Research Article

Alpha-linolenic acid regulates the growth of breast and cervical cancer cell lines through regulation of NO release and induction of lipid peroxidation

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Abstract

In the present work, we have analyzed the effect of the essential fatty acid, alpha linolenic acid (ALA) on nitric oxide release as well as induction of lipid peroxidation in breast (MCF-7 and MDA-MB-231) and cervical (SiHa and HeLa) cancer cell lines. ALA-treated cells showed a dose-dependent decrease in cell viability in both breast and cervical cancer cell lines without affecting the viability of non-cancerous transformed HEK 293 cells. Both types of cancer cells treated with ALA demonstrated a significant reduction in nitric oxide (NO) release with a simultaneous increase in lipid peroxidation (LPO). This was followed by a decrease in the mitochondrial membrane potential as well as activation of caspase 3 leading to apoptosis. Thus, ALA regulated the growth of cancer cell lines through induction of lipid peroxidation and modulation of nitric oxide release resulting in apoptosis.

Introduction

Alpha-linolenic acid (ALA 18:3), an omega-3 (n-3) fatty acid, is an essential fatty acid (EFA) that cannot be synthesized by the human body and thus must be obtained from dietary sources. ALA is found mostly in certain plant foods that include walnuts, rapeseed (canola), several legumes, flaxseed and green leafy vegetables (Barceló-Coblijn & Murphy 2009, Bougnoux *et al.* 2010). ALA is the parent n3 fatty acid and gets converted into longer chain fatty acids such as Eicosapentaenoic (EPA 20:5, ω3) and Docosahexaenoic acid (DHA 22:6, ω3) that are well known for their various functions including cardio-protection, anti-inflammatory, anticancer as well as brain development (Allayee *et al.* 2009, Burdge *et al.* 2005, Stark *et al.* 2008, Zhao G *et al.* 2004). There is a vast amount of research on EPA and DHA derived from fish oil; however, very few studies have been conducted on ALA present in plants.

A number of studies have reported that essential fatty acids selectively kill tumor cells through the generation of free radicals as well as lipid peroxidation (Das *et al.* 1999, 2000). Free radicals in the form of ROS (reactive oxygen species) and RNS (reactive nitrogen species) are known to cause oxidation of biomembranes as well as modulation of inter- and intracellular signaling networks resulting in changes of cell proliferation, differentiation and apoptosis (Das 2002, Sun *et al.* 2012). Nitric oxide, a measure of RNS, is an endogenously produced free radical that has been known to either promote or inhibit lipid peroxidation (Hogg & Kalyanaraman 1999). The enzyme responsible for the conversion of L-arginine to NO is the nitric oxide synthase (NOS) that exists in three major isoforms; inducible (NOS II/iNOS), endothelial (NOS III/eNOS) and neuronal NOS (NOS I/nNOS). In cancer cells, it has been shown that increased NO generated by iNOS contributes to tumor angiogenesis by the up-regulation of vascular endothelial growth factor (VEGF), which may increase tumor metastasis (Nakamura *et al.* 2006). Increased release of NO in the cervix has also been shown to be associated with HPV infection in cases of cervical cancer (Rahkola *et al.* 2009, Wei *et al.* 2009).

Nitric oxide has been reported to be a potent inhibitor of the lipid peroxidation chain reaction and has been shown to inhibit peroxidase enzymes that are potential initiators of the former process. Conversely, in the presence of superoxide, nitric oxide forms peroxynitrite that can initiate lipid peroxidation and oxidize lipid soluble antioxidants (Gago-Dominguez *et al.*

2005, Hofseth *et al.* 2008, Hogg & Kalyanaraman 1999, Xu *et al.* 2002). The cell membranes contain high concentrations of polyunsaturated fatty acids and are thus susceptible to peroxidation, which is a critical mechanism leading to growth inhibition and cell death (Gago-Dominguez *et al.* 2007).

In the present study, we analyzed the effect of ALA, an omega 3 fatty acid, on breast and cervical cancer cell lines in terms of cell viability, nitric oxide generation as well as status of lipid peroxidation. We observed that ALA decreased the viability of both breast and cervical cancer cells in a significant manner, albeit, at higher doses and higher exposure times. Interestingly ALA regulated the growth of both types of cancer cells through an increase in lipid peroxidation and a reduction in nitric oxide generation. This resulted in loss of the mitochondrial membrane potential of the cells leading to apoptosis through activation of the caspase 3 pathway.

Materials and Methods

Reagents

Tissue culture plasticware was purchased from BD Bio-sciences, CA, USA. Alpha linolenic acid, fatty acid-free bovine serum albumin (BSA) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylthiazoliumbromide (MTT) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Dulbecco's Modified Eagles Medium (DMEM), Penicillin and streptomycin were obtained from Gibco BRL, USA. Fetal bovine serum was purchased from Moregate Biotech, Australia. L-Glutamine, BHT and TBA were obtained from Himedia Corporation, Mumbai, India. Sulfanilamide was purchased from Qualigens, N-[1-napthyl] ethylenediamine (NEDD) was purchased from SRL and TCA was purchased from Merck.

Cell culture

The human breast adenocarcinoma (MCF-7 and MDA MB231) and cervical cancer (SiHa and HeLa) cell lines as well as the human embryonic kidney cell line (HEK293) used in this study were obtained from the National Centre for Cell Science (NCCS), Pune, India. The cells were grown in DMEM containing 2 mM L-glutamine supplemented with 10% fetal bovine serum and 100 U/ml of penicillin-streptomycin. They were incubated in a humidified 5% CO_2 incubator at 37°C.

Conjugation of Alpha linolenic acid with BSA

ALA was reconstituted in 200 µL of ethanol. For conjugation, ALA (10mM) was added to fatty acid-free bovine serum albumin (BSA) (3mM) to obtain a ~3:1 ratio of ALA: BSA (Mahadik *et al.* 1996). The conjugated omega fatty acids were incubated at 37˚C for 30 min in a CO_2 incubator and stored at -20˚C. Before use they were diluted to the required concentration with 10% DMEM.

Cell Viability Assay

Cell viability was measured using the MTT assay in breast (MCF-7 and MDA-MB-231) and cervical cancer cell lines in the presence of different concentrations of ALA and compared with the non-cancerous transformed cell line, HEK 293. The cells were seeded at a density of 1×10^5 cells/ml in 96-well plates (TPP, Europe/Switzerland) and grown for 24 h. ALA was added at different concentrations: 0-320µM for 24, 48 and 72 h. The MTT solution (5 mg/ml) was added to each well and the cells were cultured for another 4 h at 37°C in a 5% CO_2 incubator. The formazan crystals formed were dissolved in 90 µl of SDS-DMF (20% SDS in 50% DMF) (Singh *et al.* 2009). After 15 min, the amount of colored formazan derivative was determined by measuring optical density (OD) at 570 nm using the ELISA micro plate reader (Bio-Rad, Hercules, CA).

