RESEARCH ARTICLE

Distinct roles of different forms of vitamin E in DHA-induced apoptosis in triple-negative breast cancer cells

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Scope: Docosahexaenoic acid (DHA) has been shown to exhibit anticancer actions in vitro and in vivo in a variety of cancers. Here, we investigated the role for DHA in inducing apoptosis in triple-negative breast cancer (TNBC) and studied the mechanisms of action.

Methods and results: DHA induces apoptosis as detected by Annexin V-FITC/PI assay as well as induces cleavage of caspase-8 and -9, endoplasmic reticulum stress (ERS), and elevated levels of death receptor-5 (DR5) protein expression as detected by western blot assays. Chemical inhibitors of caspase-8 and -9 and small interfering RNAs (siRNAs) show DHA to induce ERS/CHOP/DR5-mediated caspase-8 and -9 dependent apoptosis. Furthermore, DHA induces elevated cellular levels of reactive oxygen species (ROS) and antioxidant; RRR-α-tocopherol (αT) blocked DHA-induced apoptotic events. In contrast to the antagonistic impact of αT, gamma-tocotrienol (γT3) was demonstrated to cooperate with DHA in inducing apoptotic events in TNBC cells.

Conclusion: Data, for the first time, demonstrate that DHA induces apoptosis in TNBC cells via activation of ERS/CHOP/DR5-mediated caspase-8 and -9 dependent pro-apoptotic events, and that different forms of vitamin E exhibit distinct effects on DHA-induced apoptosis; namely, inhibition by αT and enhancement by γT3.

Keywords:
Apoptosis / Breast cancer / Docosahexaenoic acid / ER stress / Vitamin E

1 Introduction

Triple-negative breast cancer (TNBC) is defined by a lack of expression of both estrogen and progesterone receptors as well as lack of human epidermal growth factor receptor 2 (Her-2) [1], and it has a poor prognosis due to lack of specific targeting therapy. TNBCs comprise 15–20% human breast cancer in Western countries [2]. The only therapeutic option currently available for TNBC is chemotherapy. However, prognosis for TNBC remains poor due to drug resistance and toxicity [3].

Docosahexaenoic acid (DHA), a long-chain (22 carbon) polyunsaturated fatty acid (*n*-3 PUFA), is highly enriched in oils from cold-water fishes (salmon, herring, mackerel, and sardines) and widely used as a dietary supplement. DHA has a wide range of biological activities [4]. Its anti-inflammation and cardioprotection functions have been well documented [5, 6]. Data from cell culture and animal studies as well as human clinical trials suggest that DHA can reduce tumorigenesis and tumor development in a variety of cancers, including breast cancer [7]. DHA anticancer mechanisms include inhibition of proliferation, metastasis, and angiogenesis as well as induction of apoptosis [8]. In vitro data show DHA to induce apoptosis via multiple factors, including inhibition of biosynthesis of proinflammatory molecules such as Cyclooxygenase 2 (COX-2) [9], activation of peroxisome proliferator-activated

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Abbreviations: **CHOP**, C/EBP homologous protein; **Cox2**, Cyclooxygenase 2; **DHA**, docosahexaenoic acid; **DR5**, death receptor 5; **ERS**, endoplasmic reticulum stress; **GRP78**, glucose-regulated protein 78; **H2DCF-DA**, dichlorodihydrofluorescein diacetate; **NAC**, N-acetyl cysteine; **NF-κB**, nuclear factor-kappaB; **PARP**, poly (ADP-ribose) polymerase; **PPAR**, peroxisome proliferator-activated receptor; **ROS**, reactive oxygen species; **αT**, RRR-α-tocopherol; **γT3**, gamma-tocotrienol; **TNBC**, triple-negative breast cancer; **XBP-1**, X-box binding protein 1

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receptor (PPAR) [10], suppression of nuclear factor-kappaB (NF-κB) [11], induction of reactive oxygen species (ROS) [7], and endoplasmic reticulum stress (ERS) [12]. However, a complete understanding of the mechanisms whereby DHA induces apoptosis is not fully understood. Further insights into the signaling networks modulated by DHA may provide key insights into how DHA may be used for prevention and treatment of human breast cancer.

Vitamin E is a general term used to refer to a group of eight naturally occurring compounds known as tocopherols (α, β, γ, and δ) and tocotrienols (α, β, γ, and δ) and synthetic vitamin E (*all-rac*-α-tocopherol) as well as vitamin E analogs such as RRR-α-tocopherol (αT) ether-linked acetic acid analogue (α-TEA) [13]. Both αT and *all-rac*-α-tocopherol can be purchased as acetate or succinate forms in traditional vitamin E supplements and as food additives to protect lipids from pro-oxidation. Tocotrienol forms of vitamin E are found in palm oil, cereal grains, and rice bran [13]. To date, gamma-tocotrienol (γT3), delta-tocotrienol (δT3), and tocotrienol rich fraction (TRF) have been marketed as vitamin E supplements. Despite many research efforts, the anticancer functions of natural and synthetic vitamin E compounds remain to be clearly defined [14]. Preclinical studies suggest promise of a subset of vitamin E forms as anticancer agents; namely, gamma- and delta-forms of tocopherol and tocotrienol [14–16]. However, there is no evidence that vitamin E supplements (αT or *all–rac*-αTAc) conferred any protection against breast cancer [17]. Instead, recent findings suggest that high dosage vitamin E supplements enhance the incidence of prostate cancer [18]. Thus, it is important to distinguish among the different vitamin E forms as to their anticancer actions.

In this study, we studied the mechanisms of DHA action on inducing apoptosis in TNBC and evaluated the effects of vitamin E compounds; αT and γT3, on DHAs pro-apoptotic actions. Data show that DHA induces apoptosis in TNBC cells via activation of ERS-mediated C/EBP homologous protein (CHOP)/death receptor-5 (DR5), and that αT blocks and γT3 enhances DHA pro-apoptotic actions.

2 Materials and methods

2.1 Cell culture and reagents

MDA-MB-231, SUM159, and SUM149 human TNBC cell lines [19] were used in this study. MDA-MB-231 cells were cultured in MEM media with 10% FBS as described previously [20]. SUM159 and SUM149 human breast cancer cells (Asterand, Inc. Detroit, MI) were cultured in Ham's F12 medium (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum, 5 μg/mL insulin, 1 μg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), and 10 mM HEPES buffer. For experiments, FBS was reduced to 2% to all of media and cells were allowed to attach overnight before treatments. αT (TAMA Biochemical Company, LTD, Tokyo, Japan) and γT3 (a gift from Malaysian Palm Oil Board, Kuala Lumpur, Malaysia) were dissolved in 1:4 DMSO/ethanol at 40 mM as stock solution. Equivalent levels of 1:4 DMSO/ethanol were used as vehicle control (VEH). N-acetyl cysteine (NAC, Sigma) was dissolved in H_2O. Dichlorodihydrofluorescein diacetate (H2DCF-DA) was obtained from Invitrogen. Caspase-8 and -9 inhibitors: Z-IETD-FMK and Z-LEHD-FMK were obtained from Biovision (San Francisco, CA). DHA was purchased from Sigma-Aldrich.

