

## Eleostearic Acid Inhibits Breast Cancer Proliferation by Means of an Oxidation-Dependent Mechanism

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**Abstract** Eleostearic acid ( $\alpha$ -ESA) is a conjugated linolenic acid that makes up ~60% of *Momordica charantia* (bitter melon) seed oil. Prior work found that water extract from bitter melon was able to inhibit breast cancer. Here, we investigated effects of  $\alpha$ -ESA on both estrogen receptor (ER)-negative MDA-MB-231 (MDA-wt) and ER-positive MDA-ER $\alpha$ 7 human breast cancer cells. We found that  $\alpha$ -ESA inhibited proliferation of both MDA-wt and MDA-ER $\alpha$ 7 cells, whereas conjugated linoleic acid had comparatively weak antiproliferative activity at 20 to 80  $\mu$ mol/L concentrations. We also found that  $\alpha$ -ESA (40  $\mu$ mol/L) treatment led to apoptosis in the range of 70% to 90% for both cell lines, whereas conjugated linoleic acid (40  $\mu$ mol/L) resulted in only 5% to 10% apoptosis, similar to results for control untreated cells. Addition of  $\alpha$ -ESA also caused loss of mitochondrial membrane potential and translocation of apoptosis-inducing factor as well as endonuclease G from the mitochondria to the nucleus. Additionally,  $\alpha$ -ESA caused a G<sub>2</sub>-M block in the cell cycle. We also investigated the potential for lipid peroxidation to play a role in the inhibitory action of  $\alpha$ -ESA. We found that when the breast cancer cells were treated with  $\alpha$ -ESA in the presence of the antioxidant  $\alpha$ -tocotrienol (20  $\mu$ mol/L), the growth inhibition and apoptosis effects of  $\alpha$ -ESA were lost. An AMP-activated protein kinase inhibitor (Dorsomorphin) was also able to partially abrogate the effects of  $\alpha$ -ESA, whereas a caspase inhibitor (BOC-D-FMK) did not. These results illustrate that  $\alpha$ -ESA can block breast cancer cell proliferation and induce apoptosis through a mechanism that may be oxidation dependent.

A number of studies indicate that total dietary fat intake may play a major role in the development and progression of breast cancer in humans (1, 2). However, the impact of different types of fat in the diet on breast cancer development has proven complex with results from previous studies supporting both positive and negative roles for specific fats. Some long chain polyunsaturated fatty acids (LC-PUFA) have been reported to have protective effects as shown by an inverse relationship between high fish consumption and breast cancer first noted over 30 years ago (3, 4).

We have identified bitter melon as a plant containing a LC-PUFA that seems to have potential anticancer properties. Bitter melon originated in tropical Asia, but due to its apparent health benefits, it is now grown and used medicinally in many countries and is widely available in a variety of different

supplement forms. Prior work showed that multiple types of extracts from bitter melon had *in vivo* (5–7) and *in vitro* (8) anticancer activity. Of particular interest to us is eleostearic acid ( $\alpha$ -ESA), also known as 9Z,11E,13E-octadecatrienoic acid, a LC-PUFA that makes up ~60% of bitter melon seed oil. *In vitro* studies using pure  $\alpha$ -ESA have reported anticancer activity. For example  $\alpha$ -ESA significantly reduced viability of transformed NIH-3T3 mouse fibroblast (SV-T2) and monocytic leukemia (U-937) cells (9). In additional reports, DLD-1 colorectal adenocarcinoma cells treated with  $\alpha$ -ESA *in vitro* were growth inhibited and underwent DNA laddering indicative of apoptosis (10), and both Caco-2 and HT-29 colon cancer cells had decreased viability and increased DNA fragmentation when treated with  $\alpha$ -ESA (11). In a follow-up study using Caco-2 cells,  $\alpha$ -ESA was again found to reduce cell viability and increase DNA fragmentation such as would be seen with apoptosis. Cell viability was maintained by addition of the antioxidant  $\alpha$ -tocopherol in a concentration-dependent manner, suggesting that the reduction in viability is dependent on lipid peroxidation (12). The potential for  $\alpha$ -ESA to inhibit breast cancer and possible mechanisms of action of  $\alpha$ -ESA in breast cancer has not been addressed to this point.

In this study, we investigated the anticancer effects of purified  $\alpha$ -ESA using human breast cancer cells with and without estrogen receptor by comparison of cell proliferation and apoptosis in the presence and absence of  $\alpha$ -ESA. We have also

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examined several potential pathways and mechanisms of action by which  $\alpha$ -ESA may be functioning. Clarification of the actions of  $\alpha$ -ESA may lead to a better understanding of how dietary fats impact cancer cells and to an improved ability to standardize an active ingredient of bitter melon extracts.

## Materials and Methods

### Cell culture

MDA-MB-231 cells were obtained from American Type Culture Collection and maintained in L-15 media (American Type Culture Collection) with 10% FCS (Atlanta Biologics) and pen/strep (Life Technologies). The MDA-ER $\alpha$ 7 cell line (13) is a clone of the MDA-MB-231 cell line that has been transfected with the ER $\alpha$  gene followed by selection with 200  $\mu$ g/mL of zeocin (Promega). The MDA-ER $\alpha$ 7 cell line was maintained in 200  $\mu$ g/mL zeocin-supplemented media.

