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Arachidonic Acid Signaling in Invasive and Non-Invasive Breast Cancer Cells

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by

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ABSTRACT

Breast cancer is the second largest cause of cancer-related deaths in women all over the world. Epidemiological studies suggest that the consumption of high-fat diets can promote the incidence of breast cancers in both developed and developing countries. In particular, the lipid-rich diet contains arachidonic acid (AA, a $C_{20:4}$ polyunsaturated fatty acid), which has been shown to be associated with tumor formation in breast tissues. Nevertheless, the actual mechanism by which AA induces the metastatic transformation and malignancy is not well understood. The goal of my dissertation, therefore, is to identify the molecules and unravel the pathways that participate in AA-induced carcinogenesis. In this investigation, two different breast cancer cells were used—MDA-MB-231 and MCF7. Although both cells were derived from the cancerous tissues of the human breast, they display different phenotypes. For example, the estrogen-receptor-negative MDA-MB-231 cells are highly invasive, with typical fibroblast structures, whereas MCF7 cells are estrogen receptor positive and are weakly invasive, luminal epithelial cells. Briefly, both MDA-MB-231 and MCF7 cells were treated with AA (100 μM) and a lipoxygenase inhibitor (nordihydroguaiarectin acid or NDGA, 10 μM), followed by the isolation and characterization of the AA metabolites (i.e., eicosanoid molecules) by high-performance liquid chromatography (HPLC). Immunoblot and confocal microscopy were performed to elucidate the protein expression and intracellular targeting of 5-lipoxygenase (5-LOX), the enzyme that utilizes AA to synthesize LTB_4 and other leukotrienes (LTs). Metastatic migration was analyzed by wound-healing and matrigel-mediated invasion assays. The involvement of lipid rafts (LRs) in inducing migration/invasion of MDA-MB-231 cells was evaluated by treating the

cells with an LR disruptor, methyl-β–cyclodextrin (MBCD). The analysis of LR components (i.e., caveolin-1, flotilin and cholesterol) was also monitored.

The results suggest that while MDA-MB-231 cells produced high levels of prostaglandins (i.e., PGE_2 and PGD_2), MCF7 cells synthesized excess hydroxyeicosatetraenoic acid (HETEs) compounds. Interestingly, MDA-MB-231 cells when stimulated with AA showed increased synthesis of LTB_4 (~4-fold) and decreased the production of PGE_2 and PGD_2 (~2-fold). In contrast, AA suppressed the synthesis of PGE_2, PGD_2, LTB_4, and HETE compounds in MCF7 cells, suggesting that AA affects the eicosanoid synthesis in MDA-MB-231 and MCF7 cells differently. Furthermore, I found that the AA treatment stimulated the expression of 5-LOX and promoted the migration and invasion by MDA-MB-231 cells. Interestingly, NDGA significantly blocked the AA-induced 5-LOX activation and cellular migration/invasion. AA also increased the phosphorylation of NFκB and produced excess IL-6 and IL-8. Again, AA showed minimal or no effects on 5-LOX, cytokines, and NFκB activation in MCF7 cells, which suggests that AA activates the different sets of molecules/pathways in non-invasive and invasive breast cancer cells, respectively. Because AA stimulated the synthesis of LTB_4 in MDA-MB-231 cells, I propose that the autocrine and paracrine signaling caused by LTB_4 activates NFκB-mediated cytokine release, which promotes cellular inflammation and malignancy in invasive breast cancers. The results with MBCD that blocked the invasion and migration of MDA-MB-231 cells, along with the increased synthesis of caveolin-1 and flotilin under the influence of AA, further supports the idea that the involvement of LRs is essential in AA-mediated migration and invasion of MDA-MB-231 breast cancer cells.

Finally, my results point out that the LTB_4, which is synthesized by AA, activates the inflammatory pathways that are critical for causing the metastatic invasion of breast cancer cells. In my opinion, this is a novel finding in the field of breast cancer, and it is likely that 5-LOX pathway could be an ideal target for developing new chemotherapies to treat the patients with metastatic breast cancers.

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ABBREVIATIONS

AA	Arachidonic acid
ER	Estrogen receptor
PR	Progesteron receptor
HER	Human epidermal growth factor receptor
EGFR	Epidermal growth factor receptor
TNBC	Triple negative breast cancer
COX	Cycloxygenase
LOX	Lipoxygenase
PLA2	Phospholipase A2
PG	Prostaglandin
TX	Thromboxane
LT	Leukotriene
HETE	Hydroxyeicosatetraenoic acid
NFkB	Nuclear factor kappa beta
IL	Interleukin
PARP	Poly ADP ribose polymerase
NDGA	Nordihydroguaiaretic acid
MBCD	Methyl- beta- cyclodextrin
PI3K	Phosphoinositide 3 kinase
mTOR	Mammalian target of rapamycin

MAPK	Mitogen activated protein kinase
PTEN	Phosphatase and tensin homolog
BRCA	Breast cancer type susceptibility
FAS	Fatty acid synthase
DGLA	Dihomo gamma linolenic acid
BLT	B leukotriene receptor
N-WASP	Neuronal Wiskitt-Aldrich syndrome protein
MMP	Matrix metalloproteinase
FAK	Focal adhesion kinase