2.2 Western blot analyses

Whole-cell protein extracts were prepared and examined by western blotting as described previously [20]. Proteins at 15–30 μg/lane were separated by SDS-PAGE and transferred to nitrocellulose (Optitran BA-S supported nitrocellulose, Schleicher and Schuell, Keene, NH). Antibodies to the following proteins were used: poly (ADP-ribose) polymerase (PARP), CHOP, and GRP-78 (Santa Cruz Biotechnology, Santa Cruz, CA), Caspase-8, Caspase-9, DR5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Beverly, MA). Horseradish peroxidase conjugated goat-anti-rabbit or goat-anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Rockford, IL) were used and followed by detection with ECL PLUS solution kit (PerkinElmer, Waltham, MA).

2.3 RT-PCR

mRNA levels of spliced X-box-binding protein 1 (XBP-1) was determined by RT-PCR following a previously published protocol [21]. Briefly, total RNA was extracted using RNA isolation kit (Qiagen Inc. Valencia, CA). Semi-quantitative analyses were conducted to detect spliced XBP-1 mRNA RT-PCR using the housekeeping gene β-actin as a loading control. Four microgram of total RNA was reverse transcribed to cDNA using 1 μL Superscript RTase (250 U, Invitrogen) following the manufacture's instructions. One microliter cDNA was used per PCR reaction with 15 μL Taq PCR Master Mix Kit (Qiagen Inc.) plus 10 μM oligonucleotide primer pairs (Invitrogen).

2.4 RNA interference

A scrambled RNA duplex that does not target any of the known genes was used as the nonspecific negative control for RNAi (referred to as control small interfering RNA [siRNA]). Transfection of MDA-MB-231 cells with siRNA to DR5 and CHOP (Ambion, Austin, TX) were performed in 100 mm cell culture dishes at a density of 1.5×10^6 cells/dish using Lipofectamine 2000 (Invitrogen) and siRNA duplex. After 1 day exposure to transfection conditions, the cells were recultured in 100 mm dish at 1.5×10^6 cells/dish and incubated for 1 day followed by treatments.

2.5 Quantification of apoptosis

Apoptosis was quantified by Annexin V-FITC/PI assays following the manufacturer's instructions (Invitrogen) and published procedure [21]. The Annexin V-FITC/PI assay measures amount of phosphatidylserine on the outer surface of the plasma membrane (a biochemical alteration unique to membranes of apoptotic cells) and amount of propidium iodide (PI), a DNA-binding dye that does not cross the plasma membrane of viable cells but readily enters dead cells or cells in the late stages of apoptosis. Fluorescence was measured using fluorescence activated cell sorter (FACS) analyses with a BD LSRFortessa (BD Biosciences, San Jose, CA, USA). Cells displaying phosphatidylserine on their surface (positive for Annexin-V fluorescence) were considered to be apoptotic. Annexin V/FITC was a gift from Dr. Shawn Bratton (University of Texas at Austin).

2.6 Detection of ROS

Intracellular ROS generation was detected by FACS analyses after staining the cells with H2DCF-DA, a cell-permeant ROS sensing probe for 30 min. H2DCF-DA is nonfluorescent until the acetate groups are removed by intracellular esterases and oxidation. Staining procedures followed the manufacturer's instructions and fluorescence was detected by a FACS Fortessa flow cytometer (BD Bioscience).

2.7 Statistical analyses

Apoptosis data were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of more than two treatments or a two-tailed Student *t*-test for comparison between two treatments to determine statistical differences. Differences were considered statistical significant at $p < 0.05$.

3 Results

3.1 DHA induces apoptosis in human TNBC cell lines

DHA has been reported to induce apoptosis in estrogen receptor positive, PR+, and Her-2 low-expressed MCF-7 cells; and estrogen receptor negative [22], Progesterone receptor (PR), and Her-2 overexpressed SK-BR-3 [22] human breast cancer cells [23]. Here, we evaluated the effect of DHA on inducing apoptosis in TNBC cells. Treatment of cells with DHA at physiologically relevant concentrations [24] for 24 h induced apoptosis in a dose-dependent manner as detected by Annexin V/PI analyses (Fig. 1A) and PARP cleavage, a biomarker for caspase-dependent apoptosis (Fig. 1B). It has been reported that DHA is incorporated into the cellular surface membrane within 5.2–17 min [25]. Western blot analyses reveal that DHA induces cleavage of both caspase-8 and -9 (Fig. 1B). Caspase-8 (Z-IETD-FMK) and -9 (Z-LEHD-FMK) inhibitors significantly reduced DHA-induced apoptosis as detected by Annexin V/PI analyses (Fig. 1C) demonstrating that DHA induces casapse-8 and -9 dependent apoptosis.

3.2 DHA induces ERS

DHA has been reported to induce ERS in human colon cancer cells [12]. To see if DHA induces ERS in TNBC cells, we determined the effect of DHA on the expression of ERS biomarkers. Treatment of cells with increasing doses of DHA for 1-day induced elevated levels of ERS markers Glucose-regulated protein 78 (GRP78) and CHOP protein expression as detected by western blot analyses (Fig. 2A) as well as elevated levels of spliced XBP-1 mRNA expression as detected by RT-PCR (Fig. 2B), indicating that DHA induces ERS in TNBC cells. γT3, a known ERS inducer [15, 26, 27], was used as positive control in this study (Fig. 2A).

3.3 CHOP is involved in DHA-induced apoptosis

Since CHOP has been reported to be involved in ERS-mediated apoptosis, we examined if CHOP is involved in DHA-induced apoptosis. Knockdown of CHOP using siRNA significantly reduced the ability of DHA to induce apoptosis as detected by Annexin V assay (Fig. 2C) and cleaved PARP as detected by western blotting (Fig. 2D), suggesting that DHA induces apoptosis via ERS-mediated CHOP.

3.4 DR5 is upregulated and involved in DHA-induced apoptosis via CHOP

Since several studies have shown that protein expression of the death receptor for DR5 is increased following ERS and elevation of CHOP protein expression in induction of apoptosis by different agents, such as α-TEA, γT3, and synthetic triterpenoid methyl-2-cyano-3,12-dioxoolean-1,9-dien-28-oate (CDDO-Me) [15, 21, 28], we investigated if DR5 is regulated by DHA and involved in DHA-induced apoptosis. Treatment of cells with increasing doses of DHA for 1-day induced elevated levels of DR5 protein expression (Fig. 3A). γT3 was used as positive control in this study (Fig. 3A). Knockdown of DR5 using siRNA resulted in a significant decrease in DHA-induced apoptosis as detected by Annexin V analyses (Fig. 3B) and cleaved PARP as detected by western blot analyses (Fig. 3C), indicating that DHA induces DR5-dependent apoptosis. Furthermore, data show that knockdown of CHOP using siRNA blocked the ability of DHA to upregulate DR5 (Fig. 3C), demonstrating that DHA upregulation of DR5 is CHOP dependent.

