Fatty Acids of CLA-enriched Egg Yolks Can Induce Mitochondrial Pathway of Apoptosis in MCF-7 Breast Cancer Cells

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Abstract. *Background: Fatty acids from conjugated linoleic acid (CLA)-enriched egg yolk suppressed the viability of the MCF-7 cancer line more effectively than non-enriched egg yolk. Herein we aimed to determine the molecular mechanisms by analysing the expression and activation of proteins involved in cellular stress and apoptosis signaling. Materials and Methods: Forty-eight Isa Brown laying hens (26-week-old) were fed a fortified (0.75% CLA) or a control diet (0% CLA) for 4 months. Collected eggs were used to obtain CLA-enriched (EFA-CLA) or non-enriched (EFA) fatty acid extracts for the treatment of the MCF-7 cancer cell line. Protein levels were analysed by PathScan® Stress and Apoptosis Signalling Antibody Array and western blot method. Results: Treatment with EFA-CLA led to activation of caspase signalling as main effector of apoptosis. It also increased levels of pro-apoptotic B-cell lymphoma 2 family proteins as well as promoted the release of cytochrome c, second mitochondria-derived activator of caspase and mitochondrial serine protease from mitochondria to the cytoplasm. EFA-CLA increased levels of tumour protein 53 and mothers against decapentaplegic homolog 2 tumour suppressors, and activated p38 mitogen-activated protein kinases and stress-activated protein kinase/c-Jun NH2-terminal kinase proteins. Finally, treatment down-regulated anti-apoptotic extracellular signal-regulated protein kinases 1 and 2, RAC-alpha serine/threonine-protein kinase, heat-shock protein 27, inhibitor of nuclear factor κβ, transforming growth factor beta-activated kinase 1 and survivin proteins.*

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Conclusion: Taken together, our results suggest that activation of the mitochondrial apoptotic pathway may be a potential mechanism of EFA-CLA action.

Breast cancer is the most frequently diagnosed cancer among women worldwide and one of the most common causes of death (1). Encouragingly, a study carried out in the USA between 2003 and 2012 showed an overall downward tendency in the death rate. This decrease reflects progress in earlier detection as well as improved treatments (2). However, research in cancer prevention remains insufficient.

Conjugated linoleic acid (CLA) is a term that describes a mixture of positional and geometric isomers of linoleic acid (3). Two main isomers: *cis9,trans11* and *trans10,cis12*, have been widely studied and shown to possess significant biological properties (3, 4)

CLA is commonly available in a synthetic supplement form; however, the ratio of *cis9,trans11* to *trans10,cis12* CLA isomers, most often in equal proportions (1:1), does not reflect those of natural food products. In addition, there are some concerns regarding the safety of certain *trans* CLA isomers (4). Taking all the above into consideration, it would seem preferable to obtain CLA from naturally-enriched food products. Chicken eggs can be enriched with CLA *via* feed modification, meeting the criteria of a functional food product (5, 6). Supplementation of chicken feed with a mixture of *cis9,trans11* and *trans10,cis12* CLA isomers was found to result in a favourable incorporation into egg yolk of *cis9,trans11* at a 3:1 ratio (6, 7), similar to that measured for natural, unmodified foods (8).

Our previous studies showed the beneficial effect of a fatty acid extract (EFA) from CLA-enriched eggs in reducing proliferation of breast cancer and melanoma cells (9, 10), which was stronger than that for EFA from non-enriched egg yolks. Our recently published Whole Human Genome Microarray analysis of the effect of EFA-CLA *vs*. EFA on MCF-7 transcriptome profile showed that EFA-CLA promoted the mRNA expression of some tumour suppressors

and reduced the expression of oncogenes (7). In the current study, we made an attempt to determine further the potential mechanism behind reduced viability of MCF-7 cells after treatment with EFA-CLA.

Materials and Methods

Production of CLA-enriched egg yolks. Production of CLA-enriched egg yolks was performed at the National Research Institute of Animal Production in Krakow (Poland), as described previously (11). Forty-eight Isa Brown laying hens (26 weeks old) were housed in a controlled room under 14/10 h light/dark cycle, given free access to water and commercial starter diet ('DJ' feed). After a 1-week adaptation period, an equal number of hens was randomly allocated to the control or experimental group for 4 months of the experiment. Diets were calculated to provide 2700 kcal/kg and 17% crude protein. The 0.75% dietary CLA (Tonalin FFA 80; BASF Company, Ludwigshafen, Rhineland-Palatinate, Germany) contained 80% CLA in 50:50 ratio for *cis9,trans11* and *trans10,cis12* isomers. Eggs were collected daily for 10 weeks and stored at 4°C. Yolks were separated from albumen, homogenized and frozen at –20°C. Samples were then lyophilized and again stored at –20°C until further use or analysis.

Fatty acid extraction and analysis of fatty acid methyl esters (FAME). Lipids from control and CLA-enriched yolks were extracted, derivitised to FAME and analysed by gas chromatography–mass spectrometry as described previously (8). For cell treatment, lipid extracts were subjected to basic hydrolysis (0.5 M KOH at 60°C for 15 min) and then extracted with hexane. The free fatty acids were then dissolved in ethanol (Avantor Performance Materials Poland, Gliwice, Poland) at a stock concentration of 1 g/ml and stored under nitrogen at –20°C.