Nitric Oxide assay

The concentration of NO was indirectly determined in culture supernatants as nitrite, a major stable product of NO. The cells (breast and cervical cancer cell lines) were seeded at a density of $1x10^5$ cells/ml in 96-well plates (TPP, Europe/Switzerland) for 24 h and then incubated with different concentrations of ALA (0-80 µM) for different time intervals (24, 48 and 72 h). The NO levels were estimated by the Griess reaction (Udenigwe *et al.* 2009). Briefly, 100 µL of culture supernatant was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine in 5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was measured with the ELISA micro plate reader (Bio-Rad, Hercules, CA). Nitric oxide concentration was determined using sodium nitrite ($NaNO_2$) as a standard.

RT-PCR

The total cellular RNA from control as well as cells treated with different concentrations of ALA (0-80 µM) was extracted by a one-step acid guanidine isothiocyanate-phenol method using the TRI reagent (Invitrogen). RNA was precipitated with isopropanol and the concentration was estimated using Nanodrop (Eppendorff BioPhotometer plus). 10 mg of total RNA were used for each RT-PCR reaction. 50 units of Moloney murine leukemia virus reverse transcriptase (MMuLV) (Bangalore Genei, Bangalore, India) were

added in a typical 50 µl reaction (10 µg RNA, 5X first-strand buffer, 1 mM DTT, 2.5 mM dNTPs, 50 ng/µl random primers and 15 U/µl RNAse i) and incubated for 1 h at 40°C followed by incubation at 95°C for 5 min. The purified cDNA template was amplified using different sets of primers. The primers used were β-actin-F: 5'-taccactggcatcgtgatg-gact-3'; β-actin-R: 5'-tttctgcatcctgtcggaaat-3'; iNOS-F: 5'- caga-taagtgacataagtga-3'; iNOS-R: 5'- ctatctttgttgttgtccttg-3'. PCR was performed in 25 µl volume in which 1X PCR buffer, 2.5 mM dNTPs, 1.5 mM $MgCl_2$, 1 U of Taq polymerase and 100 ng of the specific primers were added. A brief initial denaturation at 95°C for 5 min was followed by 35 cycles with the following steps: 95°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. RT-PCR products were then separated on a 1.2% agarose gel and visualized by staining with ethidium bromide. The intensities of the bands corresponding to the RT-PCR products were quantified using phosphor imager (Alpha Imager, Alpha Innotech) and normalized with respect to the β-actin product.

Lipid peroxidation assay (thiobarbituric acid reactive substance)

The generation of thiobarbituric acid reactive substances (TBARS) was measured following a published protocol (Ding *et al.* 2006) with minor modifications. Briefly, the cells were seeded at a density of $4x10^5$cells/ml in 6-well plates (TPP, Europe/ Switzerland) and were grown for 24 h, followed by treatment with different concentrations of ALA (0-80 µM). After 24 h of treatment, cells were harvested and resuspended in 120 µl of 1X PBS. They were homogenized on ice for 10 min using a micro-pestle and then centrifuged at 10,000 rpm for 10 min. Following this, 100 mM butylated hydroxytoluene (1.5 µL), 15% Trichloroacetic acid (50 µL), 0.25 mM butylated hydroxytoluene (50 µL), 0.375% thiobarbituric acid (50 µL) and 8.5% SDS (20 µL) were added. The samples were then vortexed for 5 min. This mixture was incubated at 80 ^oC for 120 min and the reaction was