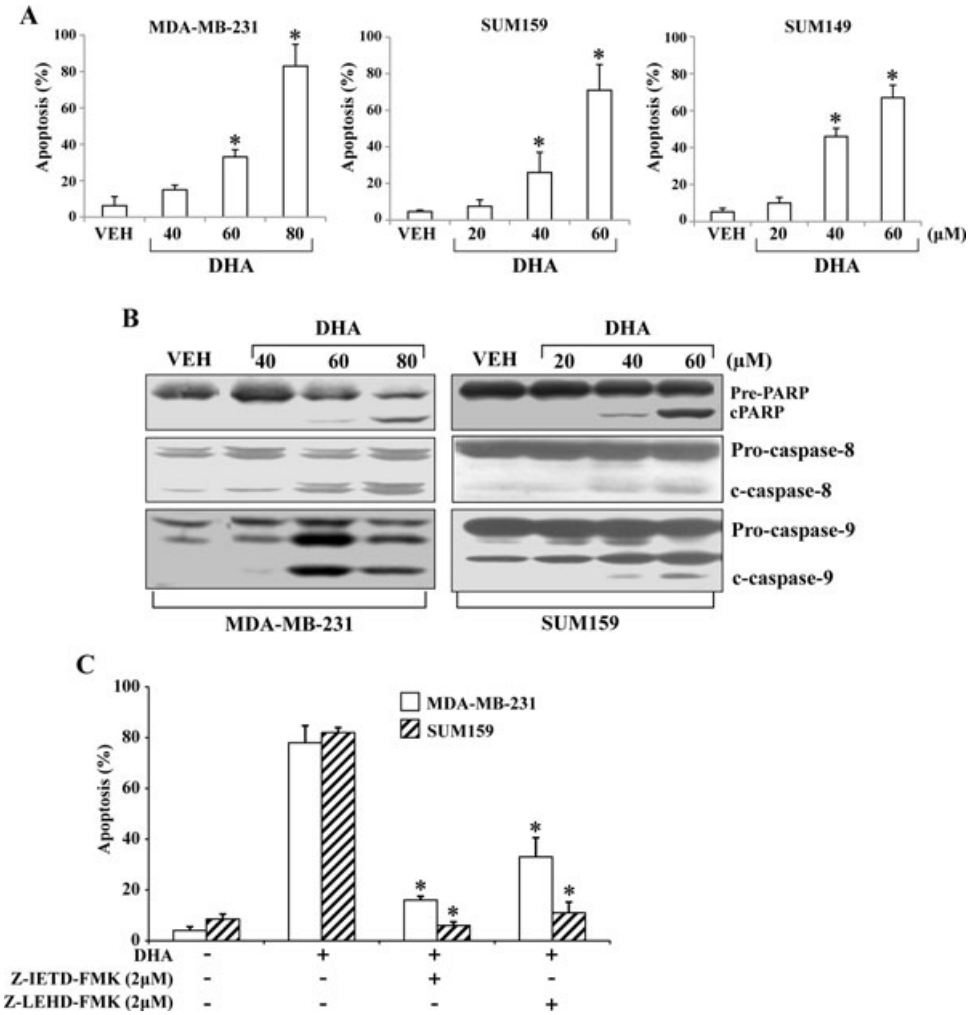

Figure 1. DHA induces apoptosis in TNBC cells. TNBC cells were treated with DHA at different concentrations for 1 day followed by Annexin V/PI analyses to determine percentage of cells undergoing apoptosis (A) and western blot to determine protein levels of biomarkers of apoptosis; cleaved PARP (pre-PARP: full-length PARP and cPARP: cleaved PARP) (B). TNBC cells pretreated with Z-IETD-FMK (caspase-8 inhibitor) or Z-LEHD-FMK (casapse-9 inhibitor) for 3 h followed by treatment of the cells with DHA at 60 μM and 80 μM for SUM 159 and MDA-MB-231 cells, respectively, for 1 day were analyzed for apoptosis using Annexin V/PI (C). Data in (A) and (C) are depicted as mean ± SD of three individual experiments. Data in (B) represents two separate experiments. *Statistically different from vehicle control (A) or DHA treatment alone (C), $p < 0.05$.

3.5 Antioxidants block DHA-induced apoptotic events

Since ROS plays an important role in DHA-induced apoptosis [4] and ROS can trigger ERS [22], it was of interest to see if ROS is involved in DHA-induced apoptotic events in TNBC cells. Thus, we evaluated the effect of antioxidants; fat-soluble αT and water-soluble NAC, on DHA-induced apoptotic events. Pretreatment of MDA-MB-231 cells with αT at different concentrations for 2 h followed by DHA treatment for 1 day significantly reduced the ability of DHA to induce apoptosis in a dose-dependent manner as detected by Annexin V (Fig. 4A). Furthermore, pretreatment of MDA-MB-231 and SUM159 cells with αT at 20 μM or NAC at 10 mM for 2 h followed by DHA treatment for 1 day significantly reduced the ability of DHA to induce apoptosis as detected by Annexin V (Fig. 4B and C) and PARP cleavage as detected by western blot analyses (Fig. 4D and E). Although treatment of MDA-MB-231 cells with αT showed an increased percentage of apoptotic cells (Fig. 4C), it is not a statistically significant difference from vehicle control. Both antioxidants markedly reduced the ERS-associated biomarkers GRP78 and CHOP, as well as DR5 (L/S) protein expression (Fig. 4D and E). Although western blot data show that DR5 protein level is increased following αT treatment, it is not different from the VEH control based on densitometric analyses performed using GAPDH levels to normalize lane loads. These data demonstrate that ROS is an upstream mediator in DHA-induced apoptotic events. Importantly, these data indicate that both water-soluble and fat-soluble antioxidants are antagonistic to DHAs anticancer pro-apoptotic properties.

