Docosahexaenoic acid monoglyceride induces apoptosis and autophagy in breast cancer cells via lipid peroxidation-mediated endoplasmic reticulum stress

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Abstract

Epidemiologic and pre-clinical studies have shown that marine n-3 polyunsaturated fatty acids (n-3 PUFAs) elicit promising chemoprevention against breast cancer. Docosahexaenoic acid monoglyceride (MAG-DHA), a docosahexaenoic acid sn-1-monoacylglycerol does not require pancreatic lipase to be absorbed, eliciting a better bioavailability when compared with other formulations such as DHA-free fatty acid, DHA-triglycerol, or DHA-ethyl ester. However, the anti-cancer actions and underlying mechanisms of MAG-DHA on breast cancer remain to be assessed. In this study, MAG-DHA induced significant growth inhibition in MCF-7 and MDA-MB-231 breast cancer cells in a dose-dependent manner. MAG-DHA treatment (80µM) led to 83.8% and 94.3% growth inhibition between MCF-7 and MDA-MB-231 cells, respectively. MAG-DHA-induced growth inhibition was tightly associated with apoptosis, as evidenced by increased active forms of caspase-3, poly (ADP-ribose) polymerase (PARP) and caspase-12. In particular, MAG-DHA-induced apoptosis was triggered by oxidative stress-mediated endoplasmic reticulum (ER) stress, as evidenced by activation of the PERK-eIF2α pathway in ER. MAG-DHA treatment also strongly suppressed the growth of E0771 murine breast cancer xenografts, significant differences of tumor volume were found between MAG-DHA group (0.271 cm³) and control group (0.875cm³) after 15 daily MAG-DHA treatments. The *in vitro* anti-breast cancer mechanism of MAG-DHA was supported by the *in vivo* xenograft model. In addition, MAG-DHA-induced ER stress concomitantly triggered autophagy in these cancer cells, and the induction of autophagy suppressed its ability to induce apoptotic cell death. Our data suggested that MAG-DHA as dietary supplement, in combination with autophagy inhibitors may be a useful therapeutic strategy in treating breast cancer.

Introduction

Breast cancer is by far the most frequently diagnosed cancer among women across the globe, with over 2 million new cases estimated in 2018. It also ranks as the leading cause of cancer-related mortality in females worldwide, leading to over 500,000 deaths per year worldwide ([Waks and Winer 2019](#)). Despite recent advances in both diagnosis and treatment, drug resistance and the development of deadly metastases of node-negative or positive breast cancer patients continue to represent a significant clinical challenge ([Harbeck and Gnant 2017](#)). Therefore, preventive strategies, novel interventions and therapeutic approaches are urgently needed.

Recently, a large body of literature has indicated that dietary interventions might be able to reduce breast cancer risk ([Anjom-Shoae and others 2020](#); [Al-Jawadi and others 2018](#)). Among the dietary factors, n-3 polyunsaturated fatty acids (n-3 PUFAs), especially docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (EPA, 20:5, n-3), naturally present in cold-water fish as well as in fish oil supplements have gained attention as possible candidates for breast cancer prevention. To date, n-3 PUFA supplements are present in foods as triacylglycerol (TAG) or in small proportions as monoacylglycerol, diacylglycerol (DAG), or phospholipid formulation ([James and M. 2015](#)). There is evidence that the influences of the chemical structures on fatty acids bioavailability have been characterized. Studies have shown that MAG-DHA, a DHA sn-1-monoacylglycerol does not require

pancreatic lipase to be absorbed, unlike DHA-triglyceride which needs to be hydrolyzed by sn-1,3-specific gastric and (colipase-dependent) pancreatic lipases as free fatty acids and monoglycerol prior to intestinal absorption. Therefore, this property confers increased absorption, and thus a better bioavailability when compared with other formulations such as DHA-triglycerol (TAG-DHA), or DHA-ethyl ester (EE-DHA) (Fortin 21 February, 2012; Fortin 17 July, 2012). Cruz-Hernandez et al. demonstrated that an enriched sn-1(3)-MAG oil was a better carrier for ω -3 long-chain PUFAs (LC-PUFAs) compared with TAG from fish oil (Cruz-Hernandez and others 2016). It was also found that MAG-DHA induced powerful inflammation resolution in various models of pulmonary diseases (Morin 2015; Khaddaj-Mallat and others 2015). In addition, recent studies have shown that MAG-DHA displayed anti-tumorigenic effects in colorectal cancer and lung cancer (Morin and others 2017; Morin 2013). However, the anti-breast cancer activity of MAG-DHA and its underlying mechanisms remain elusive. The specific objective was to evaluate the effect of MAG-DHA on tumor growth and its underlying molecular mechanisms using an *in vitro* model of breast cancer cell line MCF-7 and MDA-MB-231 and an *in vivo* mouse model of E0771 xenograft.