CHAPTER 1

GENERAL INTRODUCTION

1.1. Frequency, Distribution and Epidemiology of Breast Cancer

Breast cancer is widespread among women across the globe. A recent estimate suggests that cancer in breast tissues comprises approximately 20% of all cancers in women [1]. Worldwide, more than 1.1 million cases are diagnosed each year, and approximately 410,000 cases result in death [2]. Figure 1 shows the incidence rate and mortality rate (per 100,000 populations) of breast cancer globally as reported by the International Agency for Cancer Research in France (www.iarc.fr). However, the incidence and mortality rates are higher in the U.S., Canada, Brazil, Australia, and Europe. In 2011, 200,000 new cases of breast cancer were reported in the U.S., and the number of deaths reached almost 39,000 [Table-1, American Cancer Society]. Interestingly, recent advancements in diagnosis and treatment have resulted in approximately 2 million breast cancer survivors in the United States [3].Most of the commonly diagnosed breast cancers are non-invasive and estrogen-receptor (ER) positive and these are responsive to hormone and chemotherapy [4]. On the other hand, the invasive breast cancers are ER negative but progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) positive. Still, there are growing reports of increased incidence of double-negative breast cancer (ER positive, PR and HER-2 negative) and triple-negative breast cancer (ER, PR, and HER-2 negative) in the U.S. [5; 6]. These receptor-negative breast cancers are highly invasive and metastatic and are usually non-responsive to hormone and common chemotherapies. During metastasis, breast cancer cells migrate quickly, and this migration allows the cells to spread within the healthy tissues, distant organs, or even to the bone marrow.

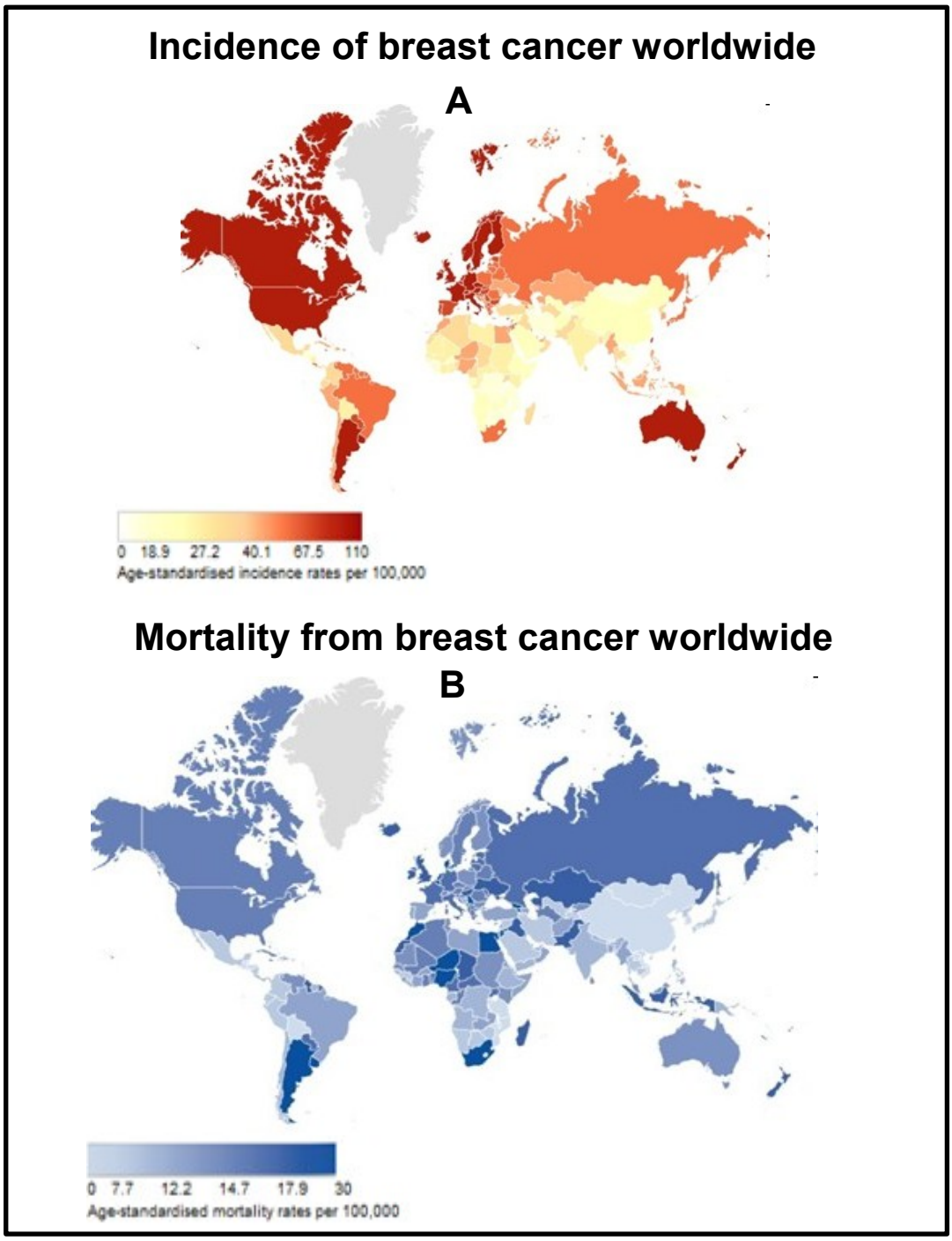


Figure 1

Breast cancer around the world

Figure 1. Breast cancer around the world

Age standardized incidence rate (Figure 1A) and age standardized mortality rate (Fig.1B) in a global perspective is shown in this figure. The incidence and mortality rates are calculated in per 100,000 people. Source: GLOBOCAN, France.