Cell culture. The human breast adenocarcinoma cell line MCF-7 (ATCC® HTB22TM) was purchased from the American Type Culture Collections (ATCC, Manassas, VA, USA). Cells were cultured in appropriate medium (Sigma-Aldrich, St Louis, MO, USA) according to the ATCC® protocol.

Cell treatment. MCF-7 cells were seeded on 96-well plates at 8×10^3 cells per well, 12-well plates at 9×10^4 cells per well, or 6-well plates at 2×10^5 cells per well. Twenty-four hours after seeding, growth medium was replaced with fresh medium containing: a) 0.5 mg/ml EFA-CLA), b) 0.5 mg/ml EFA from non-enriched egg yolks, or c) ethanol as solvent at final concentration of 0.1% as negative control (NC). Cells were treated for 48 h. Staurosporine was used as a positive control (Sigma-Aldrich) at a final concentration of 1.5 µM. Each treatment included three biological and three technical replicates.

Cytotoxicity assay. Cell viability was determined by the Crystal Violet Assay (Sigma-Aldrich and Cytotoxicity Detection Kit LDH (Roche Poland, Warszawa, Poland) according to the manufacturers' protocols.

Plasma membrane alteration in early apoptosis. Annexin-V-FLUOS Staining Kit (Roche) was used according to the manufacturer's protocol to detect induction of MCF-7 cell apoptosis after treatment with EFA-CLA and EFA.
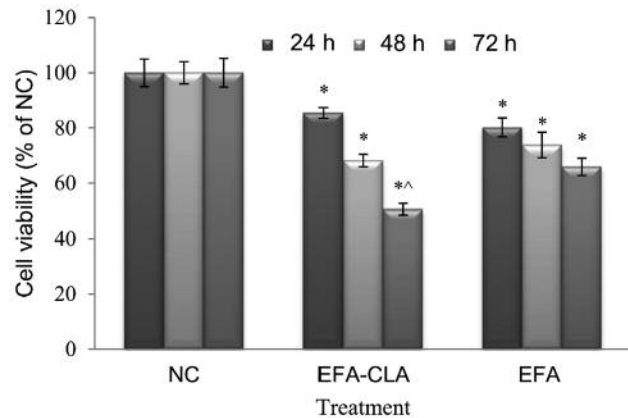

Figure 1. *Effect of fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA) on MCF-7 cell viability. Values are expressed as means±SD (n=8), standardized to the solvent-untreated control (NC) set as 100%. Statistical significance was based on t-test. Significantly different at p<0.01 vs. *NC and ^non-enriched egg yolk (EFA). Fatty acid concentration: 0.5 mg/ml.*

Stress and apoptosis signaling assay. Cell extracts were prepared and analyzed using PathScan® Stress and Apoptosis Signaling Antibody Array Kit (Chemiluminescent Readout; Cell Signalling Technology, Danvers, MA, USA) as described previously (13).

Western blot assay. Whole-cell and cellular fraction lysis were carried out using Cell Lysis Buffer and Cell Fractionation Kit (Cell Signaling Technology) with the addition of Protease Inhibitor Cocktail (BioShop, Mainway, Burlington, ON, Canada) according to the manufacturers' protocols. Total protein was quantified with Pierce BCA™ Protein Assay Kit (Thermo Fisher Scientific, Waltham MA, USA). Each western blot followed a similar procedure. Protein extract was separated on a polyacrylamide gel and transferred to a nitrocellulose filter (Bio-Rad, Hercules, CA, USA) by wet-electroblotting. Subsequently, the immobilized proteins were incubated with appropriate primary antibody to: p53 (#2527), cytochrome *c* (#11940), BH3-interacting domain death agonist (BID) (#2002), BCL2-associated X, apoptosis regulator (BAX) (#5023), BCL2-associated agonist of cell death (BAD) (#9239), second mitochondria-derived activator of caspase (SMAC/DIABLO) (#2954), mitochondrial serine protease (HTRA2/OMI) (#9745), p53 up-regulated modulator of apoptosis (PUMA) (#4976), caspase-9 (#9502), and glyceraldehyde-3-phosphate dehydrogenase GAPDH (#5174) as a reference protein (Cell Signaling Technology, Danvers, MA, USA). Finally, appropriate secondary antibody conjugated with horseradish peroxidase (#7074; Cell Signaling Technology) was applied. Detection was executed by chemiluminescence, using Clarity™ Western ECL Substrate (Bio-Rad Western blot stripping buffer (Thermo Fisher Scientific) was used to remove the antibodies from the membrane. Densitometric analysis was performed using ImageJ.