3.6 γT3 and DHA work cooperatively to induce apoptotic events

Since previous data show that γT3 induces apoptosis via activation of ERS-mediated CHOP/DR5 pro-apoptotic signaling events in MDA-MB-231 and MCF-7 human breast cancer cells [15], it was of interest to see the effect of this form of vitamin E on DHA-induced apoptosis. γT3 induced apoptosis in both

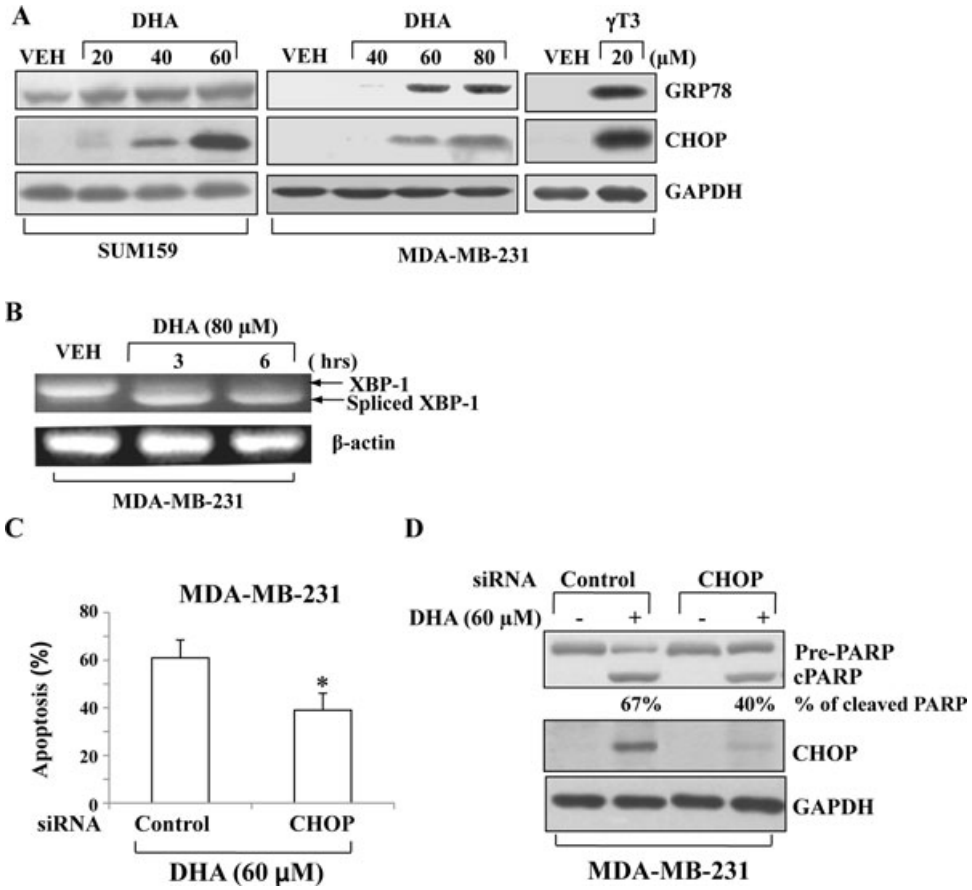

Figure 2. DHA induces endoplasmic reticulum stress (ERS). Cells were treated with DHA at different concentrations for 1 day followed by western blotting (A) or by RT-PCR (B) to determine presence of ERS-associated markers (B). MDA-MB-231 cells treated with γT3 for 1 day were used as positive control for ERS markers (A). Cells transfected with siRNAs to CHOP or control were treated with DHA for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (C) or western blot analyses to determine protein levels (D). Data in (A), (B), and (D) are representative of at least two separate experiments. Data in (C) are depicted as mean ± SD of three individual experiments. Pre-PARP: full-length PARP and cPARP: cleaved PARP. The percent of cleaved PARP (cPARP) is determined by densitometry analyses using Scion Image Software (Scion Corporation, Frederick, MD) and calculated using the formula [% of cleaved PARP = cPARP/(pre-PARP + cPARP) × 100%]. *Statistically different from control siRNA treated with DHA, $p < 0.05$.

cell lines in a dose-dependent manner at levels of 10 μM and above (Fig. 5A and B). Thus, when cells were cotreated with subapoptotic doses of γT3 (namely, treated with a level of γT3 that does not induce apoptosis within 1 day) plus DHA for 1 day to evaluate cooperative apoptotic effects, data show that the combination of γT3 plus DHA acts cooperatively to induce apoptosis as detected by Annexin V (Fig. 5C and D) and cleaved caspases 8, 9, and PARP (Fig. 5E). Furthermore, data in Fig. 5F and G show that γT3 at different concentrations enhanced DHA-induced apoptosis in both cell lines in comparison with γT3 and DHA alone. Moreover, data from western blot analyses show that combination of DHA plus γT3 enhanced ERS as detected by ERS biomarkers, GRP78 and CHOP, and ERS associated DR5 (L/S) protein expression (Fig. 6A). siRNAs to CHOP or DR5 reduced the combination pro-apoptotic effect of DHA plus γT3 (Fig. 6B), and siRNA to CHOP reduced combination (DHA + γT3) upregulation of DR5 protein expression (Fig. 6C), demonstrating that ERS-mediated CHOP/DR5 pro-apoptotic pathway is involved in apoptosis induced by the DHA plus γT3 combination.

3.7 The effects of αT and γT3 on DHA induction of ROS

Since αT and γT3 exhibited completely opposite effects on DHA-induced ROS-dependent apoptosis, it was of interest to determine the impact of γT3 and αT on DHA induction of ROS. Treatment of both cell lines with DHA for 3 h induced elevated levels of ROS (Fig. 7A). In contrast, treatment of both cell lines with γT3 for 3 h had no effect on ROS induction (Fig. 7B). Treatment of cells simultaneously with αT plus DHA for 3 h reduced ROS induction in comparison with DHA alone (Fig. 7C). While treatment of cells with γT3 plus DHA for 3 h showed neither enhancement nor diminution of DHA-induced ROS generation (Fig. 7D). These data demonstrated that; (i) γT3 is not acting as either a pro-oxidant or antioxidant in DHA-induced apoptosis, (ii) αT is acting as an antioxidant capable of blocking DHAs induction of ROS, which is critical for its pro-apoptotic effects on TNBC cells, and (iii) γT3 enhances DHA-induced apoptosis via a ROS-independent mechanism.