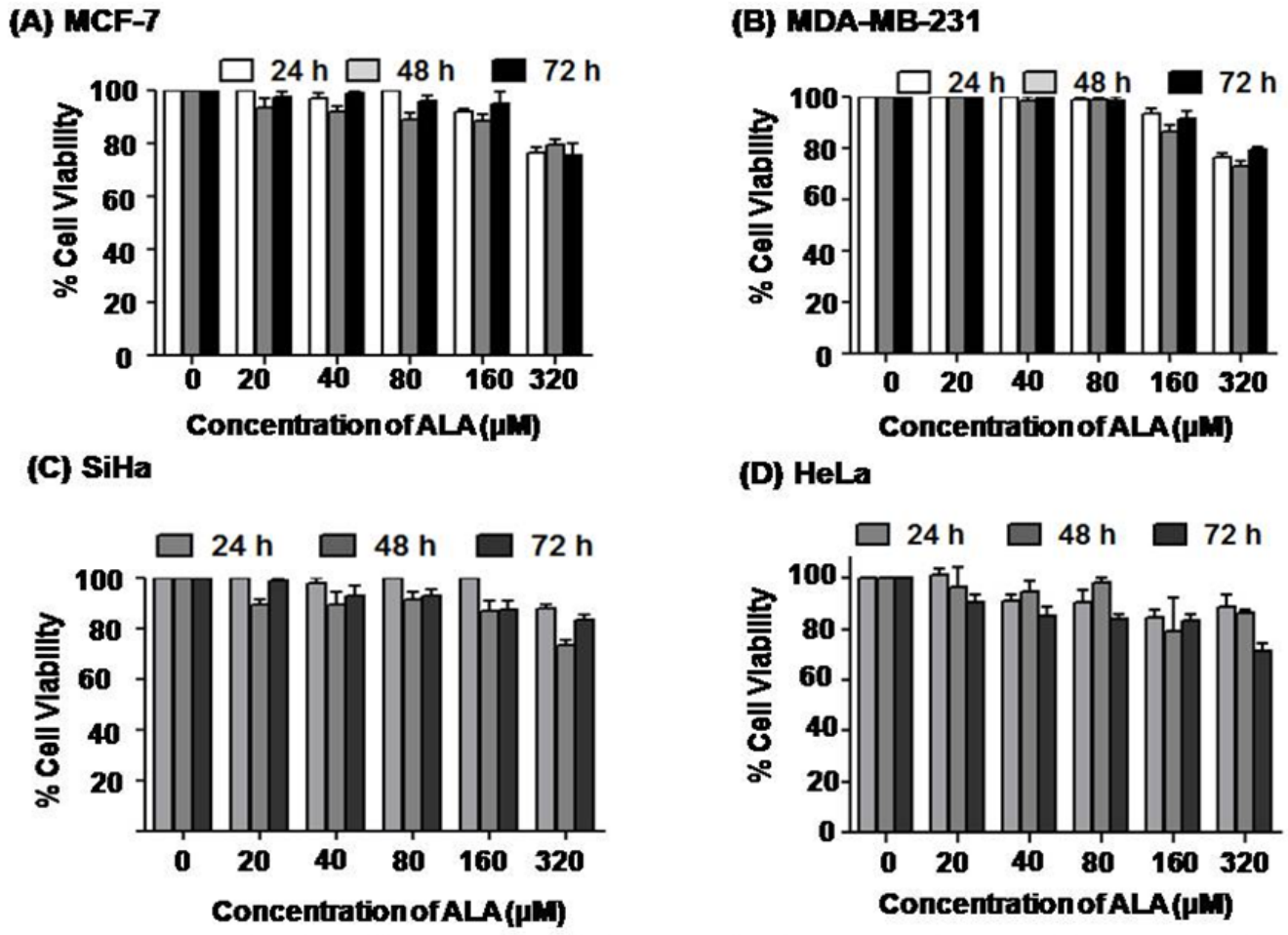

Figure 1. ALA alters the viability of breast and cervical cancer cell lines. All cell lines were treated with different doses of ALA (0-320µM) for 24, 48 and 72 h. ALA alters the cell viability of MCF-7 (A) and MDAMB231 (B) breast cancer cells as well as of SiHa (C) and HeLa (D) cervical cancer cells at 24, 48 and 72 h. All the data are presented as mean ± SEM of three independent experiments. $^*p < 0.05$ indicates statistically significant differences compared to the control untreated group.

stopped by cooling on ice for 10 min. The samples were centrifuged at 10,000 rpm for 10 min and the supernatant from each tube was transferred to a 96-well plate. The optical density was measured at 540 nm using the ELISA plate reader (Bio-Rad, Hercules CA). TBARs were calculated using 1, 1, 3, 3-tetraethoxypropane (TMP) as a standard. The resulting TBAR values were normalized by the protein concentration of each sample that was estimated by the Bradford reagent (Bio-Rad Laboratories Inc, CA, USA).

Mitochondrial membrane potential

Both breast and cervical cancer cell lines were seeded at a density of 1×10^5 cells/ml in a black 96-well plate and incubated at 37°C in a CO_2 incubator. The next day, the cells were treated with different concentrations of ALA (0-80 µM) and were incubated in a CO_2 incubator at 37°C for 24 h. The following day, the medium was removed and the cells were washed with 1X PBS and incubated with 2.5 µg/ml JC-1 staining solution (Sigma-Aldrich, St. Louis, MO) for 1 h in the dark (Wang *et al.* 2009). Fluorescence readings were measured using the Fluostar Omega microplate reader (BMG Labtech) at 520 nm for JC-1 monomers and at 590 nm for JC-1 aggregates.

Immunoblotting

Cell extracts were prepared from controls as well as cells treated with different concentrations of ALA (0-80 µM). Briefly, the cell pellets were resuspended in 40 µl lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 0.5% NP40, 50 mM NaF, 1 mM DTT, 0.1 mM PMSF, 0.5µg/ml leupeptin (Pro-pure Amersco, Solon, USA), 1 µg/ml pepstatin (Amresco, Solon, USA), 150 mM NaCl, 0.5µg/ml aprotinin (Amersco, Solon, USA), a protease inhibitor cocktail (Roche, Lewes, UK) and incubated on ice for 1 h with intermittent mixing. The extract was centrifuged for 20 min at 4°C at 12000 rpm. Protein concentration was estimated