Statistical analysis. All assays were performed in at least three independent experiments and measured in triplicate. The Shapiro-Wilk's test was applied to assess normality of distribution. An independent samples *t*-test was applied to compare unpaired means
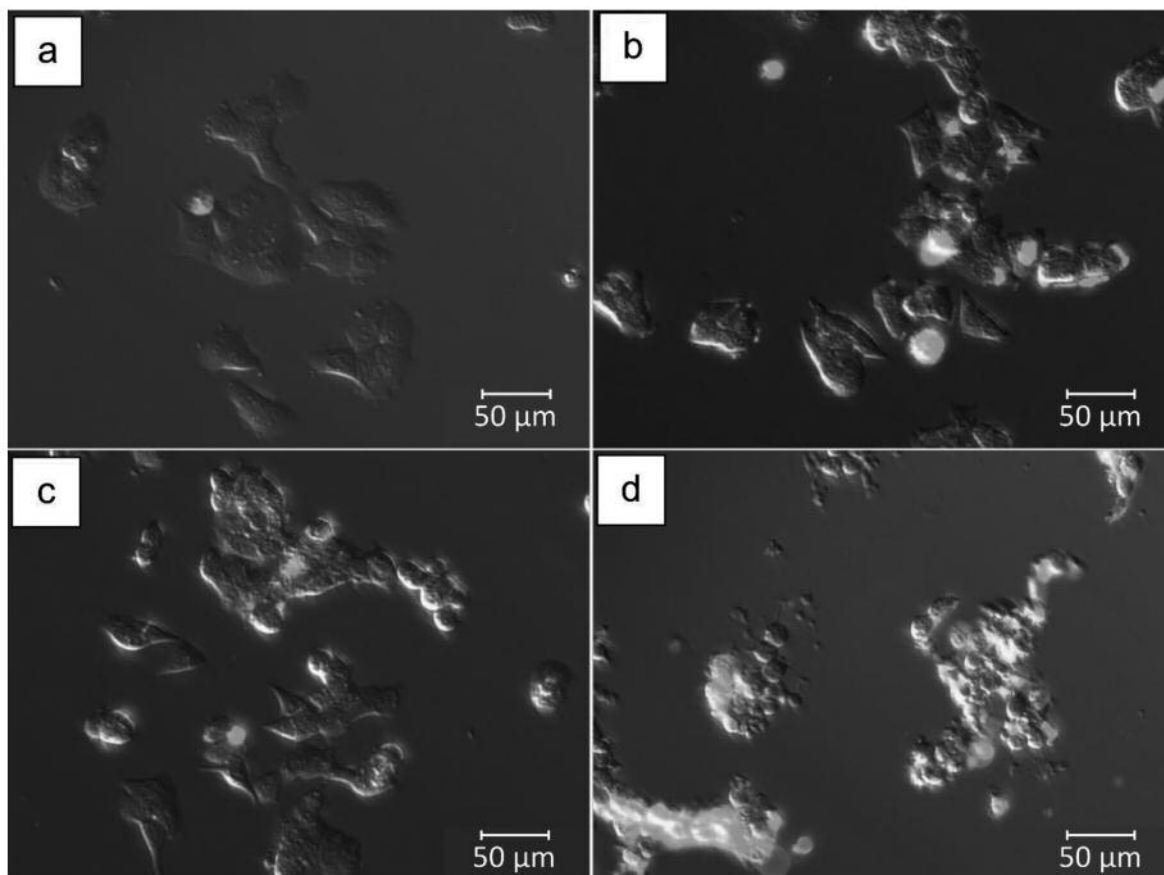
Figure 2. *Effect of fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA) on early events of apoptosis in MCF-7 cells. MCF-7 cells were untreated (a) or treated with EFA-CLA (b), non-enriched egg yolk (EFA) (c), or 1.5 μM staurosporine as positive control (d) for 72 h. Cells were stained with Annexin V-FLUOS kit. The cell cultures were subsequently analysed using a Zeiss Axio Observer Z1 fluorescence microscope. Annexin V–FLUOS-positive cells are stained green and represent apoptotic cells.*

between two groups and differences with values of $p<0.05$ were considered statistically significant. All analyses were performed using Statistica ver.12 (StatSoft, Tulsa, OK, USA).

Results

Analysis of cell viability. Both treatments with EFA-CLA and EFA reduced the viability of MCF-7 breast cancer cells compared to the NC. At 72 h post treatment, cell viability in the EFA-CLA-treated group had decreased by 50% and that of the group treated with EFA by 35% (Figure 1).

Plasma membrane alteration in early apoptosis. Results from the Annexin-V-FLUOS assay indicated early apoptosis stage for both treatment groups; however, this was more pronounced for the EFA-CLA treated group (Figure 2).

Expression of stress and apoptosis signalling proteins. The results of the PathScan® Stress and Apoptosis Signalling Antibody Array analysis are presented in Figure 3.

EFA-CLA significantly reduced the level of the pro-survival phosphorylated form of P44/42 mitogen-activated protein kinases (MAPK) [extracellular signal-regulated protein kinases 1 and 2 (ERK1/2)] to 48% that of NC ($p<0.05$). In addition, this effect was also statistically significant compared to that of EFA (94% of NC; $p<0.005$) (Figure 3). Similar results were found for the phosphorylated form of heat-shock protein 27 (HSP27). Significant decrease in protein expression was measured for both EFA-CLA- and EFA-treated cells (35% and 20% of NC, respectively; $p<0.01$) (Figure 3). Serine/threonine-protein kinase (AKT1) and survivin expression also showed a tendency to decrease, but not statistically significantly, in EFA-CLA-treated cells (81% and 94% of NC, respectively) (Figure 3). Similarly, there was a negligible decrease in the level of the active form of transforming growth factor beta-activated kinase 1 (TAK1) in EFA-CLA- and EFA-treated cells (95% and 75% of NC, respectively) (Figure 3).