Materials And Methods

2.1 Cell culture and cell viability assay

The murine E0771 cells were obtained from Roswell Park Cancer Institute (New York, USA). Normal breast cell MCF10A, human cancer cell lines MCF-7 (estrogen and progesterone receptors positive ER+/PR+) and MDA-MB-231 (estrogen and progesterone receptors negative ER-/PR-) were obtained from American Type Culture Collection (Manassas, VA, USA). E0771, MCF-7 and MDA-MB-231 cells were maintained in RPMI 1640, Dulbecco's Modified Eagle's Medium (DMEM) and L-15 medium supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin, respectively. MCF10A cells were cultured in DMEM-F12 supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 10 μ g/mL insulin, and 0.5 μ g/mL hydrocortisone. For the testing of MAG-DHA (NU-CHEK, USA) on cell viability change, the MTT assay was used. MCF10A, MCF-7 and MDA-MB-231 cells were treated with MAG-DHA complexes to BSA in a 4:1 (MAG-DHA/BSA) molar ratio at 20, 40, 60, 80 and 100 μ M or vehicle (DMSO) for 24, 48 and 72 h in medium containing 0.5% FBS. MTT reagent (at 5 mg/ml) was added to each well at final concentration 0.5 mg/ml and the plate was incubated at 37°C. After 4 h, supernatant was carefully removed and DMSO (100 μ l) was added. The absorbance was read with a microplate reader (Bio-Rad, China) at 450 nm. The relative cell viability was expressed as a percentage of the control well.

2.2 Lipid peroxidation assay

The content of MDA in cultured cells or tumor tissues was determined by MDA kit. The tests were performed according to the manufacturer's instructions (Biyotime Biotechnology, Jiangsu, China). Briefly, MCF10A, MCF-7 and MDA-MB-231 cells (1×10^6) were treated with 40 μ M MAG-DHA alone or in the co-

presence of 5 mM NAC for 48 h. Cells were collected and protein concentrations were measured. 0.2 ml of 0.091% thiobarbituric acid (TBA) reagent was added to the cell suspension (0.1 ml), and the samples were heated in boiling water for 20 min. After cooling to room temperature, the samples were centrifuged at 15,000 rpm for 10 min at 4°C. The TBA-reactive substances (RS) produced by lipid peroxidation was measured in the supernatant with a microplate reader (Bio-Rad, China) at 532 nm. The results were expressed as MDA μ M/g protein.

1. Measurements of intracellular ROS

Intracellular ROS was detected by 2,7-dichlorofluorescein diacetate reagent (DCFH-DA, MCE, China) according to the manufacturer's instructions. Briefly, MCF10A, MCF7 and MDA-MB-231 cells were seeded in confocal Petri dishes, then after treatment, cells were incubated with DCFH-DA (25 μ M) for 1 h at 37 °C and washed three times with PBS. Then, real-time fluorescent of samples was read by microscopy. Image J software was used for fluorescence signal analysis.

1. Autophagic flux measurement

To monitor autophagic flux, MCF7 and MDA-MB-231 cells were transfected with an adenovirus of tandem fluorescent mRFP-GFP-LC3B (a vital marker for autophagosomes and autolysosomes) according to the manufacturer's instructions (Hanheng biology company., Ltd, Shanghai, China). After treatment, cells were washed and monitored over time for EGFP and m-Cherry under a confocal fluorescence microscope (Leica DM 2500).

2.5 Tumor xenograft model

Murine E0771 xenografts were established in 8-week-old female C57/BL6 mice. 20 female C57/BL6 mice at 8 weeks of age were obtained from Shanghai SLAC Experimental Animal CO., Ltd (Shanghai, China) and used according to the approved protocol by the Animal Care and Use Committee of the Ningbo University (Ningbo, China). The mice were housed under aseptic conditions in standard cages in temperature- and humidity-controlled conditions with a 12 h light/dark cycle. Cultured murine E0771 cells were collected, washed twice with serum-free DMEM and counted. Each mouse received a subcutaneous injection of 2×10^6 viable cells suspended in 100 μ l of serum-free RPMI 1640 medium on day zero. All injections were given to anesthetized animals. After the formation of 100 mm³ tumors, mice were randomly assigned into 2 groups, control (untreated) and MAG-DHA-treated. Mice were administered by MAG-DHA intragastrically daily at a pharmacological dose of 617 mg/kg/day corresponding to a daily n-3 PUFA dose of 3 g/day for a 60 kg person, following dose conversion of human to mouse by the equation described by Reagan-Shaw (Reagan-Shaw and others 2007). Tumor size was measured with a caliper every 3 days, and calculated according to the following formula: tumor volume = length $\times$

width²×0.5. Mice were euthanized on week 4 and tumor tissues were removed and stored at -80°C until tissue processing.

2.6 Transmission electron microscopy (TEM) analysis for cell apoptosis and autophagy

Tumor tissues were fixed in 4% paraformaldehyde and post-fixed 2% osmium tetroxide before embedding in EPON resin for morphological studies. Ultrathin sections (100 nm on 200-mesh grids) embedded in epoxy resin were double stained with uranyl acetate and lead citrate, and observed with Philips CM10 TEM.