The mechanisms of cell migration and invasion involve complex interactions among various proteins and signaling molecules, activation of focal-adhesion kinase, actin polymerization, lammellopodia formation, and the involvement of extracellular matrix as well as integrin molecules [7].

Reports suggest that various environmental factors, carcinogens, hormones, and fatty acids such as arachidonic acid (AA, C20:4) can influence the migration of various cells, including those of breast cancer [8; 9]. A recent study on breast cancer epidemiology by Kwan et al. [10] suggested that frequent alcohol consumption may enhance the risk of breast cancer recurrence in post-menopausal and obese women. Another recent multi-ethnic cohort study by Chen et al. [11] reported that there is no evidence for the association between obesity risk variants and breast cancer risks among women across ethnic groups. Conroy et al. [12] showed that in multi-ethnic populations women with higher breast density may have an increased risk for $ER^{+}PR^{+}$ but not $ER^{-}PR^{-}$ tumors. Breast cancer is the most commonly diagnosed cancer in white females, whereas Hispanic women have the maximum incidence rate of non-treatable, triple-negative breast cancer (TNBC). The consumption of red meats, high-fat diets, and smoking are common in the U.S.-Mexico border region. It is also known that in comparison with the general population, the Hispanic community in the U.S.-Mexico border area is medically underserved and more vulnerable to various forms of cancers. The lifestyle may play an important role in the disease progression in this ethnic population.

Table-1

Estimated new breast cancer cases and deaths by age, US, 2011. Table-1 demonstrates the total number of in situ and invasive breast cancer incidences and number of deaths by age in the United States of America in 2011 (Source: American cancer Society).

Age	In Situ Cases	Invasive Cases	Deaths
Under 40	1,780	11,330	1,160
Under 50	14,240	50,430	5,240
50-64	23,360	81,970	11,620
65+	20,050	98,080	22,660
All ages	**57,650**	**230,480**	**39,520**

*Rounded to the nearest 10.

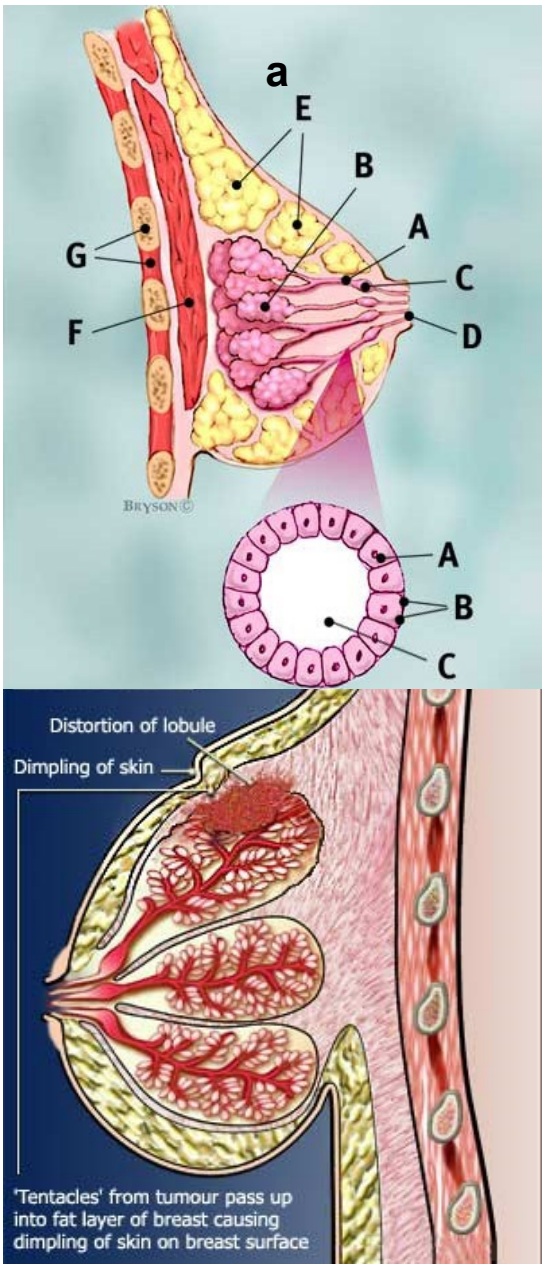
Source: Total estimated cases are based on 1995-2007 incidence rates from 46 states as reported by the North American Association for Central Cancer Registries. Total estimated deaths are based on data from US Mortality Data, 1969-2007, National Center for Health Statistics, Centers for Disease Control and Prevention.