EFA-CLA treatment resulted in a significant increase in the level of the phosphorylated form of BAD protein
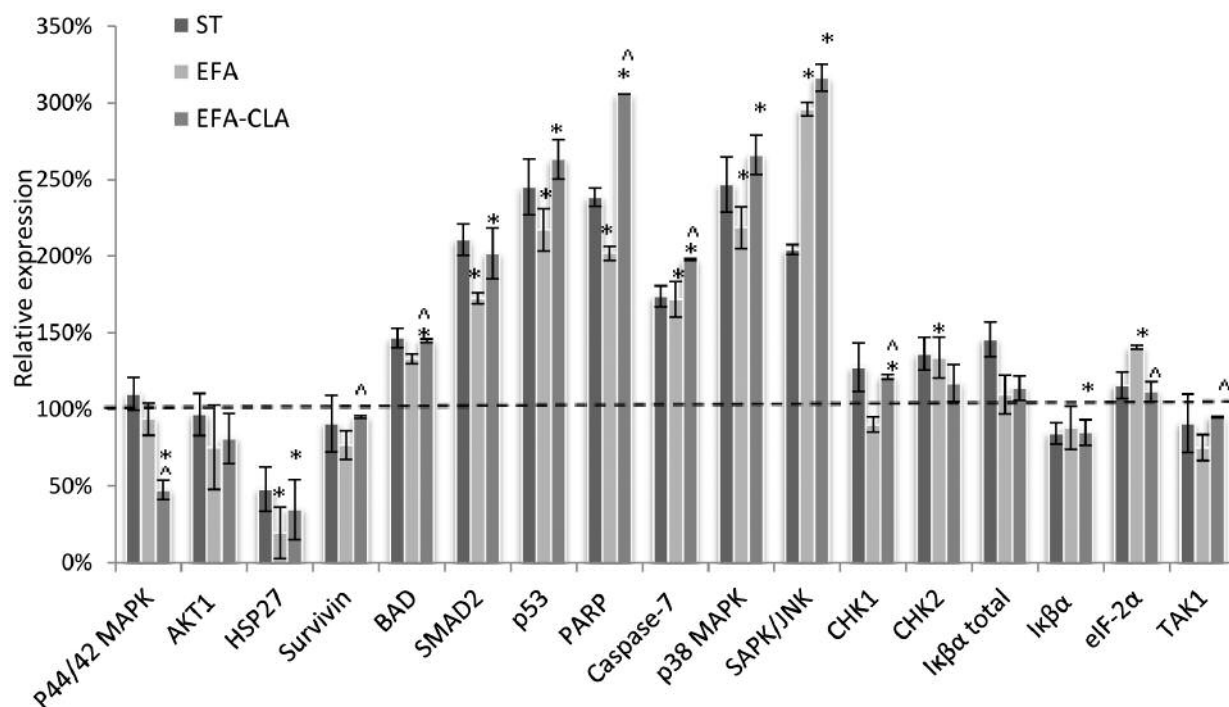
Figure 3. *Expression of stress and apoptosis signalling proteins in MCF-7 cells. MCF-7 cells were treated for 48 h with 0.5 mg/ml fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA), or from control egg yolks (EFA), or with 1.5 μM staurosporine (ST) as positive control. Cell extracts were prepared and analysed using the PathScan® Stress and Apoptosis Signaling Antibody Array Kit (Cell Signaling Technology, Danvers, MA, USA). Densitometric analysis was performed using ImageJ. Results are shown as the mean±SD normalized to the internal reference protein (α-tubulin). Expression of the solvent-treated negative control (NC) was set as 100%. Statistical significance of EFA-CLA was based on t-test. Significantly different at p<0.01 vs. *NC and ^EFA. P44/42 MAPK: Extracellular signal-regulated protein kinases 1 and 2 (ERK1/ 2); AKT1: serine/threonine-protein kinase; HSP27: heat-shock protein 27; BAD: BCL2-associated agonist of cell death; SMAD2: mothers against decapentaplegic homolog 2; p53: tumour protein 53; PARP: poly(ADP)-ribose] polymerase; SAPK/JNK: stress-activated protein kinase/c-Jun NH2-terminal kinase; p38MAPK: p38 mitogen-activated protein kinases; CHK1: checkpoint kinase 1; CHK2: checkpoint kinase 2; Iκβα: inhibitor of nuclear factor κβ; eIF-2α: eukaryotic translation initiation factor 2 subunit 1; TAK1: transforming growth factor beta-activated kinase 1.*

compared to NC (145% of NC; $p<0.05$) and to EFA-treated cells ($p<0.01$). Similar results were found for tumour suppressors phosphorylated p53 and mothers against decapentaplegic homolog 2 (SMAD2) (263% and 202% of NC, respectively; $p<0.01$) (Figure 3). EFA had a similarly significant effect on p53 and SMAD2 (217% and 173% of NC, respectively). In addition, there was an increase in the cleaved form of poly (ADP-ribose) polymerase (PARP) after both EFA-CLA and EFA treatment (306% and 202% of NC, respectively; $p<0.001$), which was significantly higher for EFA-CLA ($p<0.005$) (Figure 3). EFA-CLA had a significant effect on caspase-7 cleavage (198% of NC; $p<0.001$) as well as on p38 MAPK (266% of NC; $p<0.01$) and phosphorylation of stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) MAPKs (316% of NC; $p<0.01$) (Figure 3).

Results for EFA-CLA also showed a significant increase in phosphorylated checkpoint kinase 1 (CHK1) level (121% of NC; $p<0.05$) but a statistically insignificant increase for

CHK2 (117% of NC) (Figure 3). For inhibitor of nuclear factor κβ (Iκβα), no changes were found in total protein levels for cells treated with either EFA-CLA or EFA. However, EFA-CLA led to a significant decrease in the level of the phosphorylated form of Iκβα (85% of NC; $p<0.05$) (Figure 3). Our results showed no effect on total eukaryotic translation initiation factor 2 subunit 1 (eIF-2α) expression; however, they showed a slight increase in the phosphorylated protein form in cells treated with EFA (112% of NC; $p<0.05$) (Figure 3).