2.7 Tissue and plasma analysis of DHA

DHA levels of tissues were determined by gas chromatography, as described previously ([Bellenger and others 2004](#)). In brief, tissues were homogenized by grinding it in liquid nitrogen. Total lipids of tumor tissues were extracted using the chloroform: methanol mixture (2:1, v/v), dried under a stream of nitrogen gas and then transmethylated by heating in 14% boron trifluoride in methanol at 80°C for 20 min. Fatty acid methyl esters were then extracted in presence of 2 ml isooctane and separated by Shimadzu GC-14C gas chromatograph system (Shimadzu Corporation, Japan) equipped with a flame-ionization detector (FID) and a capillary column (DB-23, Aglient Corporation, USA). Fatty acids peaks were identified by their relative retention times comparing with those of commercial fatty acid methyl ester (NU-CHEK, USA). DHA level was expressed as a relative percentage of total analyzed fatty acids according to its peak area.

2.8 Western blotting analysis

Tumor tissues were lysed in Triton x-100 lysis buffer (20 mM Tris-HCl, pH7.6, 1 mM EDTA, 140 mM NaCl, 1% NonidetP-40, 1% aprotinin, 1mM phenylmethylsulfonyl fluoride, and 1 mM sodium vanadate). Lysates were sonicated and centrifuged at 15000 g for 10 minutes at 4 °C. Protein concentrations were determined using bicinchoninic acid (BCA) protein assay kit purchased from Biyuntian Biotech (Haimen, China). Supernatants were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in milk and incubated at 4 °C overnight with primary antibodies followed by incubation with secondary antibodies for 1 h at room temperature. Detection was performed by enhanced chemiluminescence. The primary antibodies rabbit anti-NF-E2-related factor 2 (Nrf2), rabbit anti-Heme Oxygenase 1(HO-1), rabbit anti-protein kinase R-like ER kinase (PERK), rabbit anti-phospho (p)-PERK (Thr980), rabbit anti-phospho (p)-eIF2α (Ser51), rabbit anti-eIF2α, rabbit anti-C/EBP homologous protein (CHOP), rabbit anti-cleaved caspase-12, rabbit anti-caspase-12, rabbit anti-cleaved caspase-3, rabbit anti-caspase-3, rabbit anti-cleaved PARP, rabbit anti-PARP, rabbit anti-LC3BI/II, rabbit anti-p62, rabbit anti-

Beclin-1, mouse anti- β -actin and the horseradish peroxidase-linked secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.9 Transfection of siRNAs

To study the role of Beclin-1 in MAG-DHA-induced growth inhibition of breast cancer cells, Beclin 1 specific siRNAs and the control siRNAs were transiently transfected to MCF-7 and MDA-MB-231 cells using Dharma-FECT-1 (DBA Dharmacon, Lafayette, CO), according to manufacturers' recommendation. The target sequences for Beclin-1-specific siRNAs were 5'-UGGAAUGGAAUGAGAUUAATT-3' and 5'-GCUCAGUAUCAGAGAGAAUTT-3' (GeneBank accession number [NM003766.2](#)). The efficacy of the siRNA knockdown of Beclin-1 protein expression was determined by western blotting analysis.

2.10 Statistical analysis

The results were presented as the mean $\pm$ SEM and statistical differences were evaluated by one-way ANOVA followed by Newman-Keuls test. $P < 0.05$ was considered to indicate statistical significance.

Results

3.1 MAG-DHA induced lipid peroxidation-mediated endoplasmic reticulum (ER) stress in cultured breast cancer cells

First, we examined the effects of MAG-DHA on proliferation of human normal breast cell MCF10A, human breast cancer cell MCF-7 and MDA-MB-231. Cell viability assays were performed after 24, 48 and 72 h of treatment of MAG-DHA at increasing concentrations of 20, 40, 60, 80 and 100 μ M. MCF10A cells were not sensitive to MAG-DHA treatment, cell viability was 87% upon treatment of 80 μ M MAG-DHA for 72 h (Fig. 1A, Supplementary Fig. 1). However, MAG-DHA was found to significantly reduced the viability (based on MTT assay) of MCF-7 and MDA-MB-231 cells in a concentration and time-dependent manner (Fig. 1A, Supplementary Fig. 1). Compared with MCF-7 cells, MDA-MB-231 cells were more sensitive to MAG-DHA (Fig. 1A). Cell viability rate of MDA-MB-231 was 5.7%, compared with 16.2% in MCF-7 cells upon treatment of 80 μ M MAG-DHA for 72 h (Fig. 1A). The IC₅₀ of MAG-DHA treatment was 5.4 μ M, and 29.3 μ M in MDA-MB-231 and MCF-7 cells for 72 h, respectively. The IC₅₀ of MAG-DHA treatment was 68.6 μ M (24h), 33.1 μ M (48 h) and 83.2 μ M (24h), 67.2 μ M (48 h) in MDA-MB-231 and MCF-7 cells, respectively. It was worth noting that the anti-proliferative effects of MAG-DHA in both breast cancer cells were significantly counteracted by N-acetyl-L-cysteine (NAC), a well-known antioxidant. Upon treatment of 80 μ M MAG-DHA for 72 h in the presence of NAC, cell viability rate reached to 69.7% and 82.1% in MDA-MB-231 and MCF-7, respectively (Fig. 1A, Supplementary Fig. 1). Since the unsaturated nature of MAG-DHA with 6 double bonds renders its structure more prone to free radicals by triggering lipid peroxidation, we therefore focused on examining intracellular ROS and lipid peroxidation to investigate the cellular