American Cancer Society, Surveillance Research, 2011

1.2. Mammalian Physiology, Hormonal Regulation, Tumor Immunology and Genetics

Clinically, breast cancers are identified and characterized based on their origin in the mammary tissue. The two major types of breast cancers are lobular and ductal. Figure 2 compares the normal breast with a cancerous breast. With the development of the cancerous process, the lobules are distorted, and the skin abrasion becomes evident. The lobular carcinoma develops in the milk-producing glands or lobules of the breast tissue, whereas the presence of cancer cells in the milk ducts produces ductal carcinoma. While lobular carcinomas are the ones that are mostly invasive, ductal cancers are mostly non-invasive and non-life-threatening. The spread of cancerous tissue to the lymph nodes is common in the invasive cancers, which results in the formation of swollen lymph nodes in the auxiliary region, whereas the circulatory tumor cells (CTC) can be distributed to different organs by the blood stream when the cancer cells migrate to the blood vessels present in the breast.

For diagnostic and therapeutic purposes, breast cancers are also classified according to the over-expression of the hormonal receptors or by genetic mutations. Estrogen and progesterone are the two important hormones that play a significant role in maintaining the physiological homeostasis in women. Therefore, the unregulated synthesis of these hormones and the malfunctioning of their receptors can lead to pathological conditions that result in the up- or down-regulation of many signal-producing molecules, which in turn causes abnormal cell proliferation in the breast tissue (Figures 3A and 3B). Estrogen receptors (ER) and progesterone receptors (PR) play a key role in generating the signals upon binding to estrogen and progesterone hormones.



Breast profile:
A Ducts
B Lobules
C Dilated section of duct to hold milk
D Nipple
E Fat
F Pectoralis major muscle
G Chest wall/rib cage
Enlargement
A Normal duct cells
B Basement membrane
C Lumen (center of duct)

Figure 2

Breast Physiology

Figure 2. Breast physiology

Figure 2A is a diagram of a normal breast comprising of ducts, lobules, nipple, muscle, etc. The duct cells and lumen are shown in an enlarged image. Figure 2B demonstrates the cancerous breast tissue, where lobules are distorted and skin is abrogated. (www.breastcancer.org)