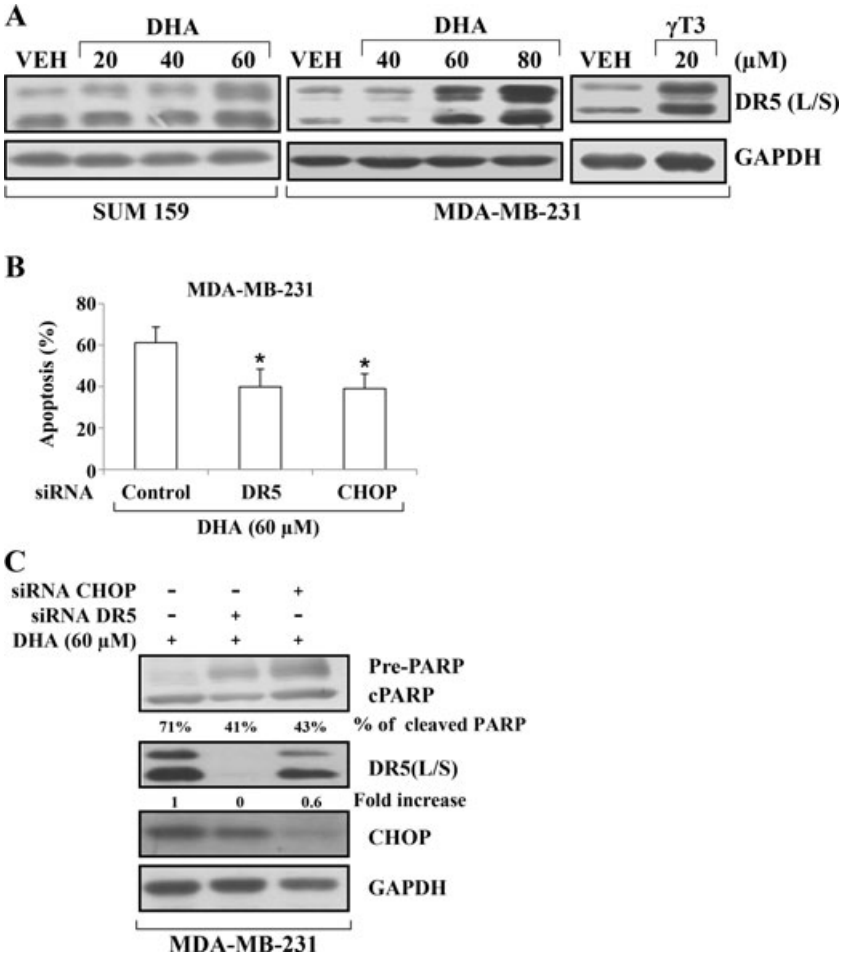

Figure 3. DR5 is involved in DHA-induced apoptosis and is regulated by CHOP. MDA-MB-231 and SUM159 cells were treated with DHA in different concentrations for 1 day followed by western blot analyses to determine protein levels (A). MDA-MB-231 cells treated with γT3 for 1 day were used as positive control for DR5 (A). MDA-MB-231 cells transfected with siRNAs to DR5, CHOP, or control were treated with DHA 60 μM for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (B) or western blot analyses to determine protein levels (C). Pre-PARP: full-length PARP and cPARP: cleaved PARP. The percent of cleaved PARP (cPARP) is determined by densitometry analyses using Scion Image Software (Scion Corporation) and calculated using the formula [% of cleaved PARP = cPARP/(pre-PARP + cPARP) × 100%]. DR5(L/S) refers to long and short forms of DR5. Fold increase of DR5 protein expression is determined by densitometry analyses using image software and normalized by GAPDH with the value for control as 1. Data in (A) and (C) are representative of at least two separate experiments. Data in (B) are depicted as mean ± SD of three individual experiments. *Statistically different from control siRNA treated with DHA, $p < 0.05$.

4 Discussion

DHA, a dietary component with known anti-inflammatory and cardioprotective benefits, is a widely used supplement. Data show that DHA at physiologically relevant levels induces apoptosis in human breast cancer cells (Fig. 1A) [23], and DHA did not induce apoptosis in normal human mammary epithelial cells (data not shown), using the same conditions as described in Fig. 1A. These data indicate that DHA exhibits a selective anticancer property and hold as a nontoxic promising anticancer agent. The novel findings in this study are: (i) DHA induces apoptosis in TNBC cells via activation of ERS-mediated CHOP/DR5 pro-apoptotic events involving both caspase-8 and -9, (ii) ROS is a necessary upstream mediator of DHA-induced apoptosis, (iii) vitamin E in the form of αT blocks DHA-induced ROS formation and apoptotic events, indicating a potential antagonistic effect of supplemental αT on DHAs anticancer action, and (iv) γT3, a form of vitamin E that exhibits anticancer activity, enhances DHA-induced apoptotic events. Taken together, our data, for the first time, demonstrate that ERS-mediated CHOP/DR5 pro-apoptotic signaling events are involved in DHA-induced apoptosis, and that two different vitamin E forms have the potential to either block or enhance the anticancer actions of DHA.

Targeting the homeostatic status of the endoplasmic reticulum has been reported to be an effective way to trigger cancer cells to undergo apoptosis [29]. Several natural compounds have been reported to induce apoptosis via induction of ERS, such as curcumin [30], γT3 [15], and epigallocatechin gallate (EGCG) [31]. DHA has been reported to induce ERS in colon cancer cells [12]. However, whether or not ERS is critical for DHA-induced apoptosis has not been addressed. CHOP, as a transcription factor for promoting DR5 transcription [32, 33], has been reported to be a critical mediator in ERS-mediated apoptosis, in part, via increased levels of DR5 [15, 21, 32, 33]. Here, for the first time, we report that DHA induces ERS in TNBC cells and ERS-mediated increased expression of CHOP is involved in DHA-induced apoptosis via upregulation of DR5, leading to activation of death receptor dependent caspase-8 and -9 apoptotic cascades.

DHA is the longest, most unsaturated, and hence, most oxidizable fatty acid commonly found in nature [34]. Its prooxidant property is one of the mechanisms of DHA action in its ability to induce apoptosis [7]. However, how ROS mediates DHA-induced apoptosis has not been identified. ROS has