mechanism by which MAG-DHA induced anti-proliferative actions in cultured cells. As shown in Fig. 1B&C, MAG-DHA treatment significantly increased ROS production in MCF-7 and MDA-MB-231 cells, but not in MCF10A cells, ROS levels increased 9 fold and 10 fold in MCF-7 and MDA-MB-231 cells, respectively. NAC could markedly suppress ROS production induced by MAG-DHA. Similarly, MAG-DHA treatment also significantly increased MDA levels in MCF-7 and MDA-MB-231 cells, but not in MCF10A cells (Fig. 1D). MDA levels increased from 0.32 and 0.29 $\mu\text{mol/g}$ to 3.77 and 3.45 $\mu\text{mol/g}$ in MCF-7 and MDA-MB-231 cells, respectively. Co-treatment of cells with NAC and MAG-DHA markedly suppressed intracellular MDA accumulation, MDA levels decreased to 0.52 and 0.34 $\mu\text{mol/g}$ in MCF-7 and MDA-MB-231 cells, respectively. Next, we determine whether MAG-DHA-induced lipid peroxidation could activate the antioxidant defense system. As shown in Fig. 1E&F, Nrf2 and HO-1, two crucial components of cellular oxidative stress response were significantly up-regulated in MCF-7 and MDA-MB-231 cells treated with MAG-DHA. These results suggest the antioxidant defense system was activated in response to MAG-DHA-mediated lipid peroxidation. In addition, Co-treatment of cells with NAC and MAG-DHA also markedly inactivated the antioxidant defense system (Fig. 1E&F). It is widely accepted that oxidative stress is linked with ER stress ([Bhandary and others 2012](#)), we therefore wondered whether MAG-DHA-induced lipid peroxidation led to ER stress. As expected, we found that the protein levels of phosphorylated PERK, a major transducer of ER stress and phosphorylated eIF2 α , a downstream target of PERK were significantly increased in MCF-7 and MDA-MB-231 cells treated with MAG-DHA, although the total amount of PERK and eIF2 α did not change (Fig. 2A&B). Simultaneously, we also found that CHOP, an ER stress-inducible transcription factors associated with cell cycle arrest and apoptosis was significantly up-regulated in MCF-7 and MDA-MB-231 cells treated with MAG-DHA (Fig. 2A&B). Co-treatment of cell with NAC markedly reversed lipid peroxidation-induced ER-stress (Fig. 2A&B). In addition, the involvement of ER stress in MAG-DHA-induced growth inhibition was further confirmed in both cells co-treated with 4-PBA, an ER stress inhibitor and MAG-DHA. As shown in Fig. 2C, MAG-DHA-induced growth inhibition was significantly counteracted by 4-PBA in both cells. Cell viability rate increased from 35.2% and 12.7% to 79.0% and 64.2% in MCF-7 and MDA-MB-231 cells treated with 40 μM MAG-DHA alone or co-treated with 4-PBA, respectively.

3.2 MAG-DHA-induced ER stress resulted in cell apoptosis and autophagy in cultured breast cancer cells

Next, in order to investigate whether MAG-DHA-induced ER stress triggered apoptosis induction, we evaluated the typical hallmarks of cells undergoing apoptosis in both breast cancer cells treated with MAG-DHA. We found that the active forms (cleaved forms) of caspase-12, a apoptosis protein specifically activated upon ER stress, cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP), two key mediators of apoptosis were significantly increased in both cells treated with MAG-DHA, and co-treatment of cells with 4-PBA prevented MAG-DHA-induced apoptosis pathway activation (Fig. 2D&E). In addition, MAG-DHA treatment significantly induced cell morphology changes in MCF-7 and MDA-MB-231 cells, and Co-treatment of cells with NAC markedly reversed this process (Supplementary Fig. 2). Evidence is accumulating that interesting functional links exist between ER stress and autophagy ([Ogata and others 2006](#); [Yorimitsu. T and others 2006](#)). Microtubule associated protein 1 light-chain 3B (LC3B- β) is a specific membrane marker for the detection of early autophagosome formation, and p62/SQSTM1 is