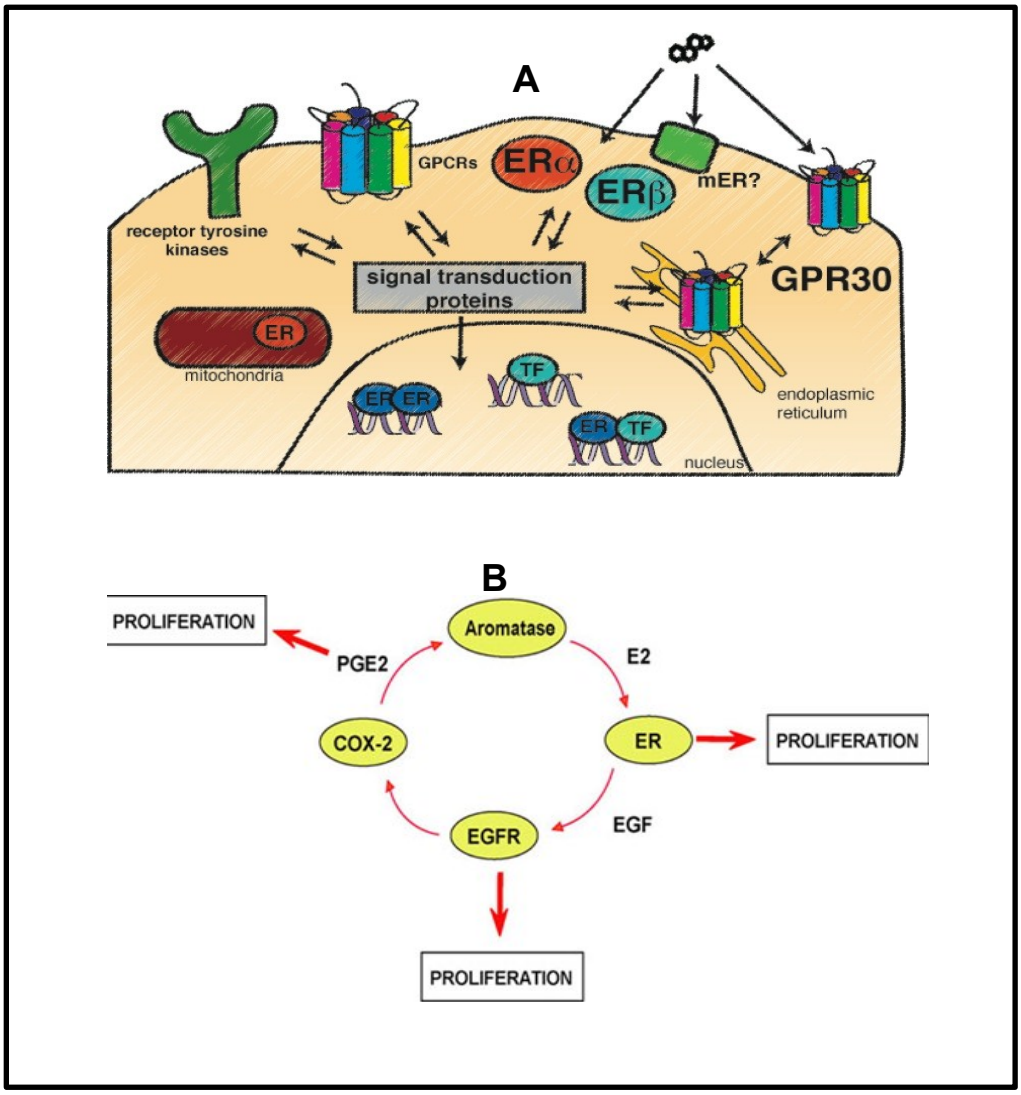


Figure 3

Estrogen signaling pathway

Fig.3. Estrogen signaling pathway

Figure 3A represents the binding of estrogen to estrogen receptor (ER). ER is localized in the nuclear membrane and acts through a G-protein-coupled estrogen receptor, GPR30. Figure 3B shows the synthesis of estradiol (E2) by aromatase gene that leads to the activation of estrogen receptor (ER) and cell proliferation during the breast cancer. ER activation is also linked to the activation of epidermal growth factor receptor (EGFR) that stimulates the cycloxygenase (COX-2) and subsequent prostaglandin E_2 (PGE_2) production. Ref: Figure 3A.The Weatherman Lab, Rose Hulman Institute of Technology; Figure 3B. [13].

ER has two different subunits, located in nuclear membrane—i.e., alpha (α) and beta (β)—and the ratio between these two receptor subunits is important for maintaining the hormonal homeostasis. GPR30, a G-protein coupled receptor also binds to estrogen or estrogen antagonist [14] and this ligand-receptor interaction is important for promoting the aggressive nature of breast tumors. Reports indicate that GPR30 expression is linked to the generation of tamoxifen resistance breast cancer cells [15]. Pandey et al. found that the interaction between connective tissue growth factor and GPR30 is essential for the metastatic migration of breast cancer cells [14].

The other major receptors such as human epidermal receptor-2 (HER2) and epidermal growth factor receptor (EGFR) are over-expressed in a large number of breast cancer patients. Different growth factors, including chemokines or eicosanoids, may bind to these receptors, and they can activate different signal-transduction molecules and promote cell proliferation, angiogenesis, invasion, and metastasis.

Breast cancer can also be categorized according to the distribution of elevated levels of hormonal receptor proteins. ER^+, PR^+, or HER^+ cancers are characterized according to the enhanced level of receptor proteins in the breast tissue. Immuno-histochemistry (IHC) and PCR analyses of a patient's samples are dependable diagnostic tools to diagnose the type of breast cancer, and accordingly the patient receives medications. If the pathological test is unable to identify any such receptor up-regulation, then the cancer is considered a triple-negative cancer, which has poor prognosis. In addition to the ER and PR, HER 2 and epidermal growth-factor receptors (EGFR) are critical for growth and malignancy. These receptors are able to bind to various ligands and can activate signaling molecules, which subsequently up-regulate the signaling of several

kinases and the transcription of various oncogenes, as well as suppressing the activities of tumor suppressor genes. ER, PR, HER, and EGFR are the most studied and diagnosed receptors in breast cancer patients.

Oncogenes are the mutated form of normal cellular genes, and the activation of oncogenes could be responsible for transforming normal cells into malignancy. The oncogenes, such as c-*myc* [16], HER-2 [17] and EGFR [18] were found to be associated with the incidence of breast cancer. There is another group of genes called tumor-suppressor genes that can function as negative regulators of the growth, invasion, and metastasis of cells [19]. These tumor-suppressor genes are vital for maintaining DNA repair, genomic stability, transcriptional regulation, and cell-cycle control [20]. The inactivation or mutation of tumor-suppressor genes is important in the development and progression of breast cancer. Some of the important tumor-suppressor genes associated with breast cancer are p53, PTEN, the retinoblastoma gene (Rb), TP53, CDH1, BRCA1, BRCA2 and BRCA3 [21; 22; 23; 24]. The BRCA1 protein interacts with other proteins such as RAD51 and BARD1 to maintain the stability of DNA. BRCA1 and BRCA2 mutations (Figure 4) are inherited in an autosomal dominant manner, and both males and females have a 50% risk of passing the mutation on to children of both sexes. The mutations in the BRCA genes are associated with 5–10% of breast cancer cases. The risk of developing breast cancer in individuals with a BRCA1 mutation may be as high as 85% [American Cancer Society]. Other than maintaining the repair mechanism, BRCA1 is also involved in embryonic development. Although mutation in the BRCA1 gene is mostly associated with breast cancer incidence, but it has also been found to be associated with fallopian tube cancer,

pancreatic cancer and male breast cancers. In breast cancer patients, more than 1,000 mutations have been detected to be associated with the BRCA1 gene, and patients who carry these kinds of mutations are more prone to develop metastasis. It is interesting that bilateral oophorectomy has been shown to have a reduced breast cancer risk in patients with BRCA1 mutation due to a reduced level of ovarian hormone availability in the system [23].