### Growth assays

Cells were harvested, counted, plated at a density of  $5 \times 10^3$  cells per well in 96-well plates, and allowed to attach overnight in an incubator maintained at 37°C without additional CO<sub>2</sub>. The following day, complete medium was removed from the wells and treatment of the cells with  $\alpha$ -ESA in complete media was then done. When cells were treated with  $\alpha$ -tocotrienol, it was given at the same time as the  $\alpha$ -ESA. The cells were then incubated for 48 h, at which time a cell proliferation assay was done. The proliferation assay was done using 10  $\mu$ L of CCK-8 reagent from the Cell Counting kit-8 as per manufacturer's instructions (Dojindo Laboratories). In this assay, a formazan dye is generated by the activity of dehydrogenases in cells that is directly proportional to the number of living cells. The plates were then incubated for 1.5 to 3 h depending on the cell line in a 37°C incubator, after which the plates were read on an ELISA reader at 450 nm. Conjugated linoleic acid (CLA) and alpha-linolenic acid (ALA) were obtained from Sigma-Aldrich and  $\alpha$ -ESA and  $\alpha$ -tocotrienol were obtained from Cayman Chemical.

### Western blots and immunofluorescence

For the Western blotting experiments, cells were plated at  $5 \times 10^5$  in six-well plates. The following day, the media was replaced with  $\alpha$ -ESA for various times as described for each experiment. Whole-cell extracts were obtained as per the manufacturer's instructions for Novagen Phosphosafe extraction reagent (Merck KGaA) and the amount of protein quantitated and standardized. Western analyses were done with actin, poly ADP ribose polymerase (PARP), and rabbit secondary antibody from Cell Signaling Tech., and ~20  $\mu$ g of protein were run in each lane. The protein was transferred to polyvinylidene difluoride membrane and checked for uniformity using Poceau S and then blocked with PBST with 5% milk. Primary antibodies were incubated in PBST with 5% milk overnight. Blots were then washed and incubated with appropriate AP-linked secondary antibodies, washed again, and incubated with ECF substrate for 45 min. Blots were then visualized with a STORM 840 (Molecular Dynamics). Immunofluorescence used apoptosis inducing factor (AIF) antibody from Millipore, endoG antibody from ProSci, Inc., and immunofluorescently labeled anti-rabbit secondary from AnaSpec. Cells were harvested, plated at a density of  $5 \times 10^3$  cells per well in CultureWells (Grace Bio-Labs), and allowed to attach overnight in an incubator. The following day, complete medium was removed from the wells and the cells were treated with  $\alpha$ -ESA for various times. Cells were then fixed and stained with AIF or endoG primary antibodies and a FITC-labeled secondary antibody. Prolong gold with antifade and 4',6-diamidino-2-phenylindole was then used to seal coverslips to slides and stain the DNA.

### Analysis of cell cycle, apoptosis, and mitochondrial transmembrane potential

Cells were plated at  $1 \times 10^6$  in 10-cm plates and allowed to adhere for 36 h. Cells were then treated as described for each assay and, 48 h

later, were analyzed for cell cycle, apoptosis, or mitochondrial transmembrane potential ( $\Delta\psi_m$ ). For cell cycle analysis, cells were harvested with 0.025% trypsin + 5 mmol/L EDTA in PBS. After washing with PBS, the cells were resuspended in 0.4 mL of PBS, and 1 mL of ice-cold absolute ethanol was added and mixed immediately. Cells were fixed at -20°C for a minimum of 2 h and then washed with PBS. Cells were incubated with 20  $\mu$ g/mL propidium iodide (PI) and 200  $\mu$ g/mL RNAase for 30 min at room temperature in the dark. Cells were analyzed on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences). Intact cells were gated in the FSC/SSC plot to exclude small debris. Cell cycle was determined using ModFit LT software (Verity Software House, Inc.). Apoptosis was evaluated using the Annexin V-FITC Apoptosis Detection kit from MBL International Corporation. Cells were harvested with 0.025% trypsin + 5 mmol/L EDTA in PBS, and 2.5% fetal bovine serum in PBS was added as soon as the cells were released from the dish. Then the cells were transferred to a centrifuge tube, washed with PBS, and incubated for 5 min at room temperature with Annexin V-FITC plus PI following the protocol included in the kit. Cells were analyzed on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences), placing the FITC signal in FL1 and the PI signal in FL2. Intact cells were gated in the FSC/SSC plot to exclude small debris. Cells in the bottom right quadrant of the FL1/FL2 dot plot (labeled with Annexin V-FITC only) are considered to be in early apoptosis, and cells in the top right quadrant (labeled with Annexin V-FITC and PI) are in late apoptosis/necrosis. Disruption of the  $\Delta\psi_m$  was analyzed with Mitocapture Apoptosis Detection kit (Biovision) used as per instructions. In apoptotic cells, Mitocapture dye does not accumulate in mitochondria but remains as monomers in the cytoplasm, and fluoresces green. The cells were analyzed by fluorescence-activated cell sorting.