degraded upon autophagy induction. Beclin-1, the first mammalian autophagy protein, plays a key role in autophagy initiation. To determine whether MAG-DHA-induced ER stress also activated autophagy, the protein expression of the autophagy markers LC3B- β / α , p62/SQSTM1 and Beclin-1 was measured with immunoblotting analyses. As shown in Fig. 3A&B, immunoblotting analyses demonstrated increased LC3B- β and Beclin-1 levels concomitantly with decreased levels of p62 in both cells treated with MAG-DHA. Simultaneously, MAG-DHA-induced autophagy was prevented by co-treatment of cells with 4-PBA. In addition, we also investigated the effect of autophagy in MAG-DHA-mediated growth inhibition using Beclin-1 specific siRNAs (Fig. 3C). Genetic silencing of Beclin-1 expression (siBeclin-1 1 and siBeclin-1 2) induced slight growth inhibition of 4.13% and 8.74% in MCF-7 cells, respectively. Similarly, siBeclin-1 1 and siBeclin-1 2 induced growth inhibition of 6.87% and 6.04% in MDA-MB-231 cells, respectively (Fig. 3D). Importantly, cell growth inhibition increased from 34.0% to 56.7% and 63.0% in MCF-7 cells co-treated with 40 μ M MAG-DHA and siBeclin-1 1 or siBeclin-1 2, respectively. Simultaneously, it was also observed that growth inhibition increased from 48.3% to 79.0% and 87.4 % in MDA-MB-231 cells co-treated with 40 μ M MAG-DHA and siBeclin-1 1 or siBeclin-1 2, respectively (Fig. 3D). Finally, we investigated the effect of autophagy in MAG-DHA-mediated growth inhibition using autophagy inhibitor chloroquine (CQ). As shown in Fig. 3E, CQ induced 8.5% and 5.2% growth inhibition in MCF-7 and MDA-MB-231 cells, respectively. Co-treatment with MAG-DHA and CQ induced growth inhibition (59.7% and 79.5%), compared with growth inhibition (38.0% and 51.4%) in MCF-7 and MDA-MB-231 cells treated with MAG-DHA alone, respectively (Fig. 3E), suggesting pharmacologic inhibition of autophagy by CQ enhanced MAG-DHA-induced growth inhibition. In addition, in order to determine the fusion efficiency of autophagosome with lysosome, a pH-sensitive, double tagged mCherry-GFP-LC3B reporter which gives rise to yellow fluorescence in autophagosomes and turns red in acidic autolysosomes because of eGFP quenching was used. As shown in Fig. 3F&G, accumulated autolysosomes (red fluorescence) and few autophagosomes (yellow fluorescence) were observed with MAG-DHA treatment in MCF-7 and MDA-MB-231 cells. Co-treatment of cells with CQ induced accumulated autophagosomes in both cells. Our data showed that suggesting that MAG-DHA may be involved in regulating autophagy initiation and promoting autophagic flux process. These results demonstrated that ER stress-triggered autophagy suppressed MAG-DHA-induced apoptotic cell death.

3.3 MAG-DHA inhibited the growth of E0771 xenografts

To evaluate the *in vivo* anti-proliferative efficacy of MAG-DHA on breast cancer, we implanted murine E0771 breast cancer cells into mice and examined the tumorigenicity of inoculated tumor cells. As shown in Fig. 4B, at day 11, the mean tumor volume is $0.086 \pm 0.017 \text{ cm}^3$. Significant differences of tumor volume were found between MAG-DHA group ($0.271 \pm 0.059 \text{ cm}^3$) and control group ($0.875 \pm 0.125 \text{ cm}^3$) after 15 daily MAG-DHA treatments. Representative macroscopic images showed MAG-DHA treatment significantly suppressed tumor growth and reduced the size of tumor xenografts in MAG-DHA-treated group compared to control group (Fig. 4A).

3.4 The *in vitro* anti-breast cancer mechanism was supported by the *in vivo* xenograft model

To determine whether the mechanism of MAG-DHA-induced cell death observed *in vitro* also can be seen *in vivo*, we evaluated the typical hallmarks of cells undergoing apoptosis in tumor tissues with immunoblotting analyses. As shown in Fig. 5A, we found that cleaved caspase-12, cleaved caspase-3 and cleaved PARP were significantly increased in tumor tissues in MAG-DHA group compared with control group. We further evaluated the ultrastructure changes of breast cancer cells from tumor tissues with transmission electron microscopy (TEM). As shown in Fig. 5B, chromatin marginalization, one of classical morphological characteristics of apoptosis was observed in tumor tissues from MAG-DHA group compared with control group. In addition, autophagy markers LC3B- β and Beclin-1 protein levels were significantly enhanced, whereas p62 expression was significantly suppressed in MAG-DHA group (Fig. 5C). The induction of autophagy by MAG-DHA treatment was confirmed by TEM analysis, as evidenced by substantial accumulation of autophagosomes with cytoplasmic organelles and other vesicles encapsulated in vacuoles in tumor tissues of MAG-DHA group (Figure. 5D). Next, Analysis of the total lipids extracted from plasma, tumor, liver and muscle tissues showed DHA was significantly elevated in these serum and tissue samples. The DHA level was $4.11 \pm 0.67\%$ in tumor tissues from MAG-DHA group compared with control group ($0.82 \pm 0.19\%$) (Fig. 6A). Also, increased MDA accumulation was observed in tumor tissues from MAG-DHA group ($0.86 \pm 0.13 \mu\text{mol/g}$) compared with control group ($0.17 \pm 0.02 \mu\text{mol/g}$) (Fig. 6B), which activated the antioxidant defense system (Fig. 6C). Furthermore, MAG-DHA-induced lipid peroxidation led to ER stress in MAG-DHA group, as evidenced by increased expression of phospho-PERK, phospho-eIF2 α and CHOP (Fig. 6D). Our data demonstrated that the proposed anticancer mechanisms of MAG-DHA based on *in vitro* cell model are confirmed by *in vivo* studies.