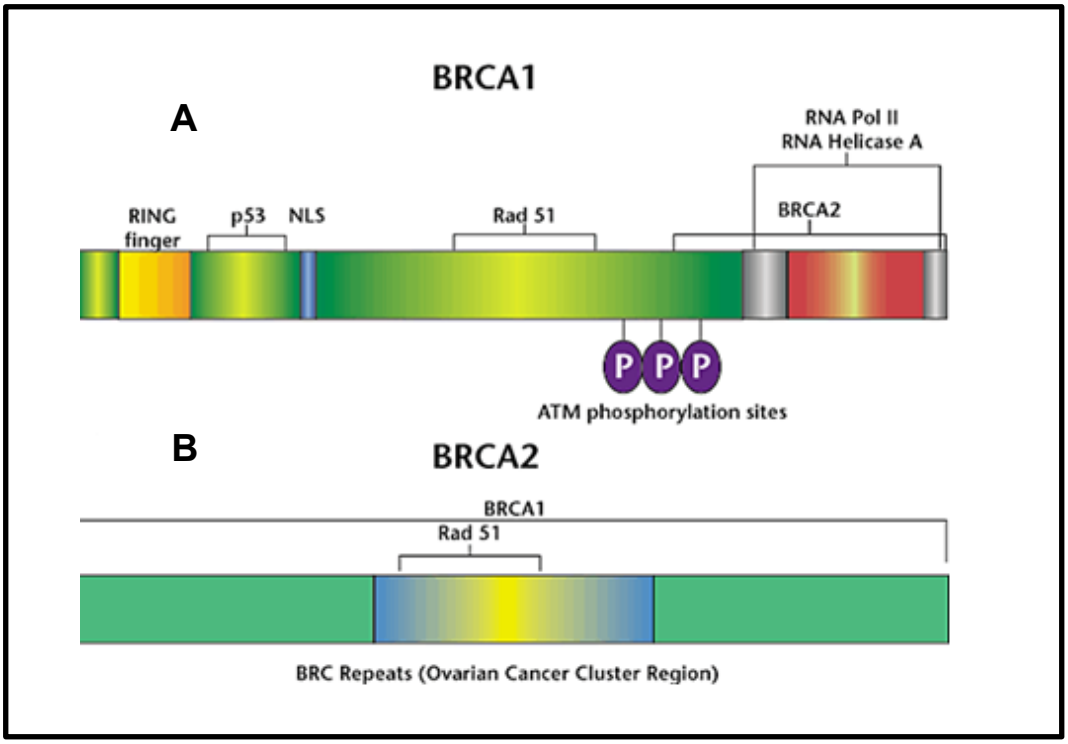


Figure 4

Breast cancer genetics

Figure 4. Breast cancer genetics

Fig.4A shows the domains of breast cancer type 1 (BRCA1) and breast cancer type 2 (BRCA2) genes. The different domains are Zn binding RING (really interesting new gene) finger domain near amino terminus; NLS, nuclear localization signal; p53 and RAD51 binding site. BRCA2 (Fig.4B) interacts with BRCA1 through a specific domain. Point mutations in RING and BRCA1 carboxy terminus domain have been found in early onset breast cancers. Serines phosphorylated by oncogene ATM in response to DNA damage [25].

Among the X-chromosome genes, tissue inhibitor of metalloproteinases-1(TIMP-1) is up-regulated in breast cancer [26]. TIMP-1 has been recently proposed as a potential biomarker for breast cancer [27].

It has been observed that tissue homeostasis is severely disturbed in breast cancer and that it is infiltrated by macrophages as well as by mast cells, which secrete cytokines, chemokines, growth factors, and matrix proteases [28]. The tumor micro-environments are filled up with immune mediators and produce chronic inflammation. The immune profiles of breast tumor patients are distinct from those of healthy individuals on the basis of tumor micro-environment and extra-cellular matrix. A recent study by Xu et al. [29] found a significant increase of CD27 and CD70 (two major co-stimulatory molecules regulating the expansion and differentiation of T-cell function) in a subpopulation of patients affected with ER^+/PR^+ breast cancer in China. The tumor cells escape from the immune surveillance mechanisms due to the chronic pro-tumorigenic inflammation. The T cells become dysfunctional or less active in malignancy, and the regulatory T cells are found in the lymphoid organs and neoplastic tissues. Bower et al. [30] reported a significant increase in $CD4^+$ T cells in breast cancer survivors, whereas B cells, natural killer cells, granulocytes, and monocytes were not altered. Several trials to develop a vaccine against breast cancer are in progress, and recently Wang et al. [31] showed a better efficacy of DEC-HER2 fusion monoclonal antibody in a mouse model to induce a high level of broad and multifunctional CD4+ T-cell immunity, CD8+ T-cell responses, and humoral immunity specific for HER2 antigen.