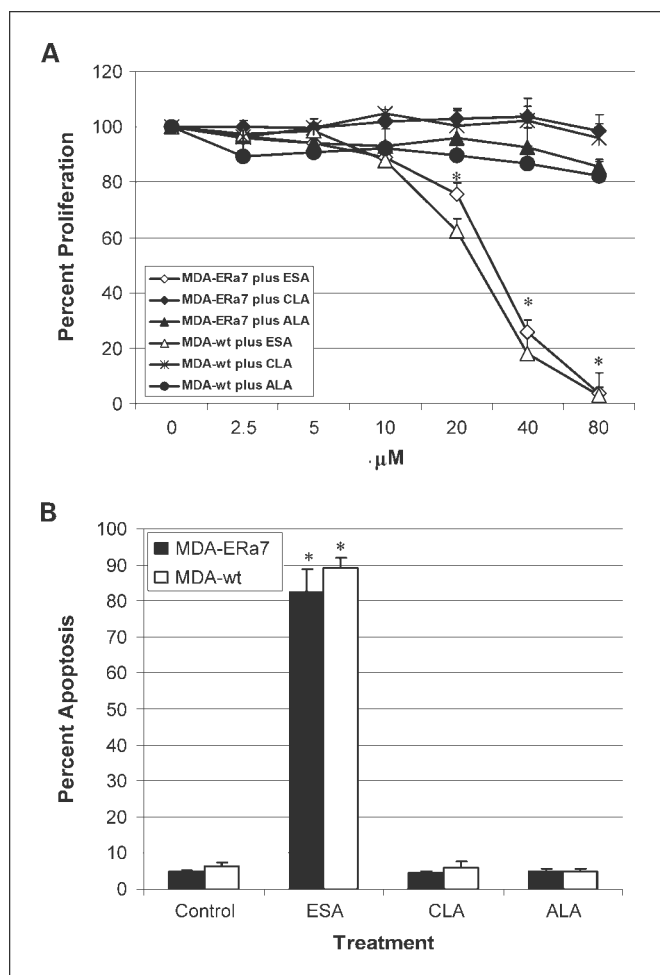
### Statistics

All data analysis was done using GraphPad Prism version 4.0. Proliferation assays were done three to eight times using triplicate wells each time and are presented as means  $\pm$  SEMs. Two-way ANOVA was used to compare the data from the proliferation assays, and one-way ANOVA was used for the remaining studies with significant differences defined as at least a *P* value of <0.05. If the one-way ANOVA was significant, then the Newman-Keuls posttest was used to compare all pairs of columns. Significance between specific groups using Newman-Keuls was defined as at least a *P* value of <0.05.

## Results

### Ability of $\alpha$ -ESA to inhibit breast cancer growth and induce apoptosis

We assessed whether  $\alpha$ -ESA possesses anticancer activity against breast cancer using estrogen receptor (ER) $\alpha$ -negative MDA-wt and ER $\alpha$ -positive MDA-ER $\alpha$ 7 cells. Our initial doses of  $\alpha$ -ESA were dictated by prior work with other cell lines (9–11). Here, we found that  $\alpha$ -ESA inhibited proliferation of both breast cancer cell lines at 20  $\mu$ mol/L (*P* < 0.05), 40  $\mu$ mol/L (*P* < 0.001), and 80  $\mu$ mol/L (*P* < 0.001), whereas CLA and ALA, though very similar to  $\alpha$ -ESA structurally, had comparatively weak antiproliferative activity at these concentrations (Fig. 1A). Prior work with MDA-MB-231 cells with CLA at higher doses found there was a significant reduction in proliferation (14); however, another study using a variety of cell lines (DLD-1, Hep G<sub>2</sub>, A549, MCF-7, and MKN-7) reported that at the lower concentrations such as what we used, there was negligible inhibition of proliferation (15). MDA-wt and MDA-ER $\alpha$ 7 cells had similar levels of inhibition that were not statistically different, illustrating that ER $\alpha$  was not involved in the effects of  $\alpha$ -ESA. Inhibition of proliferation does not necessarily equate with cell death, which was our



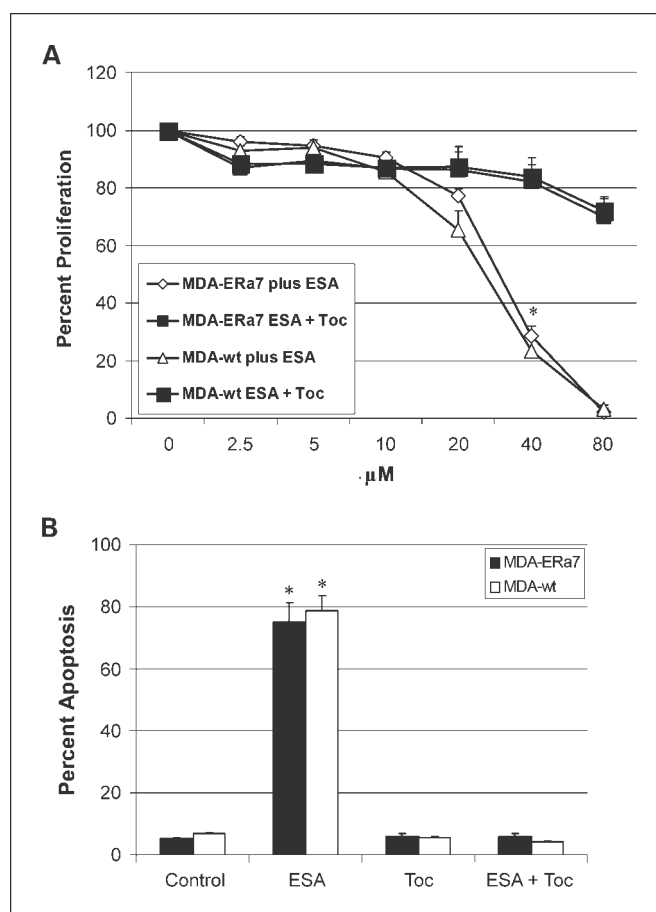
**Fig. 1.** Proliferation and apoptosis of breast cancer cell lines in response to  $\alpha$ -ESA. \*, significant differences between specific concentrations of  $\alpha$ -ESA treatment and treatment with either CLA or ALA. A, two-way ANOVA  $P < 0.0001$  for differences between the treatments. Y-axis, cell proliferation as a percent. Untreated cells given complete media instead of the treatments were considered to be 100%. X-axis, the concentrations of  $\alpha$ -ESA, CLA, and ALA. Points, mean; bars, SEM. Three to eight separate assays were done with triplicate wells in each assay. B, one-way ANOVA  $P < 0.0001$  for the difference between the treatments as a whole. Y-axis, apoptosis as a percent. X-axis, the treatments. Cells were treated for 48 h using 40  $\mu$ M concentrations of  $\alpha$ -ESA, CLA, or ALA. Untreated cells given complete media are the control. Columns, mean of four experiments; bars, SEM.

goal for treatment of breast cancer, so we examined apoptosis using a fluorescence-activated cell sorting-based Annexin V assay. To do this, we treated the cells for 48 hours with 40  $\mu$ M  $\alpha$ -ESA because this dose gave us an effect of ~80% inhibition of cell proliferation. We found that  $\alpha$ -ESA treatment resulted in a high level of apoptosis for both cell lines, 82% and 89% for the MDA-ER $\alpha$ 7 and MDA-wt cells, respectively ( $P < 0.001$ ) compared with their respective untreated control cells. CLA and ALA treatment resulted in only 5% to 10% of cells in apoptosis, which was similar to results for control cells (Fig. 1B). These results clearly illustrate that  $\alpha$ -ESA has very profound direct anti-breast cancer effects *in vitro*.