Discussion

Although most dietary lipids in foods were constituted of TAGs, however, TAGs are required to be hydrolyzed into free fatty acids and sn-2- MAG by lipolytic enzymes, mainly sn-1,3 specific pancreatic lipases in the upper part of the gastrointestinal tract in the digest process (Yen and Farese 2003), whereas MAGs are lipase independent and considered as being in a predigested form. Recent studies have found that MAG containing EPA/DHA exhibits a high degree of efficient absorption, especially under impaired absorption conditions compared to conventional dietary TAG (Caroline and others 2018; Cuenoud and others 2020). Therefore, n-3 PUFA in the MAG predigested form may offer a clinical advantage to patients struggling with lipid malabsorption/maldigestion problems. MAG is classified as non-ionic surfactants due to its composition and accounts for about 75 % of the world production of emulsifiers and stabilizers for food purposes (Ferreira-Dias and others 2001). In addition, MAG containing n-3 PUFAs is expected to have variety of application possibilities, such as incorporation into functional foods and dietary supplements, or as ingredients in pharmaceuticals, enzymatic catalysis has been used to produce purer, less degraded n-3 PUFA MAG to meet increasing market demand due to its mild reaction conditions compared to conventional chemical processes in food industry (Bornscheuer 1995). In the present study, MAG-DHA was used to study the anti-breast cancer activity and its underlying mechanisms. It was found that MAG-DHA induced lipid peroxidation-mediated ER stress, which triggered apoptosis and autophagy in cultured MCF7 and MDA-MB-231 breast cancer cells as well as in E0771 xenografts. Notably, MAG-

DHA-induced autophagy suppressed its ability to induce apoptotic cell death. In addition, we also evaluated the cytotoxicity of MGA-DHA to human normal liver cell L-02 and rat pheochromocytoma cell line PC-12. It was found that MAG-DHA slightly promoted the growth of L-02 and PC-12 in medium with 10% FBS (Supplementary Fig. 3), suggesting that MAG-DHA is not hepatotoxic and neurotoxic, and safe as dietary supplement for prevention of breast cancer.

In E0771 xenografts, it is of interest to note that DHA level was increased approximately 4.5 fold with MAG-DHA intervention (Fig. 6A). It is known that the enrichment of DHA with high unsaturation in membranes makes cancer cells more sensitive to free radicals because it makes the membrane less rigid and more vulnerable (Hishikawa and others 2017). Therefore, in the present study, the cell death induced by MAG-DHA was largely mediated by increased lipid peroxidation in cancer cells. The crucial role of lipid peroxidation in MAG-DHA-induced death of breast cancer cells was supported by the following observations: (1) Increased intracellular MDA accumulation in cultured breast cancer cells and in tumor samples in MAG-DHA group, respectively. In addition, MAG-DHA-induced lipid peroxidation also activated antioxidant defense system as evidenced by increased expression of Nrf2 and HO-1, two crucial components of cellular anti-oxidative stress response (Loboda and others 2016). (2) The cell cytotoxicity, increased MDA levels and up-regulated expressions of Nrf2 and HO-1 induced by MAG-DHA in breast cancer cells were effectively reversed by the antioxidant NAC (Fig. 1A-D). In agreement, Jing et al. demonstrated that DHA-induced cell death of breast cancer cells is associated with increased reactive oxygen species, which activates Nrf2/HO-1 signaling pathway (Tsai and others 2017). Also, van Beelen et al. reported that the hydrolysed algal oil and fish oil showed similar toxicity on colon cancer cells via oxidative stress (Beelen and others 2007).

Oxidized lipids was found to trigger ER stress in rat aortic smooth muscle cells (Haberzettl and Hill 2013). PERK, a three transmembrane protein induced the unfolded protein response upon ER stress. Activated PERK phosphorylates eIF2 α , which results in repression of global protein synthesis to restore cell homeostasis. However, elevated levels of phosphorylated eIF2 α also lead to increased translation of selected mRNAs including the transcription factor ATF4, which promotes expression of CHOP, a transcription factor involved in cell apoptosis (Cao and Kaufman 2014). In the present study, in MAG-DHA-treated breast cancer cells or in tumor tissues from MGA-DHA group, we found significant increases in PERK and eIF2 α phosphorylation levels as well as changes in CHOP expression. Furthermore, the use of antioxidant NAC significantly reversed MAG-DHA-induced ER stress in two breast cancer cells, suggesting that MAG-DHA-induced lipid peroxidation results in ER stress in breast cancer cells. In line with our results, Jakobsen et al. found that DHA induced cell cycle arrest by ER stress in human colon cancer SW620 cells (Jakobsen and others 2008). Similarly, Pettersen et al. also reported that DHA triggered oxidative-stress-mediated ER stress in human colon cancer SW620 cells (Pettersen and others 2016). However, several *in vitro* and *in vivo* studies reported that DHA provides neuroprotection against ER stress (Begum and others 2012; Begum and others 2014). This discrepancy may largely be explained by the cell-specific differences in ER stress response. Taken together, the results of the present study suggest that lipid peroxidation-induced ER stress is involved in the anti-breast cancer effects of MAG-DHA.