1.3. Lipids, Lipid Rafts and Cell Signaling in Breast Cancer

Dietary fats have been known to play a critical role in the etiology of cancer. Epidemiological studies carried out over the years have documented that diets high in animal fat and omega-6 polyunsaturated fatty acid (ω–6 PUFA) contribute to an increased risk of breast cancer [32; 33]. Dietary PUFA has a profound influence on the growth rate of transplantable human breast cancers in immunodeficient mouse [34]. It has also been shown that diets containing omega-3 polyunsaturated fatty acids (ω-3 PUFA) suppress the tumorigenesis in mouse [35]. Although dietary fatty acids and lipids have been implicated in causing cancer, the mechanisms by which they initiate this disease are not completely understood. Recent studies indicate that a high intake of dietary fat results in increased production of bile acid along with different eicosanoid molecules such as prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETE) compounds, and others. Figure 5 depicts a general scheme of AA metabolism. Eicosanoids have a regulatory effect on cell growth in cancer as well as in non-cancer cells [36; 37; 38; 39]. These compounds enhance the proliferative activity of epithelial cells by increasing ornithine decarboxylase that is involved in cell division [40].

The synthesis of various signaling molecules like protein kinase C, NFκB, MAPK, mTOR, ERK1/2, PI3K/Akt **(**Figures 6 and 7**)** play important roles in the activation of different oncogenes in invasive breast cancers that promote angiogenesis, invasion, and migration.

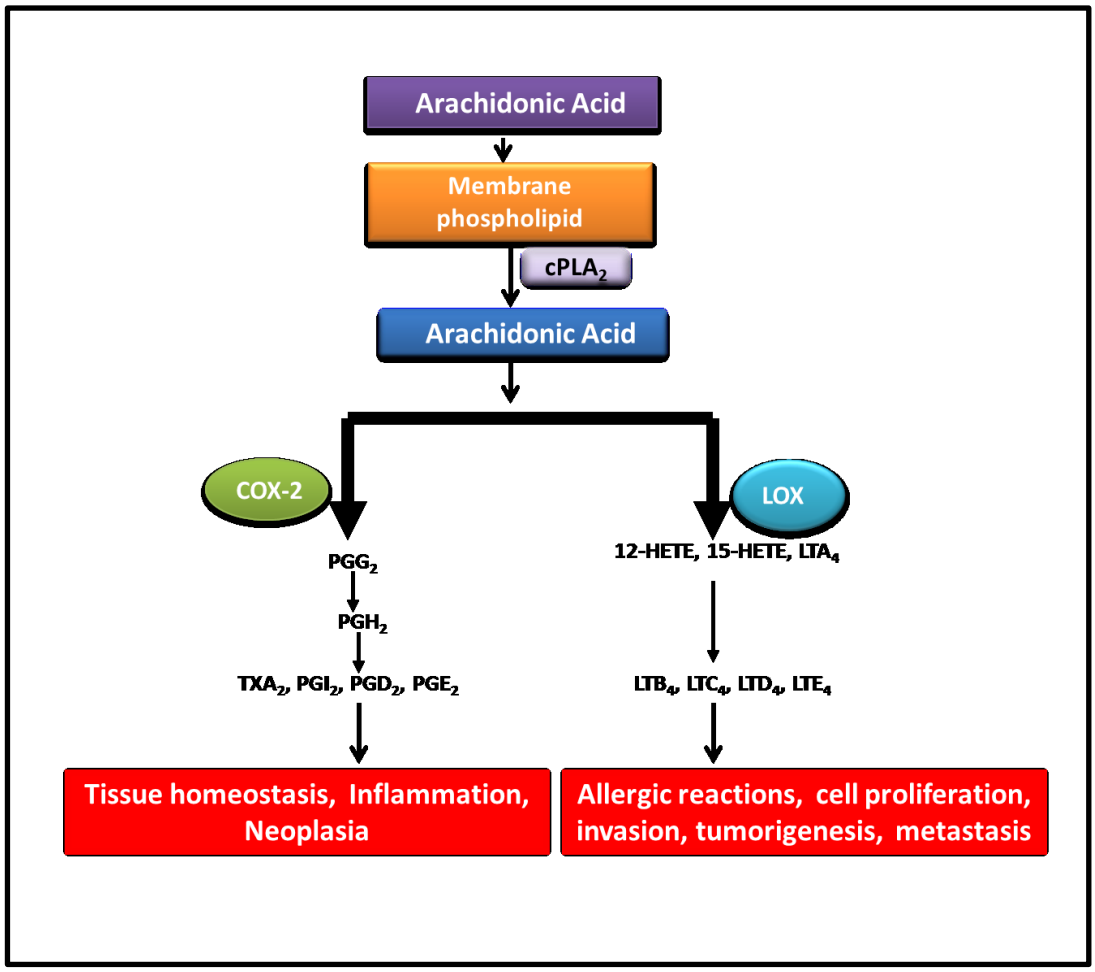


Figure 5

Arachidonic acid signaling/metabolism

Figure 5. Arachidonic acid signaling/metabolism

Arachidonic acid (AA), present in the lipid rich diet, and incorporated into membrane phospholipids, forming arachidonoyl-phospholipid complex. This complex is hydrolyzed by cytoplasmic phospholipase A_2 ($cPLA_2$) and releases free and unesterified AA. Free AA under influence of cycloxygenase (COX) and lipoxygenase (LOX) produces different inflammatory molecules like prostaglandins (PGG_2, PGH_2, PGI_2, PGE_2 and PGD_2), thromboxanes (TXA_2), leukotrienes (12-HETE, 15-HETE, LTA_4, LTB_4, LTC_4 and LTD_4).