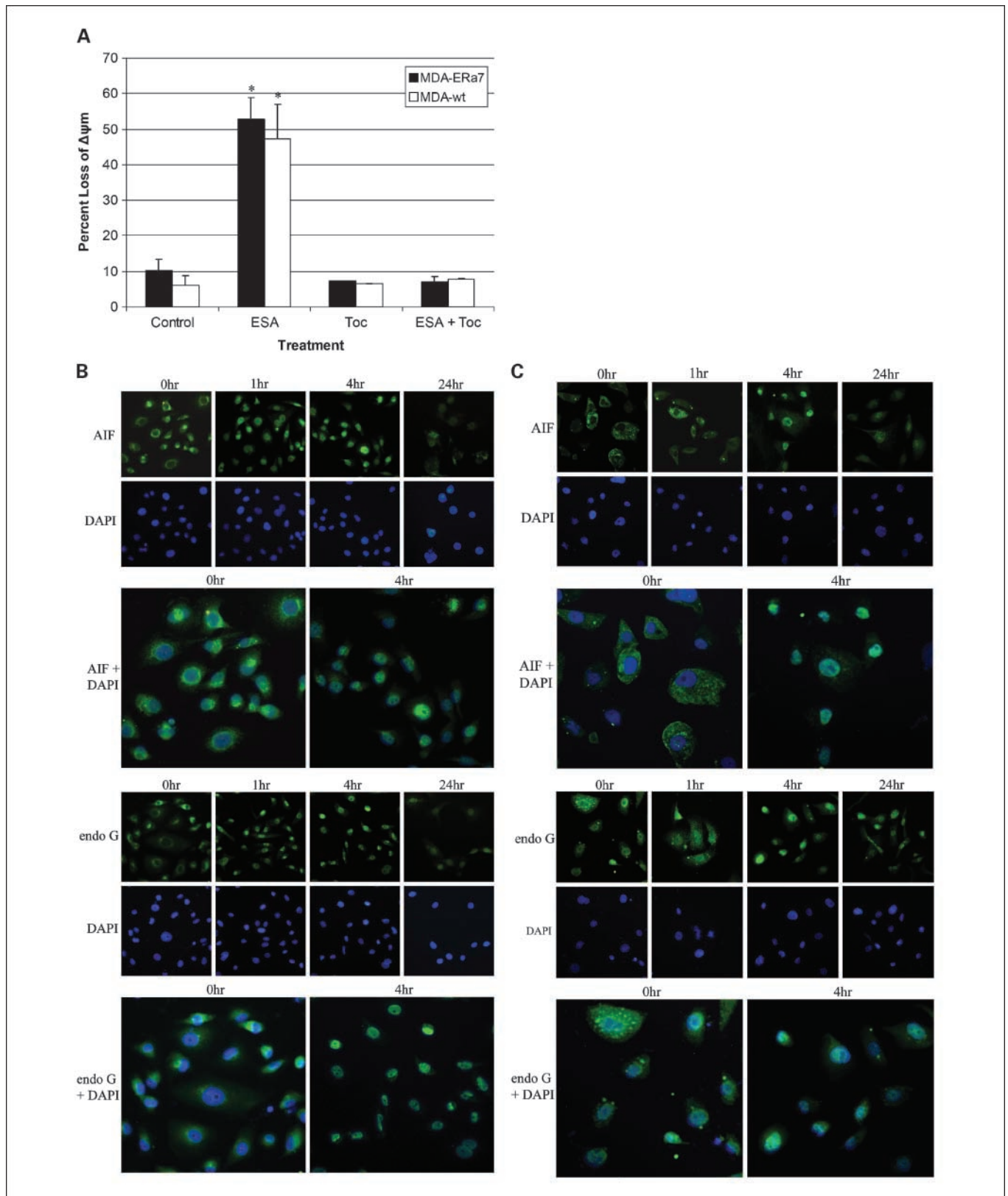
#### Ability of antioxidants to block $\alpha$ -ESA effects

To determine potential mechanisms of action for  $\alpha$ -ESA, we investigated the potential for lipid peroxidation to play a role in the antiproliferative effect that  $\alpha$ -ESA exerted on the breast

cancer cells. To do this, we treated the cells with  $\alpha$ -tocotrienol, a form of vitamin E that has antioxidant activity (16). We hypothesized that the use of an antioxidant should reduce reactive oxygen and nitrogen species and lipid peroxidation that would in turn prevent inhibition of breast cancer by  $\alpha$ -ESA. Figure 2A shows that when the cells were treated with  $\alpha$ -ESA in the presence of 20  $\mu$ M  $\alpha$ -tocotrienol, growth inhibition by  $\alpha$ -ESA was attenuated with the cells retaining 70% and 72% of their proliferation potential for the MDA-ER $\alpha$ 7 and MDA-wt cells, respectively, at the  $\alpha$ -ESA concentration of 80  $\mu$ M/L. The differences were statistically significant at 20  $\mu$ M/L ( $P < 0.01$ ), 40  $\mu$ M/L ( $P < 0.001$ ), and 80  $\mu$ M/L ( $P < 0.001$ ). To further characterize the dependence of  $\alpha$ -ESA actions on an oxidation-dependent mechanism, we measured apoptosis in the presence or absence of 20  $\mu$ M  $\alpha$ -tocotrienol. Treatment with 20  $\mu$ M/L  $\alpha$ -tocotrienol alone did not alter