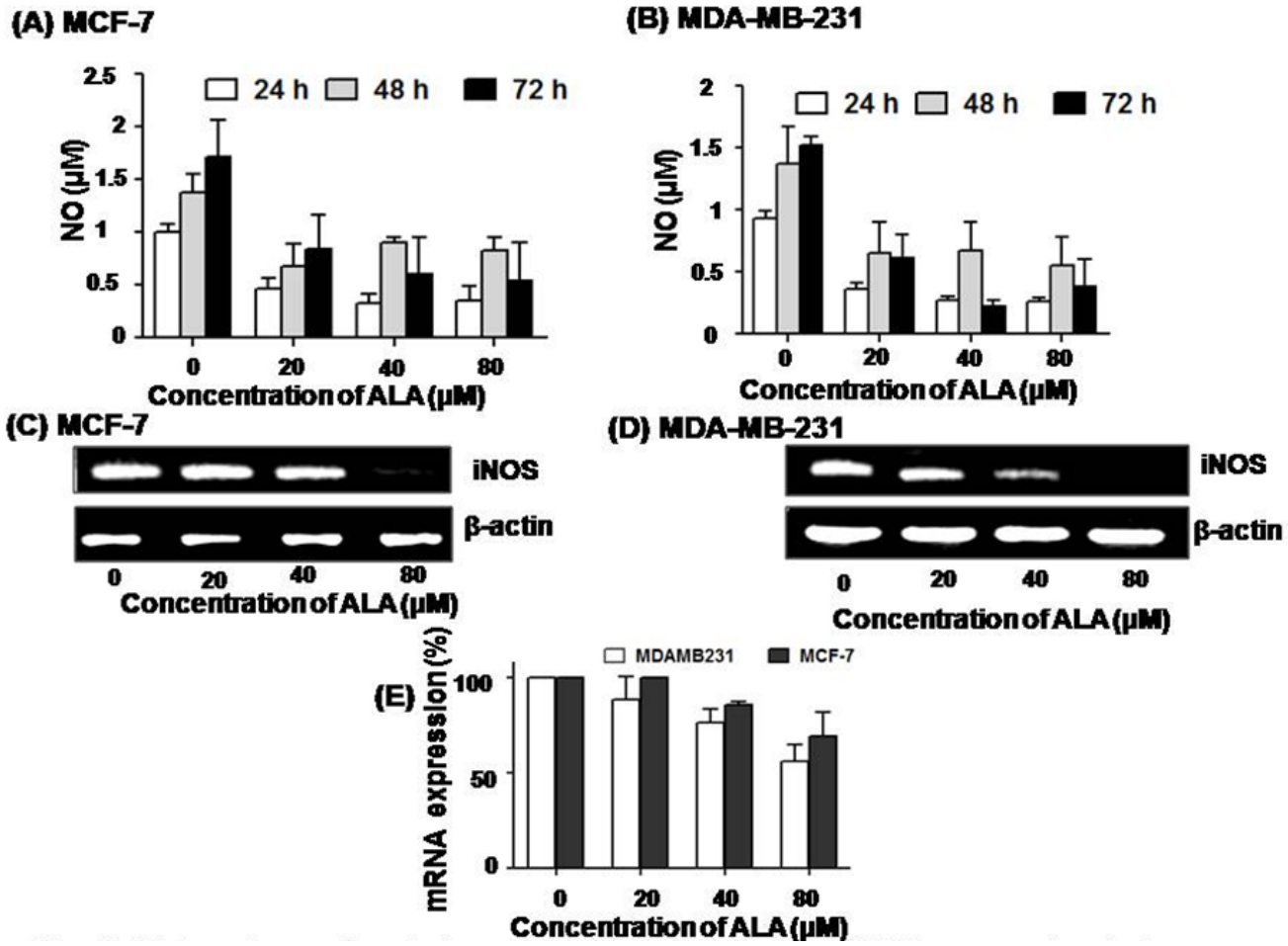

Figure 2. ALA reduces the nitric oxide release as well as iNOS expression in breast cancer cell lines. Cells were treated with different concentrations of ALA (0-80 µM) for 24, 48 and 72 h and the nitric oxide release was measured in MCF7 (A) and MDA-MB-231 (B) cells. ALA treatment reduces the iNOS expression at mRNA level in MCF-7 (C) and MDAMB231 (D) cells. β-actin was used as the loading control. Densitometric analysis of iNOS expression is shown (E). Values are represented as mean ± SEM of five independent experiments, each conducted in triplicates. $^*p < 0.05$ indicates statistically significant differences compared to the untreated control cells.

using the Bradford reagent (Biorad Laboratories Inc, CA, USA). Equal amounts of protein were loaded on a 10% SDS-polyacrylamide gel and transferred electrophoretically to an Amersham Hybond-P PVDF membrane (GE Healthcare, UK) in sodium phosphate buffer (pH 6.8). The membrane was blocked in 5% BSA in TBST and incubated at room temperature for 1 h with rabbit polyclonal antibody for caspase 3 and mouse monoclonal antibody for tubulin (Santacruz, CA, USA) at 1:500 and 1:2000 dilutions, respectively. The membrane was washed in TBST and incubated with donkey anti-rabbit IgG HRP conjugate at 1:5000 (for caspase) and donkey antimouse IgG HRP conjugate at 1:3000 (for tubulin) dilutions. Proteins were visualized using a chemiluminescence kit (Amersham ECL Advance western blotting detection kit, GE Healthcare, UK) and densitometric analysis of X-ray films was performed on the Alpha Imager using the Alpha Ease FC software, Alpha Innotech.

Statistical analysis

All experiments were performed in triplicates and repeated at least three times. The data are presented as mean ± SD. Statistical analysis was conducted with the Graph Pad 4 prism program using one-way ANOVA. The p values used for comparisons were < 0.05.

Results

ALA alters the cell viability of breast and cervical cancer cell lines

Omega 3 fatty acids including, ALA, have been known to inhibit the growth of cancer cells (Das *et al.* 1998, Horia & Watkins 2005, Kim *et al.* 2009, Sagar & Das, 1995). In the present report, we have treated breast (MCF7 and MDA-MB-231) and cervical (SiHa and HeLa) cancer cell lines with different concentrations (0 -320 μM) of ALA for 24, 48 and 72 h. It was observed that in breast cancer cell lines, there was an apprecia-

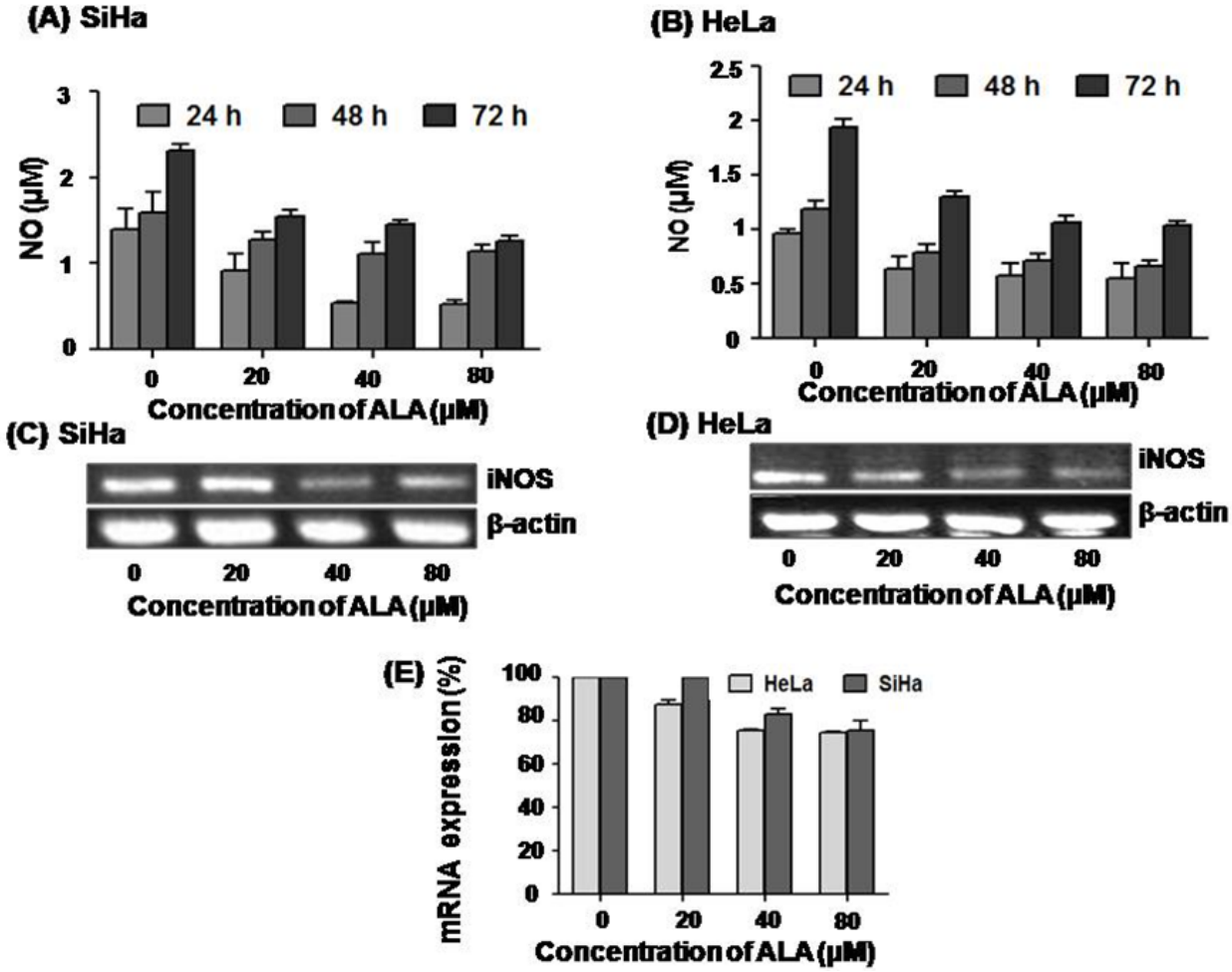

Figure 3. ALA reduces nitric oxide as well as iNOS levels in cervical cancer cell lines. The cells were treated with different concentrations of ALA (0-80 μM) for 24, 48 and 72 h and the nitric oxide release was measured in SiHa (A) and HeLa (B) cells. ALA treatment reduces the iNOS expression at mRNA level in SiHa (C) and HeLa (D). β-actin was used as the loading control. Densitometric analysis of iNOS expression has been shown (E). Values are represented as mean ± SEM of five independent experiments, each conducted in triplicates. $^{*}p < 0.05$ indicates statistically significant differences compared to the untreated control cells.