Western blot analysis of protein levels in cell lysate. Western blot results showed the effect of EFA-CLA treatment on proteins known to be involved in mitochondrial-induced apoptosis. P53 and cytochrome *c* were found at significantly higher levels in whole-cell lysate from cells treated with EFA-CLA (Figures 4 and 5). Further analysis of membrane and cytoplasmic fractions showed significant accumulation of cytochrome *c* in the cytoplasm at the expense of the

Figure 4. *Expression of p53 in MCF-7 cells (whole-cell lysate). MCF-7 cells were treated with solvent alone as negative control (NC) or treated with fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA), or from control egg yolks (EFA), or 1.5 μM staurosporine (ST) as positive control for 48 h. Cell extracts were prepared using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of Protease Inhibitor Cocktail (BioShop, Mainway, Burlington, ON Canada). Protein extract was separated on a polyacrylamide gel and transferred to a nitrocellulose filter (Bio-Rad, Hercules, CA, USA) by wet-electroblotting. The immobilized proteins were incubated with primary antibody to tumor p53 protein (Cell Signaling Technology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) was used as a reference protein. Detection was performed by chemiluminescence, using Clarity™ Western ECL Substrate (Bio-Rad).*

Figure 5. *Expression of BH3-interacting domain death agonist (BID), apoptosis regulator BAX (BAX), BCL2-associated agonist of cell death (BAD) and p53 up-regulated modulator of apoptosis (PUMA) proteins in MCF-7 cells. MCF-7 cells were treated with solvent alone as a negative control (NC) or treated with fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA), or from control egg yolks (EFA), or 1.5 μM staurosporine (ST) as positive control for 48 h. Cell extracts were prepared using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of Protease Inhibitor Cocktail (BioShop, Mainway, Burlington, ON Canada). Protein extract was separated on a polyacrylamide gel and transferred to a nitrocellulose filter (Bio-Rad, Hercules, CA, USA) by wet-electroblotting. The immobilized proteins were incubated with primary antibodies to BID, BAX, BAD and PUMA (Cell Signaling Technology, Danvers, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) was used as a reference protein. Detection was performed by chemiluminescence, using Clarity™ Western ECL Substrate (Bio-Rad). WCL, Whole-cell lysate; CYT, cytoplasmic cellular fraction; MEM, membranous cellular fraction.*

membranous fraction (Figure 5). BAD protein was shown to be accumulated in the membranous fraction (Figure 6) together with other pro-apoptotic proteins from the BCL2 family, notably BID, BAX and PUMA (Figure 6). Our results also showed an increased level in whole-cell lysate of SMAC/DIABLO and HTRA2/OMI proteins, which promote caspase activity (Figure 5). Finally, protein levels of caspase-9 were measured due to being directly related to the mitochondrial pathway. Our results showed the presence of the cleaved form of caspase-9 (Figure 7).

Discussion

In the current study, we made an attempt to determine potential mechanisms behind reduced viability of MCF-7 cells after treatment with EFA-CLA (Figure 1).

Cells integrate multiple signals from a variety of sources before following either pro- or anti-apoptotic pathways (Figure 8). To examine whether apoptosis was the primary cause of cell death, we analysed the levels of proteins associated with apoptotic events. Furthermore, we verified the effect of EFA-CLA on selected proteins involved in cellular stress signalling with respect to their integration into apoptosis induction.

One of the earliest stages of apoptosis includes changes on the cell surface. Phosphatidylserine is an important phospholipid membrane component present in the inner side of the plasma membrane. During apoptotic events, phosphatidylserine is translocated to the surface where it acts as a signal for macrophages to engulf apoptotic cells (14, 15). Annexin V, a calcium-dependent phospholipid-binding protein,
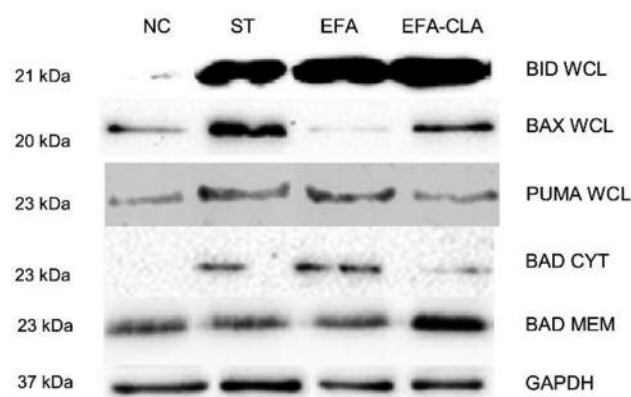
has high affinity for phosphatidylserine, and its fluorochrome-labelled form is used in detection of apoptotic changes using assay kits. Our results clearly showed the induction of apoptosis after treatment with both fatty acid extracts, with the effect of EFA-CLA being more pronounced (Figure 2).

To further verify our initial findings, western blot was carried out targeting proteins associated with apoptotic events (Figures 3-7). Our results for EFA-CLA-treated MCF-7 cells showed an increased accumulation of p53 protein in cell lysates, compared to NC and EFA-treated cells (Figures 3 and 4). Accumulation of p53 protein in cancer cells has been associated with suppressed growth and division (16). As a transcription factor, p53 is responsible, among others, for the expression of mitochondrial-induced pro-apoptotic proteins, *i.e.* BAX, BID, BAD and PUMA (17-19). Indeed, our results confirmed that EFA-CLA, simultaneously with increasing p53, increased the levels of BAX, BID, BAD and PUMA proteins (Figure 3, 6).