**Fig. 2.** Effects of the antioxidant  $\alpha$ -tocotrienol on proliferation and apoptosis of breast cancer cells in response to  $\alpha$ -ESA. \*, significant differences between specific concentrations of the treatments. A, two-way ANOVA  $P < 0.0001$  for differences between the treatments. Cell were plated, allowed to adhere overnight, treated with  $\alpha$ -ESA in the presence or absence of 20  $\mu$ M/L  $\alpha$ -tocotrienol (Toc), and 48 h later, the proliferation assays were done as described above. Y-axis, cell proliferation as a percent. Untreated cells given complete media instead of the treatments were considered to be 100%. X-axis, the concentration of  $\alpha$ -ESA. Points, mean; bars, SEM. Four separate experiments were done with triplicate wells in each assay. B, one-way ANOVA  $P < 0.0001$  for the difference between the treatments as a whole. Y-axis, apoptosis as a percent. Untreated cells given complete media are the control. X-axis, the treatments. The concentration of  $\alpha$ -ESA was 40  $\mu$ M/L and the concentration of  $\alpha$ -tocotrienol was 20  $\mu$ M/L. Cells were treated for 48 h. Columns, mean from four experiments; bars, SEM.



**Fig. 3.**  $\alpha$ -ESA treatment effects on the mitochondrial membrane potential and translocation of AIF and endoG. One-way ANOVA  $P < 0.0001$  for the difference between the treatments as a whole. **A**, cells were treated with  $\alpha$ -ESA in the presence or absence of 20  $\mu\text{mol/L}$   $\alpha$ -tocotrienol (Toc) for 48 h. Y-axis, cells with disruption of the mitochondrial membrane in percent. The concentration of  $\alpha$ -ESA was 40  $\mu\text{mol/L}$  and the concentration of  $\alpha$ -tocotrienol was 20  $\mu\text{mol/L}$ . **B**, MDA-ER $\alpha$ 7 cells and **C**) MDA-wt cells were treated with 40  $\mu\text{mol/L}$   $\alpha$ -ESA for the times shown above each panel. Untreated cells were given complete media. Green, positive staining for AIF and ENDOG; blue, positive staining for 4',6-diamidino-2-phenylindole (DAPI) staining of DNA. The cells were observed and photographed with an oil lens at  $\times 630$  magnification.



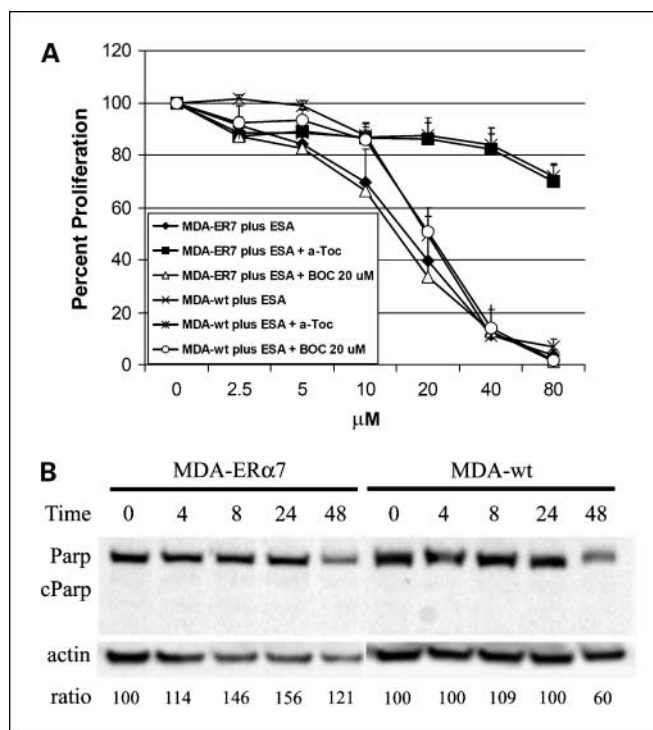
apoptosis levels compared with untreated controls. In addition, the ability of  $\alpha$ -ESA to induce apoptosis was blocked by  $\alpha$ -tocotrienol (Fig. 2B), leading to levels of apoptosis similar to that of untreated controls.

### The intrinsic apoptosis pathway and $\alpha$ -ESA

To identify what parts of the apoptosis pathway are important for the effects of  $\alpha$ -ESA, we determined whether the  $\Delta\psi_m$  was affected by  $\alpha$ -ESA treatment. Cells were incubated with 40  $\mu\text{mol/L}$   $\alpha$ -ESA for 48 h, and then the  $\Delta\psi_m$  was determined using a Mitocapture Apoptosis Detection kit (Biovision). In apoptotic cells, Mitocapture dye does not accumulate in mitochondria, rather, it remains as monomers in the cytoplasm and fluoresces green; in contrast in healthy cells, it accumulates in the mitochondria and fluoresces red. Tumor cell lines tend to undergo an increased rate of both growth and death compared with nontumor cells leading to some loss of  $\Delta\psi_m$ . However, treatment with  $\alpha$ -ESA led to a significant difference from controls ( $P < 0.001$ ) with 53% and 48% of the treated MDA-ER $\alpha$ 7 and MDA-wt cells, respectively, having disruption of the mitochondrial membrane potential ( $\Delta\psi_m$ ; Fig. 3A). The addition of  $\alpha$ -tocotrienol returned the  $\Delta\psi_m$  to the level of the control cells. The loss of  $\Delta\psi_m$  can precipitate translocation of AIF and endonuclease G (endoG) out of the mitochondria and into the nucleus resulting in apoptosis. To test for this, we treated cells with  $\alpha$ -ESA for various time periods and then stained them for AIF and endoG. As shown in Fig. 3B (MDA-wt) and C (MDA-ER $\alpha$ 7), the majority of both AIF and endoG are in the cytoplasm with a small amount in the nucleus before  $\alpha$ -ESA treatment, consistent with where you would expect to find mitochondria before  $\alpha$ -ESA treatment. However, as soon as 1 hour after the addition of  $\alpha$ -ESA for the MDA-wt and after 4 hours for the MDA-ER $\alpha$ 7 cells, the nuclei of the cells were brighter green than before  $\alpha$ -ESA treatment, suggesting that both AIF and endoG translocated from the mitochondria to the nuclei after treatment with  $\alpha$ -ESA. Integration of these results suggests that  $\alpha$ -ESA is activating apoptosis in part via the intrinsic pathway.