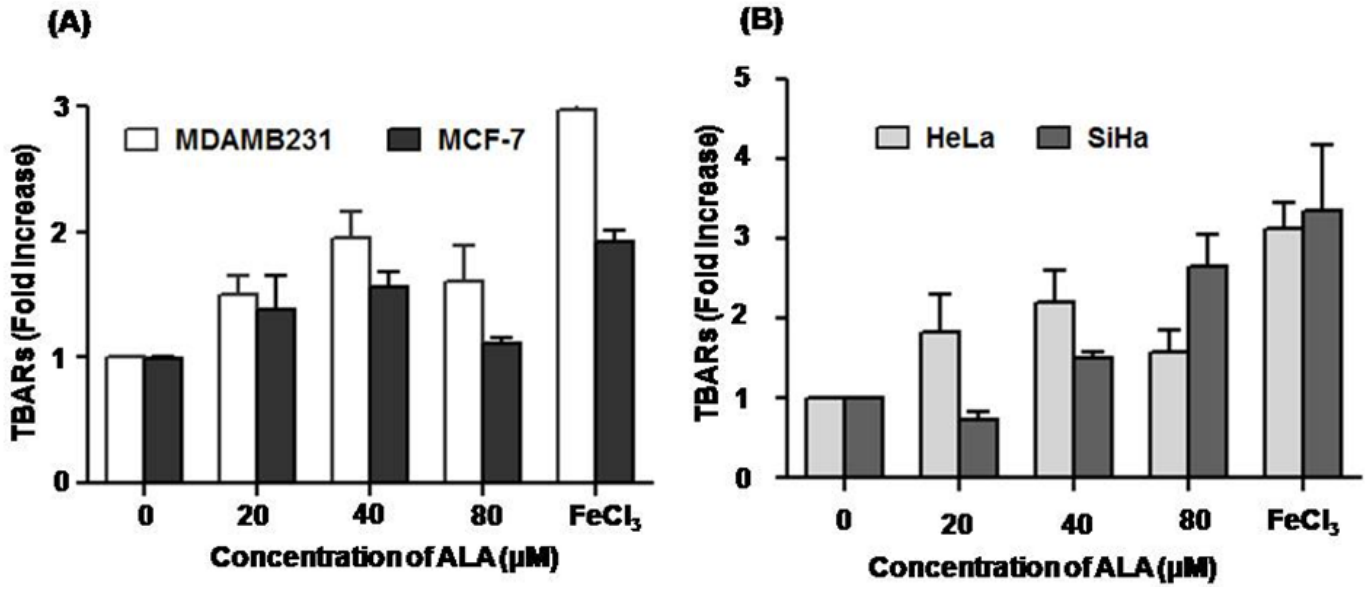

Figure 4. ALA increases lipid peroxidation in breast and cervical cancer cells. All the cell lines were treated for 24 h. ALA induced lipid peroxidation in breast (**A**) and cervical cancer (**B**) cells, as shown by TBARs assay. The TBAR values were normalized by the protein concentration of each sample estimated by the Bradford reagent. Values are represented as mean ± SEM of five independent experiments. **p<0.001* indicates statistically significant differences compared to the untreated control.

ble decrease in cell survival ($p<0.0001$) above 80 μM of ALA at 24 h (Figure 1A and B). However, in MCF7 cells, the viability was found to significantly decrease at 48 h post-ALA treatment at the relatively lower dose of 20 μM, whereas at 72 h, the cell viability was similar to that observed at 24 h (Figure 1A). On the other hand, MDA-MB-231 cells showed a decrease in viability above 80 μM at 48 h that was observed at 24 h as well. However, the percentage viability was reduced at 48 h compared to that at 24 h. There was no appreciable difference in MDA-MB-231 cells post-ALA treatment at 72 h compared to the 24 h treatment period (Figure 1B). These data suggest that both breast cancer cell lines seem to be more sensitive to ALA treatment at 48h.

In the cervical cancer cell line SiHa, ALA was found to decrease the viability after a 160 μM dose at 24 h ($p<0.0001$). However, after incubating the cells with ALA for 48 h and 72 h, the viability started decreasing at 20 μM and 40 μM, respectively (Figure 1C). In HeLa cells, ALA decreased the cell viability after 40 and 160 μM at 24 and 48h, respectively. However, at 72h, there was a significant decrease in viability at a lower dose (20 μM) (Figure 1D). These results suggest that cervical cancer cells respond to ALA treatment at higher exposure times (beyond 24h) at lower concentrations. Interestingly, in HEK 293 cells (non-cancerous), ALA did not show any toxicity up until 320 μM, implying that it was specific only for the cancerous cell lines (Figure 1S).

ALA decreases the nitric oxide levels in breast and cervical cancer cell lines

Nitric oxide plays a dual role in cancer; it can either promote or suppress it (Crowell *et al.* 2003, Lechner *et al.* 2005). To test the effect of ALA on nitric oxide levels, both the breast and cervical cancer cell lines were treated with different concentrations of ALA (0-80 μM) at different time intervals (24-72h). It was observed that at 40 and 80 μM, ALA significantly reduced the levels of NO by ~3.0 and ~2.8-fold ($p<0.05$), respectively, in MCF7 cells in 24h (Figure 2A). Similarly, in MDA-MB-231 cells, there was a ~3.4 and 3.6-fold ($p<0.05$) decrease in NO at 40 and 80 μM concentration of ALA, respectively, in 24h (Figure 2B). On incubating the cells until 48 and 72h, the trend in the decrease in NO was similar to that observed at 24h, however, it was more significantly decreased in 24h of treatment. The intracellular nitric oxide levels were also measured using the fluorescent dye DAF-FM; both cell lines showed an appreciable decrease (Figure 2Sa). The decrease in NO was further confirmed by analyzing the effect of ALA on iNOS expression at mRNA level that showed a significant dose dependent decrease compared to the untreated control cells in both MCF-7 (Figure 2C) and MDA-MB-231 (Figure 2D) cell lines. Densitometric analysis of iNOS expression was performed using the phosphorimager (Figure 2E) and normalized with respect to β-actin as an internal control.