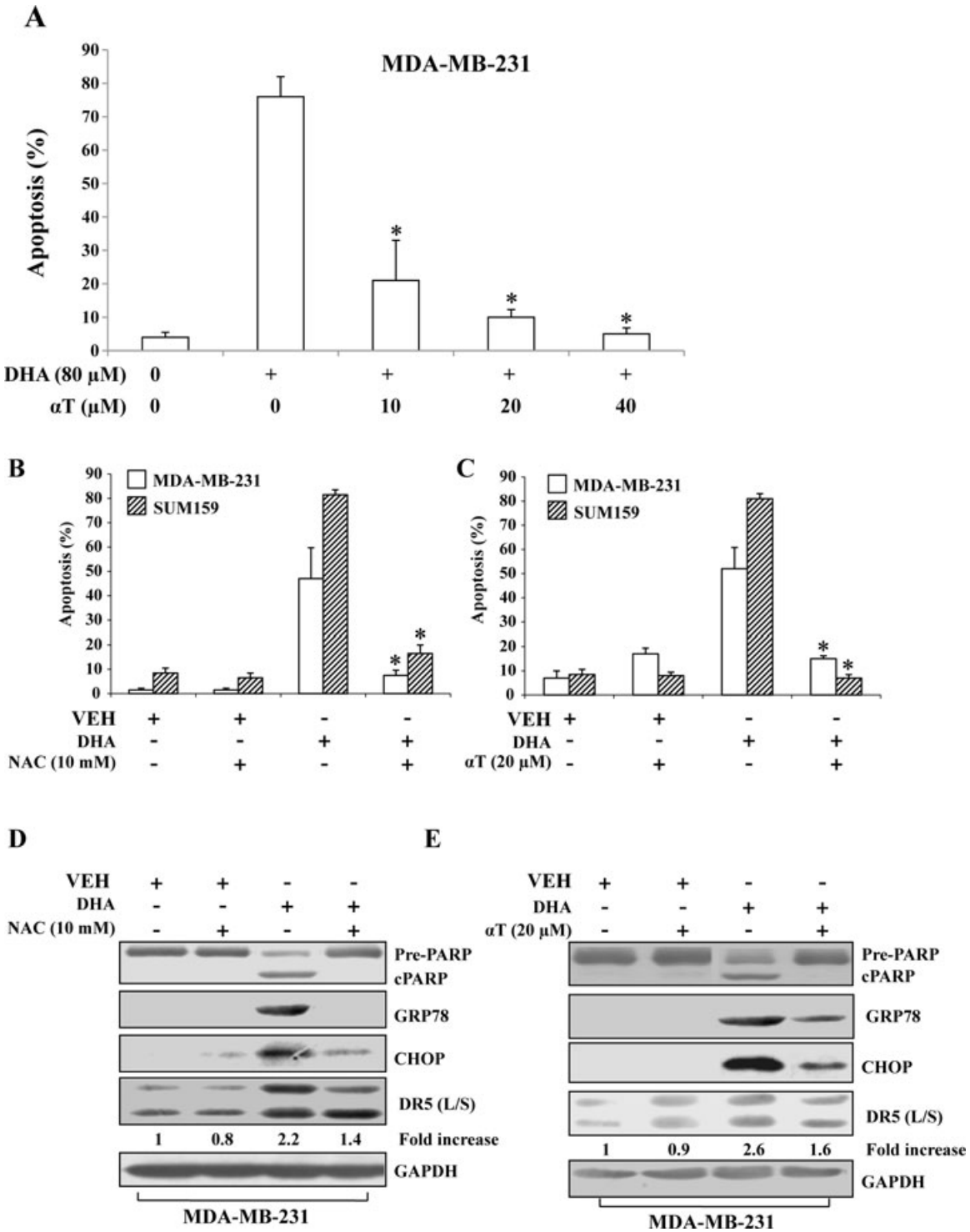

Figure 4. Antioxidants block DHA-induced apoptotic events. Pretreatment of MBA-MB-231 cells with αT at indicated concentrations for 2 h followed by DHA treatment for 1 day significantly reduced the ability of DHA to induce apoptosis as detected by Annexin V/PI analyses (A). MDA-MB-231 and SUM159 cells pretreated with αT or NAC for 2 h were treated with DHA 80 μM and 60μM, respectively, for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (B and C) and western blot analyses to determine protein levels (D and E). Pre-PARP: full-length PARP and cPARP: cleaved PARP. DR5(L/S) refers to long and short forms of DR5. Fold increase of DR5 protein expression is determined by densitometry analyses using Scion Image Software (Scion Corporation) and normalized by GAPDH with the value for control as 1. Data in (A), (B), and (C) are depicted as mean ± SD of three individual experiments. Data in (D) and (E) are from two separate experiments. *Statistically different from DHA treatment alone, $p < 0.05$.

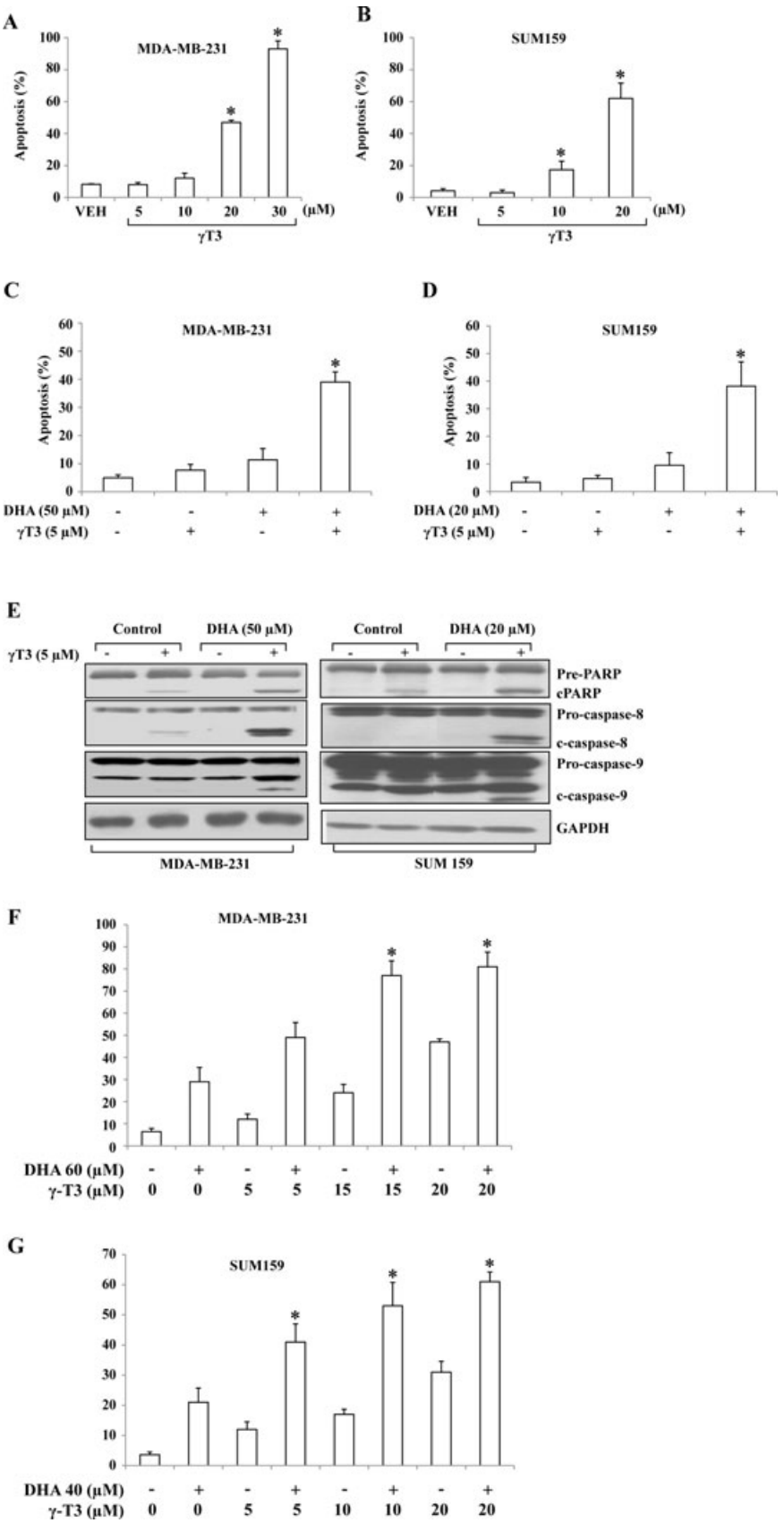

Figure 5. γT3 and DHA work cooperatively to induce apoptosis. MDA-MB-231 and SUM159 cells were treated with different doses of γT3 as indicated for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (A and B). The cells were treated with subapoptotic doses of γT3 and DHA for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (C and D) or western blot analyses to determine protein levels (E). MDA-MB-231 and SUM159 cells were cotreated with different doses of γT3 plus apoptotic doses of DHA as indicated for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (F and G). Pre-PARP: full-length PARP and cPARP: cleaved PARP. Data in (A), (B), (C), (D), (F), and (G) are depicted as mean ± SD of three individual experiments. Data in (E) are representative of data generated in at least two separate experiments. *Statistically different from vehicle control (A and B) or from single treatments (C, D, F, and G), $p < 0.05$.

