apoptotic effect contributes to the more noticeable antiproliferative inhibitory effect elicited by OBF treatment. Since growth inhibitory activity and cell death induction are typical properties of anticarcinogenic agents [Kanduc et al., 2002], our data strongly indicate the enhanced chemopreventive properties of RA-rich butterfat.

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