In the SiHa cervical cancer cell line, it was observed that ALA significantly reduced the levels of

NO by ~2.6 and 2.7-fold ($p<0.0001$) at 40 and 80 µM, respectively (Figure 3A). On the other hand, in HeLa cells, the NO levels were reduced by ~1.7 and 1.8-fold ($p=0.0010$) at 40 and 80 µM, respectively, in 24 h (Figure 3B). In this case, after treating the cells with ALA for 48 and 72h, the trend in the decrease in NO was similar to that observed at 24h, however, it was more significantly reduced after 24h of treatment. The intracellular nitric oxide levels were measured by using the fluorescent dye DAF-FM; SiHa showed a significant decrease in NO levels compared to HeLa (Figure 2Sb). Moreover, the decrease in NO was supported by the corresponding decrease in iNOS expression at the mRNA level in a dose-dependent manner, compared to the untreated control cells in both HeLa (Figure 3D) and SiHa (Figure 3C) cells. Densitometric analysis of iNOS expression was performed using the phosphorimager (Figure 3E).

ALA increases lipid peroxidation in breast and cervical cancer cell lines

Since ALA decreased the nitric oxide release in our study and NO is known to either inhibit or promote lipid peroxidation (Cauwels *et al* 2005, Miles *et al.* 1996), we analyzed the effect of ALA on lipid peroxidation in both breast and cervical cancer cell lines. It was observed that ALA increased lipid peroxidation at all doses in both types of cancer cell lines; the increase was more significant at 40 µM. At this dose, ALA increased the lipid peroxidation by ~1.6 ($p=0.0038$) and ~2-fold ($p=0.0002$) in MCF-7 and MDA-MB-231, respectively (Figure 4A). On the other hand, in SiHa

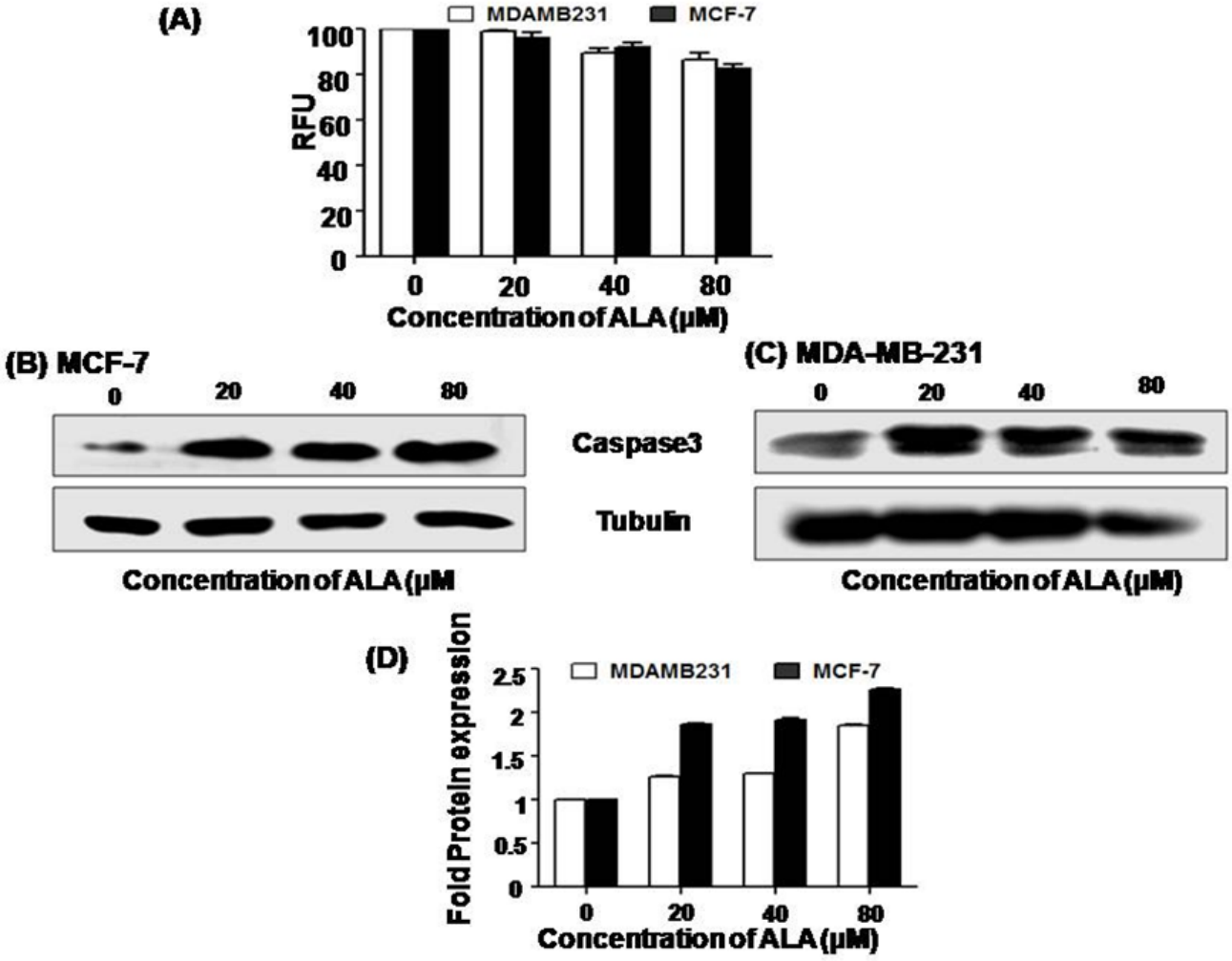

Figure 5. ALA induces apoptosis in breast cancer cells. Breast cancer cells, MCF-7 and MDAMB231, (1 x 10^5cells/well) were treated with ALA for 24 h. Decrease in mitochondrial membrane potential was analyzed by MARS data analysis software 2.10R3 (BMG Labtech) (A). All the data are presented as means ± SEM of three independent experiments. $p < 0.05$ indicate statistically significant differences compared to the control untreated group. Caspase 3 (17/21 kDa) expression was determined in ALA treated MCF-7 (B) and MDAMB231 (C) cell lines. The histogram depicts densitometric analysis of western blots of caspase 3 (D). Values are represented as mean ± SEM of three independent experiments *p<0.001* indicate statistically significant differences compared to the untreated control cells.