### Lack of caspase involvement in $\alpha$ -ESA anticancer affects

To determine if the caspase cascade and PARP are required for  $\alpha$ -ESA-initiated apoptosis, we performed proliferation assays using the broad spectrum caspase inhibitor BOC-D (OMe)-FMK. One-hour pretreatment of the cells at 37°C with 20  $\mu\text{mol/L}$  BOC-D(OMe)-FMK did not alter the ability of  $\alpha$ -ESA to inhibit proliferation of either the MDA-ER $\alpha$ 7 or the MDA-wt cells (Fig. 4A), although treatment with  $\alpha$ -tocotrienol blocked the anticancer effects of  $\alpha$ -ESA as before (Fig. 2A). To further clarify if the caspase cascade was involved, we performed Western blot analysis for cleaved PARP that represents one of the steps at the bottom of the caspase cascade. Full-length PARP was readily observed but cleaved PARP was not detected after 0, 4, 8, 24, or 48 hours of incubation with  $\alpha$ -ESA (Fig. 4B) for either the MDA-ER $\alpha$ 7 or the MDA-wt cells. The amount of full-length PARP did not change when normalized to actin for the MDA-ER $\alpha$ 7 cells, although the amount of full-length PARP did decline at the 48-hour time point in the MDA-wt cells. Taken together, these data indicate that  $\alpha$ -ESA can block proliferation through a caspase-independent mechanism.



**Fig. 4.**  $\alpha$ -ESA does not depend on caspase for apoptosis induction in MDA-wt or MDA-ER $\alpha$ 7 breast cancer cells. *A*, cells were treated with various concentrations of  $\alpha$ -ESA in the presence or absence of BOC-D-FMK at 20  $\mu\text{mol/L}$ , and 48 h later, the proliferation assays were done. *Y*-axis, cell proliferation as a percent. Untreated cells given complete media instead of the treatments were considered to be 100%. *X*-axis, the concentration of  $\alpha$ -ESA. *B*, Western blot of PARP and cleaved PARP. MDA-ER $\alpha$ 7 and MDA-wt cells were treated with 40  $\mu\text{mol/L}$   $\alpha$ -ESA for the times shown above the lanes. *Left*, the specific proteins stained for. *Top*, the cell lines.

### Cell cycle blockage by $\alpha$ -ESA

The ability of  $\alpha$ -ESA to cause alterations in the cell cycle was also investigated. Cells exposed to 40  $\mu\text{mol/L}$   $\alpha$ -ESA but not similar concentrations of either CLA or ALA increased the percentage of cells in the G<sub>2</sub>-M phase (Fig. 5). The percent of MDA-ER $\alpha$ 7 cells in the G<sub>2</sub>-M phase increased from 22% to 39%, and the percent of MDA-wt cells in the G<sub>2</sub>-M phase increased from 12% to 23% in the presence of 40  $\mu\text{mol/L}$   $\alpha$ -ESA for 48 hours. The percentage of cells in the G<sub>2</sub>-M phase under both untreated and treated conditions was different for the two cell lines but the increases in the G<sub>2</sub>-M phase following  $\alpha$ -ESA treatment was similar for both lines.

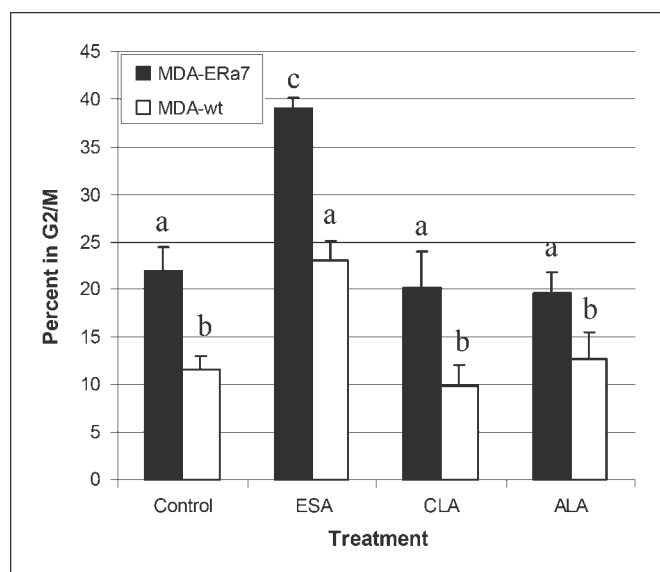
### AMPK activation and $\alpha$ -ESA function

AMPK is a serine/threonine protein kinase, which serves as an energy sensor in eukaryotic cells (17), and has been implicated in inhibition of cell proliferation. The AMPK inhibitor pyrazolopyrimidine partially blocked the antiproliferative effects of  $\alpha$ -ESA for both the MDA-wt and the MDA-ER $\alpha$ 7 cell lines (Fig. 6). The greatest blockage occurred with a pyrazolopyrimidine concentration of 1.0  $\mu\text{mol/L}$  and an  $\alpha$ -ESA concentration of 40  $\mu\text{mol/L}$ , and resulted in the percent proliferation increasing from 27% to 61% in the MDA-ER $\alpha$ 7 cells ( $P < 0.01$ ) and from 31% to 70% in the MDA-wt cells ( $P < 0.01$ ), implicating AMPK in  $\alpha$ -ESA-mediated effects.