BAX, BID, BAD and PUMA, among many others, belong to the BCL2 family. Activity and interdependencies of BCL2 family proteins play a pivotal role in the regulation of cell death
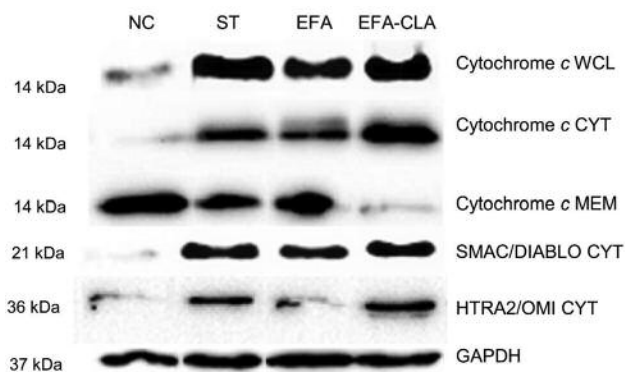
Figure 6. *Expression of cytochrome c, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (SMAC/DIABLO) and mitochondrial serine protease (HTRA2/OMI) in MCF-7 cells. MCF-7 cells were treated with solvent alone as a negative control (NC) or treated with fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA), or from control egg yolks (EFA) or 1.5 μM staurosporine (ST) as positive control for 48 h. Cell extracts were prepared using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of Protease Inhibitor Cocktail (BioShop, Mainway, Burlington, ON Canada). Protein extract was separated on a polyacrylamide gel and transferred to a nitrocellulose filter (Bio Bio-Rad, Hercules, CA, USA) by wet-electroblotting. The immobilized proteins were incubated with primary antibodies to cytochrome c, SMAC/DIABLO and HTRA2/OMI (Cell Signaling Technology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) was used as a reference protein. Detection was performed by chemiluminescence, using Clarity™ Western ECL Substrate (Bio-Rad). WCL, Whole-cell lysate; CYT, cytoplasmic cellular fraction; MEM, membranous cellular fraction.*

Figure 7. *Expression of caspase- 9 in MCF-7 cells (whole-cell lysates). MCF-7 cells were treated with solvent alone as negative control (NC) or treated with fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA), or from control egg yolks (EFA), or 1.5 μM staurosporine (ST) as positive control for 48 h. Cell extracts were prepared using Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA, USA) with the addition of Protease Inhibitor Cocktail (BioShop, Mainway, Burlington, ON, Canada). Protein extract was separated on a polyacrylamide gel and transferred to a nitrocellulose filter (Bio-Rad, Hercules, CA, USA) by wet-electroblotting. The immobilized proteins were incubated with primary antibodies to caspase-9 (Cell Signaling Technology). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology) was used as a reference protein. Detection was performed by chemiluminescence, using Clarity™ Western ECL Substrate (Bio-Rad).*

(20). Activation of PUMA indicates that it plays a critical role in the p53 tumour-suppressor pathway. It was shown that disruption of the PUMA gene reduced p53-mediated apoptosis and tumour suppression (21). In addition, *Puma*-knockout mice showed abnormal response to apoptotic stimuli (22). Our results also showed the accumulation of BAD protein in the membranous fraction (Figure 6). Phosphorylated BAD normally remains sequestered by 14-3-3 in the cytosol and once dephosphorylated, it will be translocated to the mitochondrial membrane to release cytochrome *c* (23). Thus, our results suggest the accumulation of pro-apoptotic, non-phosphorylated form of BAD. Interestingly, at the same time our data from the Stress and Apoptosis Signalling Array showed an increased level of the phosphorylated form of BAD in the cytosol (Figure 3). Fernando et al. showed that phosphorylated BAD exerted cell cycle-inhibitory effects by targeting cyclin D1 (24). Thus, the presence of both forms suggests that BAD may act *via* both pro-apoptotic and antiproliferative mechanisms to prevent cancer cell growth (Figure 8).

Our results showed an increased level of cytochrome *c* after treatment of MCF-7 cells with EFA-CLA (Figure 5), as well as its translocation from the membrane to the cytosol (Figure
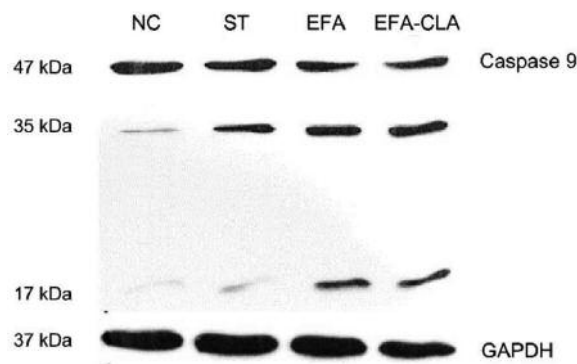
5). In addition, we found a similar relationship for SMAC/DIABLO and HTRA2/OMI; specifically, their increased accumulation in the cytosolic fraction (Figure 5). Interestingly, for all our results, the effect was stronger for EFA-CLA compared with EFA (Figures 4-7). Pro-apoptotic BCL2 family members (including BID, BAX, BAD, BCL2-like protein 11 (BIM), and PUMA] are normally localised in the cytoplasm; however, during apoptosis induction, they can be activated and translocated to the outer mitochondrial membrane. As a result, they participate in the release of cytochrome c, as well as SMAC/DIABLO and HTRA2/OMI proteins from the mitochondria to the cytoplasm, consequently activating caspases. To our knowledge, this is the first study supporting this mechanism for the action of EFA-CLA.