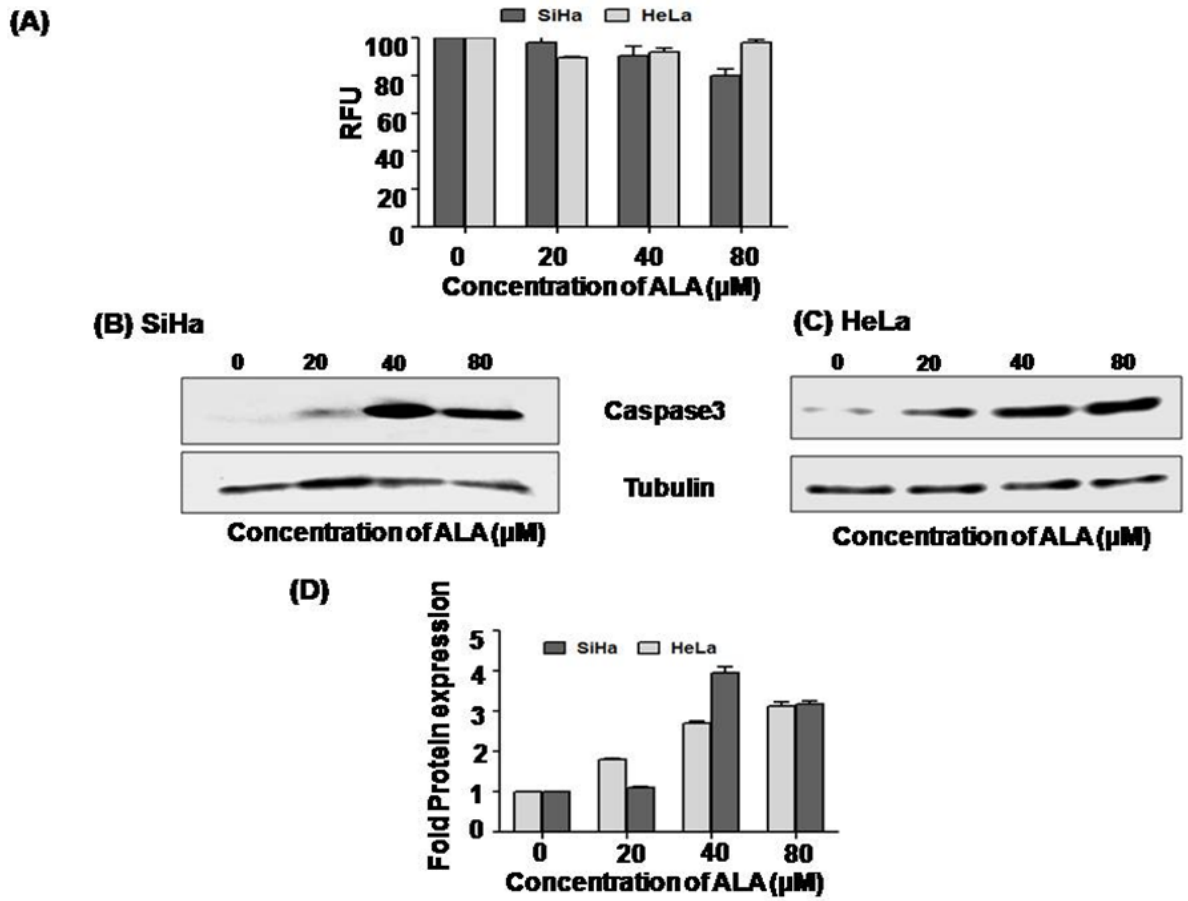

Figure 6. ALA induces apoptosis in cervical cancer cells. ALA decreases the mitochondrial potential in the cervical cancer cell lines SiHa and HeLa. The data was analyzed by the MARS data analysis software 2.10R3 (BMG Labtech). (A) All data are presented as means ± SEM of three independent experiments. $p < 0.05$ indicate statistically significant differences compared to the control untreated group. Caspase 3 (17/21 kDa) expression was determined in ALA-treated SiHa (B) and HeLa (C) cell lines. The histogram depicts densitometric analysis of western blots of caspase 3 (D). Values are represented as mean ± SEM of three independent experiments. *p<0.001* indicate statistically significant differences compared to the untreated control cells.

cells, ALA showed a dose-dependent increase in lipid peroxidation (LPO) wherein at 80 μM concentration, a ~2.6-fold increase in LPO was observed. However, in HeLa cells, there was a dose-dependent increase in LPO until 40 μM ALA treatment wherein there was a ~2.2-fold (p=0.0017) increase in lipid peroxidation (Figure 4B).

ALA induces apoptosis in breast and cervical cancer cell lines

The loss of mitochondrial membrane potential is the hallmark of apoptosis (Wang *et al.* 2009). Since ALA decreased NO and increased LPO, we wanted to analyze whether ALA induced apoptosis in both cancer cell types. Thus, we evaluated the effect of ALA on the mitochondrial membrane potential in both breast and cervical cancer cells. It was observed that it significantly reduced (p<0.0001) the mitochondrial membrane potential in breast cancer cell lines (MCF-7 and MDA-MB-231) in a dose-dependent manner (Figure 5A). This was supported by a corresponding increase in the expression of caspase 3 (17/21 kDa) in ALA-treated MCF7 (Figure 5B) and MDA-MB-231 (Figure 5C) cells compared to the untreated control cells. Densitometric analysis of caspase 3 expression was performed by the phosphorimager (Figure 5D) and normalized with respect to tubulin as an internal control.

On the other hand, in SiHa cells, there was a dose-dependent decrease in the mitochondrial membrane potential that correlated with a dose-dependent increase in caspase 3 expression. In HeLa cells, the decrease in mitochondrial membrane potential was observed more significantly at 20 μM of ALA. However, there was a significant dose-dependent increase in caspase 3 expression in HeLa suggesting that ALA induced apoptosis in cervical cancer cells (Figure 6A). Densitometric analysis of caspase 3 expression was performed by the phosphorimager (Figure 6D).

All these results suggested that ALA induced apoptosis in both breast and cervical cancer cell lines through activation of caspase 3 and a decrease in the mitochondrial membrane potential.