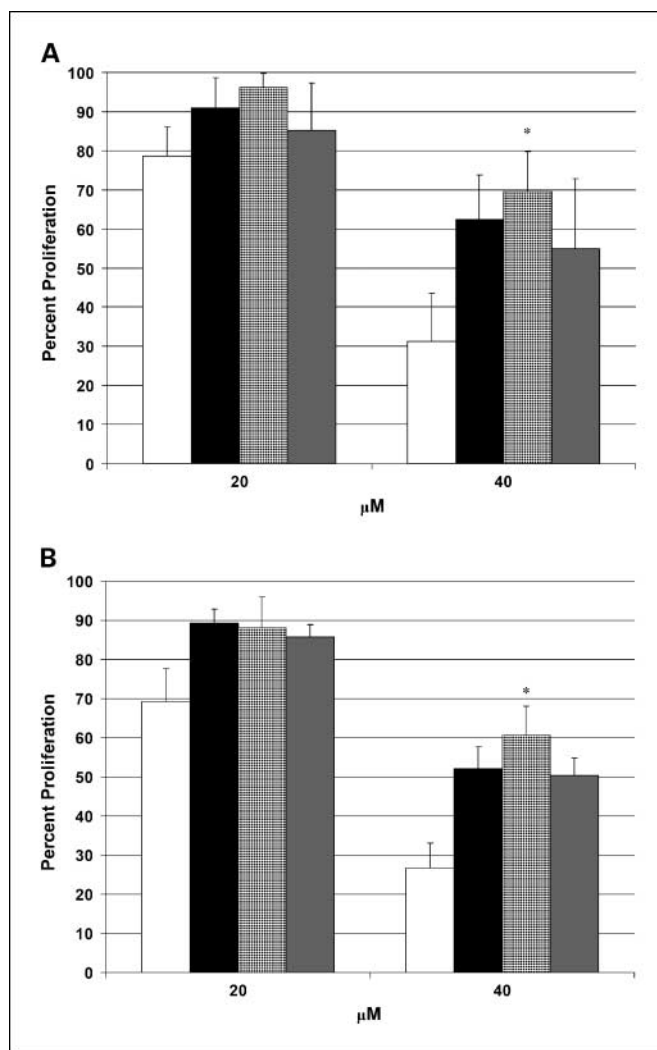
## Discussion

We are the first to show direct inhibition of human breast cancer cell proliferation by  $\alpha$ -ESA, a LC-PUFA that makes up ~60% of bitter melon seed oil. We found that  $\alpha$ -ESA was able to inhibit proliferation as well as to induce apoptosis regardless of whether the cells were ER responsive or ER unresponsive (Fig. 1A). In addition, the concentration of  $\alpha$ -ESA needed for inhibition was considerably lower than that of CLA. Previous studies found that bitter melon seed or fruit extracts were capable of anticancer activity in a rat colonic aberrant crypt foci model (18), a mouse skin carcinogenesis model (19), a DLD-1 (colorectal adenocarcinoma) mouse xenograft model (10), and a mouse mammary tumor model (6). Therefore, although a number of studies have suggested that various bitter melon components/extracts have anticancer activity, no prior work has evaluated the specific effects of  $\alpha$ -ESA in relation to breast cancer.

We have investigated the mechanism of how pure  $\alpha$ -ESA may inhibit tumorigenesis by means of a number of *in vitro* assessments. Our work has identified two potential parts of the mechanism. First, there seemed to be a direct effect on human breast cancer cells via lipid peroxidation. Lipid peroxidation has been implicated as a possible anticancer mechanism for LC-PUFAs. For example, it was shown that eicosapentaenoic acid (EPA), a LC-PUFA found in fish oil, inhibited DLD-1 human colon cancer xenograft growth through a lipid peroxidation mechanism (20). A separate study found that  $\alpha$ -tocopherol blocked the ability of fish oil to suppress N-methylnitrosourea-induced mammary tumors in Sprague-Dawley rats (21). Additionally, female nude mice implanted with MDA-MB-231 human breast cancer cells, the parental cell line for our MDA-ER $\alpha$ 7 cell line, exhibited decreased tumor growth when fed increasing amounts of fish oil compared



**Fig. 5.**  $\alpha$ -ESA treatment results in a G<sub>2</sub>-M cell cycle block. One-way ANOVA  $P < 0.0001$  for the difference between the treatments as a whole. Letters, significant differences between specific treatments. Y-axis, cells in G<sub>2</sub>-M shown as a percent. Untreated cells given complete media instead of the treatments are the control. X-axis, the treatments. Columns, mean from four experiments; bars, SEM.