Our results also showed a decrease in the level of phosphorylated HSP27 as well as of survivin (Figure 3). HSP27 is a mediator of cell stress that confers resistance to adverse environmental conditions. It inhibits the release of cytochrome *c* from the mitochondria and increases the expression of AKT (25) (Figure 8). Signalling Array results also showed that EFA-CLA reduced the levels of phosphorylated AKT1 and ERK1/2 MAPKs (Figure 3). ERK1/2 MAP and AKT kinases are major signalling nodes that primarily transmit growth and proliferation signals (26, 27). In addition, AKT phosphorylates pro-apoptotic BAD
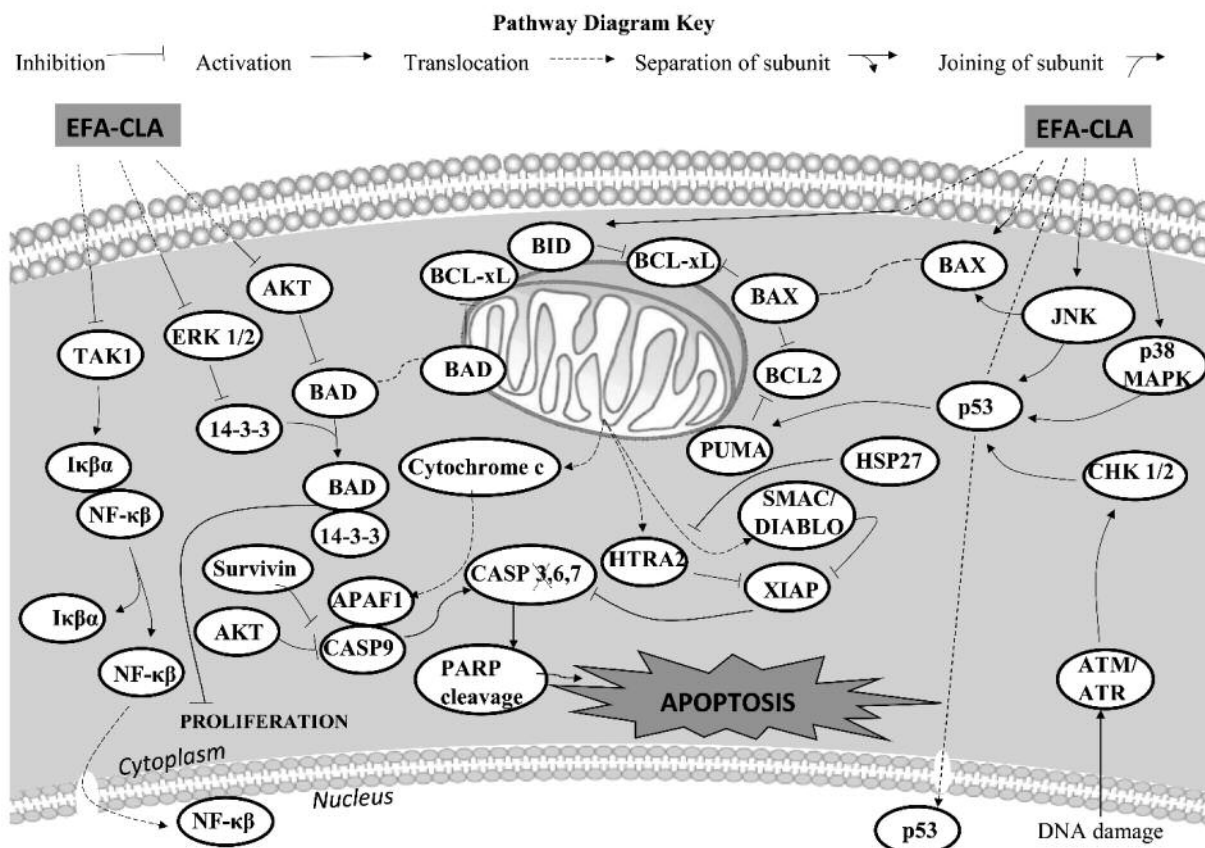
Figure 8. *Mechanism of fatty acid extract from conjugated linoleic acid-enriched egg yolks (EFA-CLA)-induced apoptosis in MCF-7 breast cancer cells [modified from (13)].*

protein at Ser136 and inhibits its ability to induce apoptosis (23, 28). Survivin, on the other hand, binds and inhibits caspase-3 and promotes cell division (29-32) (Figure 8).

Our results also showed a significantly increased level of phosphorylated SMAD2 (Figure 3), which is a key mediator of transforming growth factor beta 1 (TGFβ1) signalling. Available literature suggests that TGFβ1 induces apoptosis *via* the mitochondrial pathway, including activation and translocation of BAX and BAD into mitochondrial membranes, release of cytochrome c, as well as activation of caspase-3 and PARP (33). Further research on pro-apoptotic effects of TGFβ1 in BME-UV1 bovine cancer cell line showed the activation of classic SMAD signalling pathway. Specifically, TGFβ1 stimulation led to SMAD2 phosphorylation of Ser465/467 followed by SMAD2 translocation to the nucleus, which initiated transcription of specific genes (34).