Discussion

Omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs) are the essential fatty acids that are important for human health. Various studies suggest that the dietary fatty acids play an important role in carcinogenesis wherein the n-3 fatty acids have anti-carcinogenic potential and n-6 fatty acids are pro-cancerous (Barceló-Coblijn & Murphy 2009, Sinclair *et al.* 2002, Stark *et al*. 2008). Alpha-linolenic acid (C18:3*n*-3, ALA), the most abundant n-3 PUFA, is an essential fatty acid in the human diet and is present in green leaves, oil, seeds (flaxseed, canola, perilla) and nuts. It has been shown to reduce the growth of various cancers including breast and cervical cancer (Horia & Watkins 2005, Kim *et al.* 2009, Sagar & Das 1995). Even though epidemiological studies showing a direct correlationsip between ALA intake and cancer response are limited (De Stefani *et al.* 1998, Franceschi *et al.* 1996), there are studies showing association between low levels of ALA in adipose tissue of patients with high risk of breast cancer (Klein *et al.* 2000). There are reports suggesting that high ALA diets can inhibit the growth of spontaneous or carcinogen induced mammary tumors (Fritsche *et al.* 1990, Hirose *et al*.1990, Kamano *et al.* 1989, Munoz *et al.* 1995). The average intake of ALA in European countries, USA and Canada has been shown to range between 0.8 and 2.2 g/d (Burdge & Calder 2005). In the UK the intake of ALA has risen from a mean of 1.4 g/d in 1987–8 to 2.1 g/d according to the British Adult Diet Survey (Henderson *et al.* 2004). Since, the conversion of ALA into longer ω-3 PUFAs is generally considered low, it has been shown that a moderate consumption of walnuts (4 walnuts/day for 3 weeks) markedly increases the blood levels of ALA and its metabolic derivative, EPA.

In the current study, we have focused on analyzing the effect of ALA on modulation of growth of breast and cervical cancer cells in terms of regulation of nitric oxide and lipid peroxidation. ALA decreased the nitric oxide levels in both cancer cell types. It is well-known that high levels of NO have both genotoxic and angiogenic properties (Nakamura *et al.* 2006). Increased NO production catalyzed by the iNOS enzyme in tumor cells plays a critical role in tumor angiogenesis, cancer progression and metastasis (Narayanan *et al.* 2003). ALA has been reported to decrease iNOS expression in the LPS-stimulated macrophage cell line RAW 264.7 (Ren *et al.* 2007, Udenigwe *et al.* 2009). Our results showed that ALA not only decreased the iNOS expression at the mRNA level but also reduced the intracellular levels of NO in both the breast and cervical cancer cell lines. Thus, the observed decrease in NO in both cancer cell lines by ALA reinstates its antineoplastic potential.

PUFAs have been shown to initiate free radical production and the generation of lipid peroxide products, selectively in tumor cells (Das 2002, Sun *et al.* 2012). Several studies with ALA have shown that it increased lipid peroxidation in breast cancer cells (Menéndez *et al.* 2001, Pardini 2006). Moreover, an inverse correlation has been reported between lipid peroxidation and cell proliferation (Das 2002). In line with this, we found that ALA increased the lipid peroxidation with a simultaneous decrease in cell proliferation in both breast and cervical cancer cell lines. Recently, it was demonstrated that peroxidized products of n-3 PUFAs suppress iNOS induction and NO production in a peroxidation-dependent manner (Araki *et al.* 2011). Thus, the observed decrease in NO in both types of cancer cell lines may be partly due to NO suppression by peroxidized products of ALA. A strong association between decreased NO levels and increased lipid peroxidation has been reported in several papers. For example, it was found that patients suffering from fibromyalagia had higher serum levels of TBARS (particularly, malondialdehyde) and lower levels of nitrite compared to the control groups (Ozgocmen *et al.* 2006). Another report has shown that a decrease in the level of NO in rats treated with alloxan-induced diabetes was associated with increased levels of lipid peroxides (Mohan & Das, 2001). Thus, increase in lipid peroxides may lead to increased free-radical generation that may inactivate NO, resulting in its low levels.

Mitochondria play an important role during apoptosis (Wang *et al.* 2009). Reactive oxygen species can directly activate the mitochondrial permeability transition and result in loss of mitochondrial membrane potential ($\Delta\Psi$), which results in the release of cytochrome c (cyt c) and activation of the caspase pathway (Cao *et al.* 2010, Kim *et al*. 2005, Lee *et al.* 2008, Sun *et al.* 2012). Our results showed that ALA reduced nitric oxide levels and increased lipid peroxidation in both breast and cervical cancer cells; this may be responsible for the observed apoptosis (Figure 7). Conversely, increased nitric oxide has been reported to inhibit lipid peroxidation by scavenging lipid peroxyl radicals (Hogg & Kalyanaraman 1999). Increased NO has also been shown to prevent activation of caspase 3 resulting in inhibition of apoptosis (Maejima *et al.* 2005, Mahidhara *et al.* 2003, Kim *et al* 1997, Zhou *et al.* 2005). Thus, the increased LPO in the presence of ALA leads to a decrease in NO resulting into disruption of the mitochondrial membrane potential and activation of caspase 3 causing apoptosis (Figure 7). Taken together, our data suggest that ALA

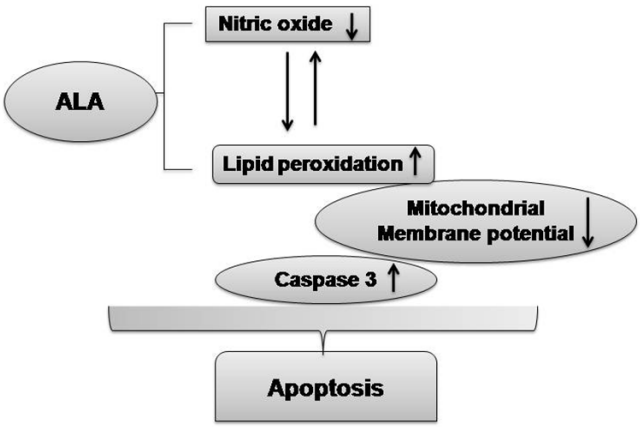

Figure 7. A proposed model for the mode of action of ALA. ALA reduces the levels of nitric oxide and induces lipid peroxidation. The increase in lipid peroxidation results in the decrease in mitochondrial membrane potential and caspase-3 activation leading to apoptosis.

regulates the growth of breast and cervical cancer cells through regulation of lipid peroxidation as well as nitric oxide generation that may lead to apoptosis.

Conclusion

Omega 3 fatty acids are known to exert anticancer effects through various mechanisms. One of them is through the generation of free radicals while another is through lipid peroxidation (Sun *et al.* 2012). Most of the research work to date has analysed the significance of EPA and DHA in cancer with very few data reported on ALA. Our paper has tried to delineate the anticancer properties of ALA in terms of its potential to regulate lipid peroxidation as well as nitric oxide generation that in turn result in the control of carcinogenesis. However, more studies are required in the future to elucidate the role of PUFAs in governing the inter-relationship between the nitric oxide and lipid peroxidation status of cells for the regulation of cancer growth.

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Competing interests

The authors declare no conflict of interest.

Author Contributions

RKG designed the study and drafted the manuscript. RD has carried out the major experiments and contributed in manuscript writing. PM and SS have helped in lipid peroxidation and RT-PCR experiments. All the authors have read and approved the final version of the manuscript.

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