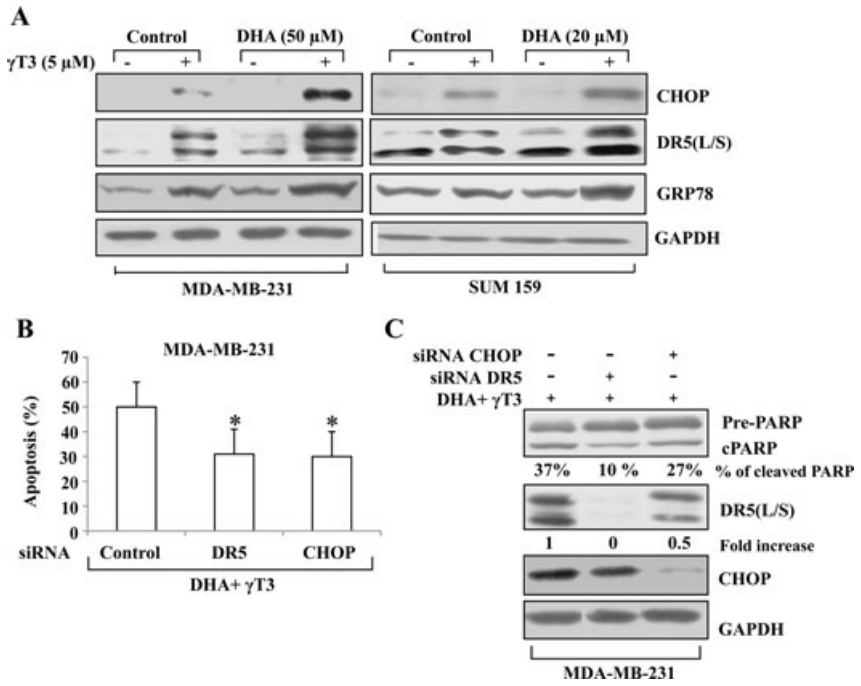

Figure 6. ERS is involved in γT3 plus DHA induced apoptosis. MDA-MB-231 and SUM159 cells were cotreated with γT3 plus DHA for 1 day followed by western blot analyses to determine the ERS marker protein expression (A). Cells transfected with siRNAs to DR5, CHOP, or control were treated with a combination of γT3 plus DHA for 1 day followed by Annexin V/PI analyses to determine apoptosis induction (B) and western blot analyses to determine protein levels (C). Pre-PARP: full-length PARP and cPARP: cleaved PARP. The percent of cleaved PARP(cPARP) is determined by densitometry analyses using image software and calculated using the formula [% of cleaved PARP = cPARP/(pre-PARP + cPARP) × 100]. DR5(L/S) refers to long and short forms of DR5. Fold increase of DR5 protein expression is determined by densitometry analyses using image software and normalized by GAPDH. Data in (B) are depicted as mean ± SD of three individual experiments. Data in (A) and (C) are representative of data generated in at least two separate experiments. *Statistically different from control siRNA transfected cells treated with γT3 plus DHA, $p < 0.05$.

been reported to trigger ERS, leading to apoptosis [35]. Here, for the first time, we report that ROS mediates DHA-induced apoptosis via upregulation of ERS-mediated CHOP/DR5 in TNBC cells. Evidence supporting this conclusion include: (i) DHA induces increased levels of ROS, (ii) antioxidants αT and NAC blocked the DHAs ability to induce apoptosis and increase levels of ERS biomarkers GRP78 and CHOP, and DR5, and (iii) αT blocked DHAs ability to increase levels of ROS.

An additional important finding in this study is that different vitamin E forms exhibit opposite roles in DHA-induced apoptosis. αT functions as an antioxidant and directly antagonizes DHAs anticancer actions. In striking contrast, γT3 exhibits neither pro-oxidant nor antioxidant properties, and exhibits a marked beneficial combination effect on DHAs pro-apoptotic actions. These findings further support the notion that different forms of vitamin E possess distinctly different anticancer properties, and second, they provide important insights into how DHAs application in the clinic for cancer prevention or treatment will need to take into account other dietary influences. Further studies will be required to ascertain if limitation of αT consumed from supplements and food sources, and/or supplement with γT3 impacts DHAs anticancer efficacy.

It appears that the pro- or antioxidant properties of vitamin E depend on not only the form of vitamin E, but also the cell type and experimental conditions. For example, Kannappan et al. reported that γT3 induced upregulation of DR5 via ROS in human colon cancer cells [36], and Nowak et al. reported that γT3 protected against oxidant injury in renal proximal tubular cell [37]. While our data show that γT3 exhibited neither pro- nor antioxidant properties in TNBC (Fig. 7B), even when apoptotic doses were used (up to 20 μM, data not shown).

How γT3 cooperates with DHA to induce apoptosis is not entirely clear. Data showing that γT3 did not enhance DHA-induced ROS (Fig. 7D), rules out the possibility that γT3 enhances DHA-induced apoptosis via pro-oxidation impact. Recently, we have studied the role of ceramide in γT3-induced ERS-mediated apoptosis. Data show that γT3 induces elevated intracellular levels of ceramide and that the chemical inhibitors of de novo ceramide synthesis pathways were capable of blocking γT3-induced apoptosis and ERS-mediated CHOP/DR5 pro-apoptotic events (unpublished data), indicating that ceramide generated from de novo ceramide synthesis pathway are involved in γT3-induced apoptosis. Since both ceramide generated from de novo ceramide synthesis pathway [38] and ROS can trigger ERS [35], it is possible that γT3 cooperates with DHA to induce apoptosis via a combination of increased ROS and increased ceramide. Further studies will be needed to address this possibility. Based on the data present here and our unpublished studies, a schematic diagram of proposed signaling events in DHA-induced apoptosis of TNBC and the distinct role of αT and γT3 on DHA pro-apoptotic actions are depicted in Fig. 8.