Many studies have reported that n-3 PUFAs induced apoptosis by triggering the intrinsic mitochondrial and ER pathways(Fukui and others 2013; Liu and Ma 2014). In the present study, by using western blotting and TEM assay, we confirmed that MAG-DHA induced apoptosis in the *in vivo* tumor xenografts. It has been reported that ER stress has been found to activate caspase-3 apoptotic signaling via caspase-12 (Faitova and others 2006).Our results showed that increased expression of active form of caspase-12, caspase-3 and PARP was also observed in MAG-DHA-treated breast cancer cells as well as in tumor tissues from MAG-DHA group, and the use of 4-PBA protected the cells from MAG-DHA-induced cell death, further confirming the involvement of ER stress in MAG-DHA-induced apoptotic cell death. It is speculated that MAG-DHA may preferentially increase ROS in or near the plasma membrane lipid rafts, and trigger lipid peroxidation-mediated ER stress, which leads to cell apoptosis. At the same time, we could not exclude the involvement of mitochondrial pathway in MAG-DHA-induced apoptosis. In line with our results, Connolly et al. reported that dietary DHA, the sole n-3 PUFA found in golden algae oil, depressed the growth of mammary carcinoma in athymic mice through apoptosis induction(Connolly and others 1999).

Autophagy is known as a catabolic process that removes damaged cellular components to maintain cellular integrity and stability under certain conditions, such as in response to nutrient deprivation, organelle damage, or other stresses (Glick and others 2010). However, excessive or prolonged autophagy may also induce cell death (Platini and others 2010). In this study, we found that MAG-DHA not only induced apoptosis but also induces autophagy as evidenced by increased autophagosome formation in the *in vivo* tumor xenografts. In line with our results, n-3 PUFAs have been found to induce cell autophagy in human pancreatic cancer cells, human multiple myeloma cells and breast cancer cells (Chen and others 2014; Eliseo and others 2017; Fukui and others 2013; Niso-Santano and others 2015).In addition, recent investigations have revealed that ER stress can either stimulate or inhibit autophagy (Rashid and others 2015; Senft and Ronai 2015). In this study, it was found that MAG-DHA-induced ER stress triggered autophagy and protected breast cancer cells from MAG-induced apoptotic cell death, suggesting that a combination of MAG-DHA with autophagy inhibitor may provide a useful strategy in increasing the therapeutic efficacy in breast cancer.

Conclusion

As summarized in Fig. 7, our results demonstrated that MAG-DHA exerted its anti-proliferative effects in cultured breast cancer cells and in *in vivo* tumor xenografts. The prevention of MAG-DHA against breast cancer was strongly associated with lipid peroxidation-mediated ER stress, which induced cell apoptosis and autophagy. Whereas MAG-DHA induced autophagy activation suppressed apoptotic cell death. Our study also emphasizes the ongoing need for additional preclinical studies investigating the molecular targets of MAG-DHA in breast cancer cells as well as the urgent need for new clinical studies evaluating the potential role of dietary MAG-DHA supplementation mainly in combination with autophagy inhibitors, chemo-and radio-therapeutic anticancer regimens to improve clinical outcome and survival of breast cancer patients.

Declarations

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Competing interests

All authors declare no competing interest.

Authors' contributions

All authors read and approved the final manuscript. Zu-quan Zou, Jin-jie Zhang and Wen-ge Yang designed overall research experiments. Tian-tian Wang, Yong Yang and Feng Wang performed the experiments and data analysis. Yong Yang collected and analyzed references. Tian-tian Wang wrote the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

MAG-DHA-induced apoptotic cell death was associated with lipid peroxidation. (A) MCF10A, MCF-7 and MDA-MB-231 cells were treated with MAG-DHA in indicated concentrations alone or in the co-presence of 5mMN-acetyl-L-cysteine (NAC) for 72 h, and cell viability was analyzed using MTT assay. (B&C) MCF10A, MCF-7 and MDA-MB-231 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 5 mM NAC for 48 h, and cellular ROS was stained with DCFH-DA and analysed with fluorescent microscopy. (D) MCF10A, MCF-7 and MDA-MB-231 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 5mM NAC for 48 h, and the content of MDA was determined by MDA kit. (E&F) MCF-7 and MDA-MB-231 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 5mM NAC, and the protein expression of oxidative stress response marker Nrf2 and HO-1 was monitored by western blotting analysis. Results are presented as the mean ± SEM from three independent experiments, * P < 0.05; ** P < 0.01 by Newman-Keuls test.