**Fig. 6.** Response of breast cancer cells to  $\alpha$ -ESA with AMPK inhibitor. A, the effects on MDA-Era7 cells; B, MDA-wt cells. Two-way ANOVA  $P < 0.0001$  for differences between the treatments for both cell lines. \*, significant differences between specific concentrations of the treatments. Cells were plated, allowed to adhere overnight, pretreated with  $\alpha$ -ESA for 1 h, and then treated with  $\alpha$ -ESA at 20 or 40  $\mu$ mol/L in the presence of 0.0  $\mu$ mol/L ( $\square$ ), 0.5  $\mu$ mol/L ( $\blacksquare$ ), 1.0  $\mu$ mol/L ( $\text{hatched}$ ), and 2.0  $\mu$ mol/L ( $\text{dotted}$ ) Dorsomorphin (pyrazolopyrimidine) for 48 h. The proliferation assays were done using the Cell Counting kit-8 as per manufacturer's instructions (Dojindo Laboratories). Y-axis, cell proliferation as a percent. Untreated cells given complete media instead of the treatments were considered to be 100%. X-axis, the concentration of  $\alpha$ -ESA. Columns, mean; bars, SEM. Three separate experiments were done with triplicate wells in each assay.

with corn oil-fed controls (22). Interestingly, the addition of antioxidants to the diet decreased this protective effect of fish oil (22). Moreover, an *in vitro* study with MDA-MB-231 cells found that doxorubicin inhibition of cell proliferation was increased by treatment with another fish oil-associated LC-PUFA, docosahexaenoic acid and that the affect was abolished by  $\alpha$ -tocopherol (23). Despite these studies, it has been shown that some antioxidants such as vitamin E and  $\beta$  carotene may be able to function as prooxidants under certain conditions (24, 25) and  $\alpha$ -tocotrienol may therefore be oxidizing  $\alpha$ -ESA. Thus, although these studies support our findings that  $\alpha$ -ESA action may be dependent on an oxidation mechanism, it

will require additional work before we can definitively say that lipid peroxidation plays an important role in the anticancer actions of LC-PUFA.

The idea that oxidative stress could lead to anticancer effects may be initially counterintuitive because it is well-documented that in certain model systems, antioxidants have tumor inhibitory effects. However, it has been shown that reactive oxygen and nitrogen species can have multiple roles in the cell, i.e., low levels allow normal protective signaling, intermediate levels lead to cellular damage and tumorigenesis, and high levels can result in an oxidative burst and cell death (for review, see ref. 26). It is possible that  $\alpha$ -ESA inhibits breast cancer cell proliferation through a similar oxidation-dependent mechanism characteristic of an oxidative burst.

A second aspect of how  $\alpha$ -ESA may inhibit breast cancer growth is through activation of AMPK. Work with antidiabetic drugs supports this potential mechanism of action. For example, a chemical dubbed "exercise in a pill" by the media, aminoimidazole carboxamide ribonucleotide (AICAR), activates AMPK and is being investigated as an antidiabetic drug. AICAR administration increased running endurance by 44% in normally sedentary mice (27). In addition, the percent of white adipose tissue relative to body weight was significantly decreased in AICAR-treated mice compared with untreated controls. As mentioned above, body fatness has been implicated in the risk of postmenopausal breast cancer, and as such, a reduction in white adipose tissue as seen with AICAR or other AMPK-activating mechanisms may result in inhibition of breast cancer. Metformin is a first line treatment for type 2 diabetes that has been implicated as a potential antineoplastic agent for breast cancer (28). Metformin also functions in part by activation of AMPK. Previous studies found that stimulation of AMPK by metformin resulted in a significant repression of cell proliferation in both ER $\alpha$ -negative MDA-MB-231 and ER $\alpha$ -positive MCF-7 and T47D breast cancer cells (29). In addition, the World Cancer Research Fund/American Institute for Cancer Research report found that exercise, such as what AICAR may simulate, was linked to a probable decrease in breast cancer risk (30). It is possible that AICAR and similar biologically active compounds could decrease breast cancer risk by simulating exercise. Here, we found that inhibition of AMPK partially blocked the effects of  $\alpha$ -ESA (Fig. 6), suggesting that  $\alpha$ -ESA may be functioning in part through a mechanism similar to AICAR and metformin.

The question of how much  $\alpha$ -ESA would be required for *in vivo* anticancer actions is challenging to address because it has not yet been investigated in humans. However, 100  $\mu$ mol/L EPA was capable of inhibiting proliferation of multiple cell lines including the MDA-MB-231, T-47D, MCF-7, KPL-1, and MKL-F (31, 32). In addition, when human subjects with a history of colorectal adenomas were given EPA at 2 grams per day for 3 months, levels of EPA increased in normal colonic mucosa, and this was associated with significantly reduced proliferation and increased mucosal apoptosis (33). It is likely that  $\alpha$ -ESA will exert an even greater ability to inhibit breast cancer *in vivo* than EPA because the MDA-wt cells were inhibited 42% at 100  $\mu$ mol/L concentrations with EPA (31), which is considerably less than the 97% inhibition we found with 80  $\mu$ mol/L  $\alpha$ -ESA (Fig. 1A). In addition, previous work with  $\alpha$ -ESA has shown that a portion of the amount ingested does arrive at distant tumor sites (10). *In vivo* work with tung oil (70%  $\alpha$ -ESA) in a DLD-1 xenograft model illustrated that 1% of dietary intake was enough to have significant effects on tumor growth (10). Work with the rats that were treated with azoxymethane to induce colonic aberrant crypt foci using bitter melon seed extract (~60%  $\alpha$ -ESA) found that rats fed the extract had significantly fewer aberrant crypt foci compared with control rats at doses of 0.01%, 0.1%, and 1% of total caloric intake (18). Therefore, it should be possible to attain anti-breast cancer effects with  $\alpha$ -ESA, although more studies are clearly needed.

In summary, many studies indicate that the type of dietary fat may be pivotal for inhibition of breast cancer. Here, we have characterized how  $\alpha$ -ESA, a LC-PUFA, impacts breast cancer cell lines *in vitro*. This work should be useful first for defining preclinical studies and eventually in making dietary recommendations to women concerning their intake of  $\alpha$ -ESA, which humans cannot synthesize *de novo* and as such must be obtained from dietary sources. Although bitter melon extract is available commercially, the specific actions of  $\alpha$ -ESA on breast cancer have not been fully studied. The combination of known health benefits with an active market for supplements has led to many claims of health benefits for bitter melon extract but few scientifically proven actions. Our study has provided novel information concerning the use of  $\alpha$ -ESA in cancer inhibition.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Cancer Prevention Research

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