In summary, our data show that DHA induces apoptosis in TNBC cells via activation of ERS-mediated CHOP/DR5 pro-apoptotic events, in which ROS is an upstream mediator. αT, acting as a classic antioxidant, blocks DHA pro-apoptotic action, while γT3 enhances DHA pro-apoptotic action via a ROS-independent mechanism. These studies not only provide novel insights into a better understanding of the

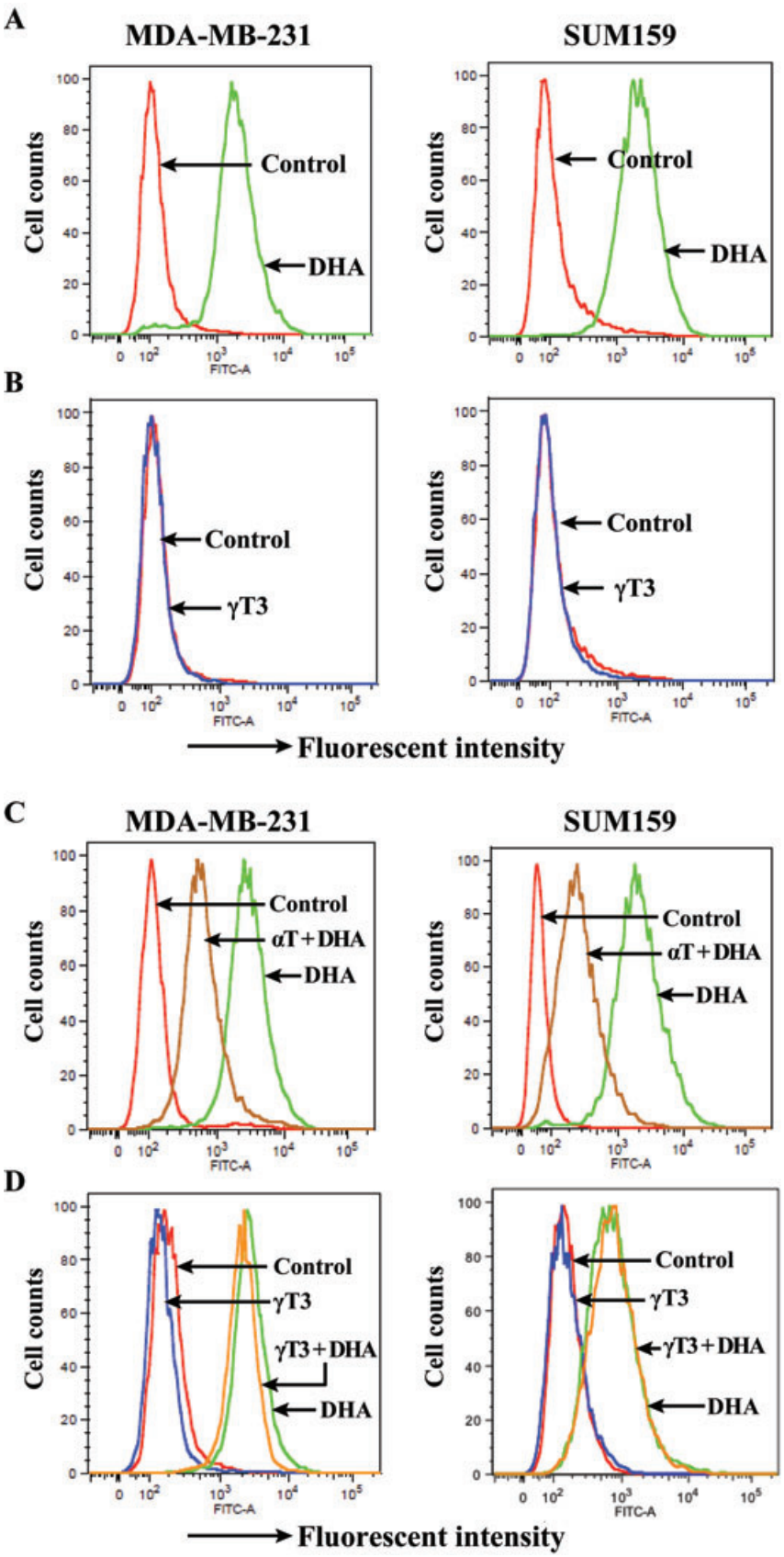

Figure 7. DHA induces ROS. MDA-MB-231 and SUM159 cells were treated with DHA at 60 and 40 μM, respectively (A), γT3 at 5 μM (B), αT at 20 μM + DHA at 60 and 40 μM, respectively (C), and γT3 at 5 μM + DHA 50μM and 20μM, respectively (D) for 3 h. ROS intensity was determined by FACS analyses after staining the cells with special ROS indicator dye H2DCF-DA.

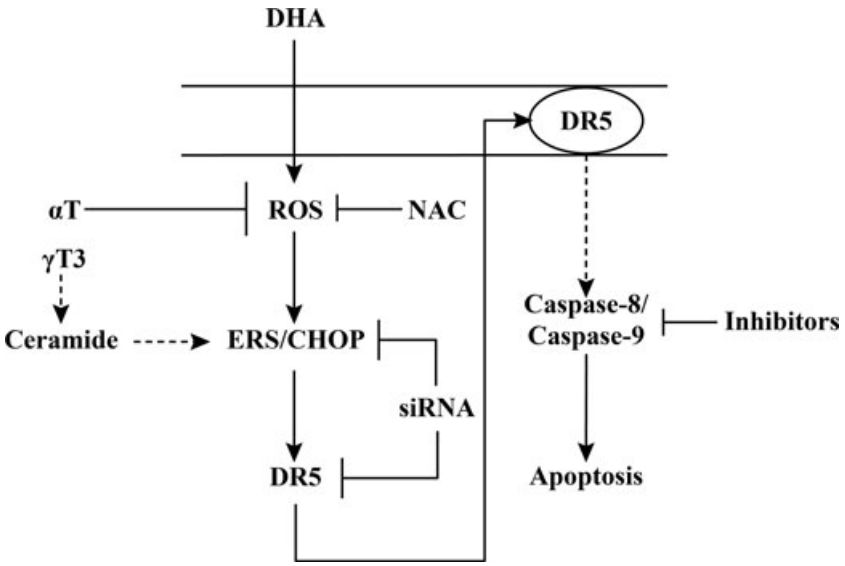

Figure 8. Proposed signaling events in DHA-induced apoptosis of TNBC and the role of αT and γT3 on DHA pro-apoptotic actions. Based on data presented here and other data generated by our lab (unpublished data), we propose the following signaling events in DHA-induced apoptosis and the role of αT and γT3 in DHA pro-apoptotic actions: (i) DHA alone induces elevated levels of ROS, leading to induction of ERS, (ii) DHA upregulates DR5 protein expression via ERS-mediated CHOP, (iii) DHA upregulation of DR5 triggers caspase-8 and -9 dependent apoptotic cascade, (iv) αT blocks DHAs ability to induce ROS thus blocking DHAs pro-apoptotic actions via its antioxidant property, and (v) γT3 cooperates with DHA to produce ERS and downstream pro-apoptotic actions. Unbroken arrows indicate data generated in the present study; broken arrows indicate postulated signaling pathways.

mechanisms underlining DHA-induced apoptosis but also provide *in vitro* evidence to support the use of γT3, and avoidance of αT, to improve DHA anticancer actions. These data provide a sound rationale for further *in vivo* study.

A. X., W. Y., B. G. S., and K. K. conceived and designed the study, analyzed the data, and contributed to the writing of the manuscript. A. X. conducted all the experiments and R. T. conducted western blot data in Fig. 6A.

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Potential conflict of interest statement:

U.S. and International patents on α-TEA, an anticancer vitamin E analog, are held by the Research Development Foundation. B. G. S., K. K., and W. Y. are listed as inventors. Authors have no conflict of interests with the vitamin E forms used in this study.

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