Figure 2

MAG-DHA-mediated lipid peroxidation induced ER stress and cell apoptosis. (A&B) MCF-7 and MDA-MB-231 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 5 mM NAC, and the protein expression of the ER stress markers such as p-PERK, PERK, p-eiF2α, eiF2α and CHOP (G&H) was monitored by western blotting analysis. (C) MCF-7 and MDA-MB-231 cells were treated with MAG-DHA (40μM) alone or in the co-presence of 2.5 mM 4-PBA (an ER stress inhibitor) for 72 h, and cell viability was analyzed using MTT assay. (D&E) MCF-7 and MDA-MB-231 cells were treated with 40 μM MAG-DHA alone or in the co-presence of 2.5 mM 4-PBA, and the protein expression of the apoptotic markers such as cleaved caspase-12, caspase-12, cleaved caspase-3, caspase-3, cleaved PARP and PARP was measured. Results are presented as the mean ± SEM from three independent experiments, * P < 0.05; ** P < 0.01 by Newman-Keuls test.

Figure 3

MAG-DHA-induced ER stress triggered cell autophagy. (A&B) MCF7 and MDA-MB-231 cells were treated with 40 μ M MAG-DHA alone or in the co-presence of 2.5 mM 4-PBA, and the protein expression of the autophagic markers such as LC3B-I/II, p62 and Beclin-1 was monitored by western blotting analysis. (C) Protein expression levels of Beclin-1 after siRNA treatment against Beclin-1 in MCF7 and MDA-MB-231 cells. (D) siScramble and Beclin-1 siRNA-treated MCF7 and MDA-MB-231 cells were treated with MAG-DHA (40 μ M) for 48 h. and cell viability was analyzed using the MTT assay. (E) MCF7 and MDA-MB-231 cells were treated with MAG-DHA (40 μ M) alone or in the co-presence of 5 μ M chloroquine (CQ) for 48 h, and cell viability was analyzed using MTT assay. (F&G) MCF7 and MDA-MB-231 cells were transfected with an adenovirus of tandem fluorescent mRFP-GFP-LC3B. Cells were treated with MAG-DHA (40 μ M) alone or in the co-presence of 5 μ M chloroquine (CQ) for 48 h, and fluorescence was read by confocal microscope. Results are presented as the mean $\pm$ SEM from three independent experiments, * P < 0.05; ** P < 0.01 by Newman-Keuls test.

Figure 4

Effects of MAG-DHA on the growth of E0771 cell xenografts. (A) Representative tumor photographs showing tumor formation at 28 days after cell implantation. (B) E0771 cells (2×10^6 cells/100 μ l serum-free RPMI 1640) were subcutaneously injected into C57/BL6 mice as described in the Materials and Methods section. Tumor size was measured every 3 days, and tumor volume was calculated on the basis of the following formula: tumor volume = (length $\times$ width $\times$ 2) $\times$ 0.5. The points represent mean tumor volume $\pm$

SEM obtained from 10 control(Ctrl) mice or 10 MAG-DHA-treated animals.* P < 0.05; ** P< 0.01 compared to the corresponding control.

Figure 5

MAG-DHA intervention induced apoptosis and autophagy in the E0771 cell xenografts. (A) The apoptotic markers such as cleaved caspase-12, caspase-12, cleaved caspase-3, caspase-3, cleaved PARP and PARP were monitored by western blotting analysis in tumor tissues from control and MAG-DHA group. (B) The tumor tissues were fixed and analyzed for apoptosis induction using the transmission electron microscopy (TEM). White arrows indicated chromatin marginalization in E0771 cells from MAG-DHA group.(C) The autophagic markers such as LC3, p62 and Beclin-1 were monitored by western blotting analysis in tumor tissues from controland MAG-DHA group. (D) The tumor tissues were fixed and analyzed for autophagosome formationusing TEM. White arrows indicated autophagosome formation in E0771 cells from controland MAG-DHA group.

Figure 6

MAG-DHA intervention induced lipid peroxidation, activated anti-oxidative stress response and triggered ER stress in E0771 xenografts. (A) DHA levels in plasma and tissues from control and MAG-DHA group. (B) The content of MDA was measured by MDA kit in tumor tissues from control and MAG-DHA group. (C) The sensors of oxidative stress Nrf2 and HO-1 were monitored by western blotting analysis in tumor tissues from control and MAG-DHA group. (D) The ER stress markers such as p-PERK, PERK, p-eiF2α, eiF2α and CHOP were measured by western blotting analysis in tumor tissues from control and MAG-DHA group. Results are presented as the mean ± SEM from three independent experiments, * $P < 0.05$; ** $P < 0.01$ compared to the corresponding control.

Figure 7

Schematic depicting the anti-cancer mechanism of MAG-DHA on breast cancer. The prevention of MAG-DHA against cultured breast cancer cells or in vivo tumor xenografts was strongly associated with lipid peroxidation-mediated ER stress, which induced cell apoptosis and autophagy. Notably, ER-induced autophagy suppressed MAG-DHA-mediated apoptotic cell death.

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