Activation of p38 MAPK and SAPK/JNK MAPKs (Figure 3) occurs during dual phosphorylation mechanism in response to cell stressors. The functional consequence of their activation is regulation of inflammatory response, cell-cycle arrest and cellular stress-induced apoptosis (Figure 8). It has been shown that SAPK/JNK and p38 contribute to apoptotic signal transduction in response to various stressors (35, 36). The literature indicates that SAPK/JNK can contribute to the activation of both c-JUN/AP1 external apoptosis pathway, as well as the mitochondrial apoptosis pathway discussed here. Both mechanisms may act independently or co-operate with one another to induce cell death (37).

CHK1 and CHK2 kinases (Figure 3) play an important role in DNA damage checkpoint control. They also affect the post-translation modifications of p53 protein (Figure 8). In response to stressors (*e.g.* DNA damage), ATM serine/threonine kinase (ATM) and ATR serine/threonine kinase (ATR) kinases together with CHK1 and CHK2 phosphorylate p53 on Ser15 and Ser20. This leads to inhibition of p53 interaction with MDM2 and inhibition of p53 ubiquitination. As a result, the level of tumour-suppressor protein p53 increases (38, 39). Our results showed that treatment with EFA-CLA increased the expression of kinases CHK1 (statistically significant) and CHK2 (statistically insignificant) (Figure 3), supporting the hypothesis of pro-apoptotic activity of EFA-CLA.

IκBα forms a complex with nuclear factor kappa B (NF-κβ) transcription factor, inhibiting NF-κβ activity associated with the up-regulation of anti-apoptotic gene expression (Figure 8). On the other hand, phosphorylation of IκBα results in NF-κβ release and its translocation to the nucleus, where it can function as a transcription factor (39). Although there were no significant changes in the level of total IκBα, our results showed a significant decrease in the phosphorylated form of IκBα in EFA-CLA-treated cells (Figure 3). In addition, our results showed a decrease in the expression of the active TAK1 form. Activated TAK1 promotes phosphorylation of IκBα protein and mitogen-activated protein kinase kinase 4 (MKK4), MKK3/6 and mitogen-activated protein kinase kinase kinase 14 (40). Taken together, EFA-CLA treatment seems to target the activation of NF-κβ *via* reducing the level of phosphorylated IκBα, again supporting the hypothesis of its pro-apoptotic properties.

Cytochrome *c*, SMAC/DIABLO and HTRA2/OMI proteins activate the caspase-dependent mitochondrial pathway (42-45) (Figure 8). In the cytoplasm, cytochrome *c* interacts with apoptotic peptidase-activating factor 1 protein and pro-caspase-9 to form a so-called apoptosome (46). Once formed, the apoptosome can activate inactive pro-caspase-9 (47), followed by activation of effector caspases, including caspase-3/7 (37, 48). Interestingly, our results for EFA-CLA showed a slight decrease in the level of pro-caspase-9 (Figure 7) and an increase in the level of its active form (Figure 7). This effect was also observed for EFA, however, less pronounced (Figure 7). SMAC/DIABLO and HTRA2/OMI play a role in promoting apoptosis by inhibiting the activity of IAP (IAP binding protein), an inhibitor of caspase-3 (49). Although our results clearly indicate activation of apoptotic events, we were unable to detect the presence of caspase-3, as the MCF-7 cell line lacks caspase-3 owing to a functional deletion in the CASP3 gene (50). However, some studies suggest that apoptosis can occur without the activation of caspase-3 and *via* the activation of caspase-7 (51, 52), or even *via* other proteases as cell death executioners (53, 54). Those hypotheses find confirmation in our results as treatment with EFA-CLA showed increased levels of activated caspase-7 as well as the cleaved form of PARP (Figure 3).

Finally, PARP is a DNA repair and apoptosis enzyme that is inactivated when cleaved at Asp214 by caspase-3 or caspase-7 (55). Thus, finally we verified the induction of apoptosis by EFA-CLA. The results shown here indicate that the EFA-CLA fatty acids studied by us induce the mitochondrial apoptosis pathway. Although in the literature there is information on the induction of apoptosis in relation to synthetic CLA isomers (56, 57), we present the first report of induction of apoptosis due to EFA from a CLA-enriched product (Figure 8).

The American Dietetic Association recognizes beef (as well as lamb, turkey, and dairy product) as a functional food due to its CLA content (58). In this context, our study strengthens the position of CLA-enriched eggs as a functional product. We admit that our results are based on an in vitro model which requires further verification *in vivo*; however, we showed for the first time the potential molecular mechanism behind inhibition of growth of MCF-7 cancer cells treated with extract from CLA-enriched yolks *via* mitochondrial-induced apoptosis (Figure 8). The signaling pathway proposed here might also explain reduced viability of cancer cells by other CLA-enriched products. It would be beneficial to test this hypothesis on other cancer cell lines.

Conclusion

In conclusion, our results, for the first time, show the complete mechanism for the effect of EFA-CLA on the MCF-7 breast cancer cell line: the activation of intrinsic, mitochondrial apoptotic pathway, supported by the JNK signaling pathway. Our conclusions are strengthened by the down-regulation of pro-survival proteins by EFA-CLA treatment. Taken together, our findings support the hypothesis that CLA-enriched eggs might be an easily available functional food product with a potential chemopreventative activity.

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