Table-2

Important eicosanoids and their functions. The physiological and pathological effects generated by different eicosanoids are listed in.

Important eicosanoids	Physiology	Pathology
PGD2	anti-inflammation, ovulation, implantation and menstruation	dysmenorrhea, endometriosis and cancer
TXB2	thrombotic tendency	arteriosclerosis
HETE	activates neutrophil and monocytes	stimulate proliferation of cancer cells
LTB4	immune-mediated inflammatory reactions of anaphylaxis	stimulate proliferation of cancer cells

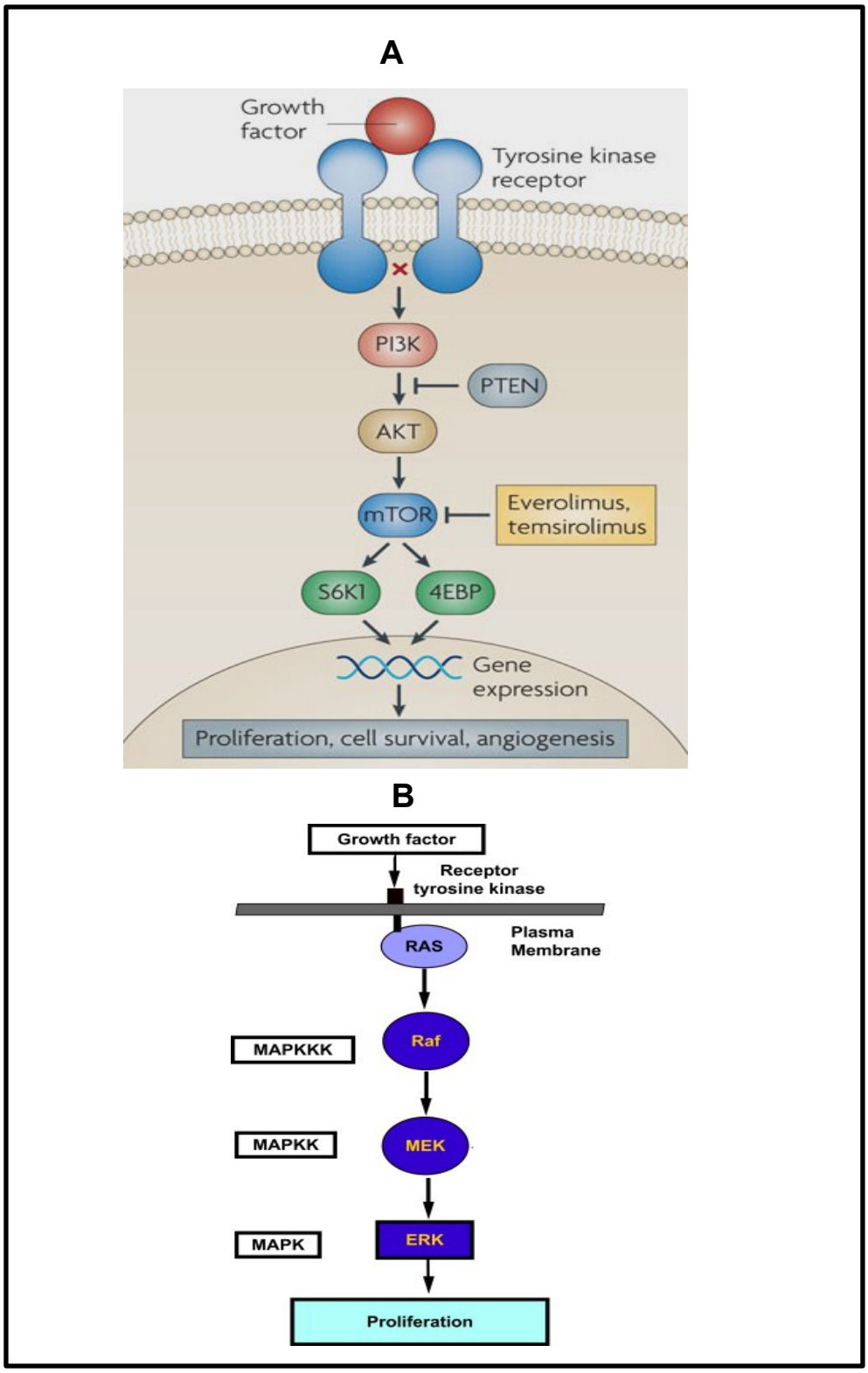


Figure 6.

PI3K, mTOR, Akt and MAPK pathway in cancer

Figure 6: PI3K, mTOR, Akt and MAPK pathway in cancer. Figure 6A demonstrates the activation of phosphoinositide-3-kinase (PI3K)/Akt pathway by ligand binding with a tyrosine receptor kinase. Phosphotase and tensin homolog (PTEN) is a tumor suppressor gene which can block this activation. Akt activation causes the stimulation of mammalian target of rapamycin (mTOR), which is responsible for activation of several oncogenes. Everolimus is an anti-cancer drug which inhibits mTOR. [41].

Figure 6B depicts the Ras/Raf/MEK/MAPK pathway. External growth factor and/or stress response elements may activate the membrane bound receptor tyrosine kinase which subsequently activates Ras/Raf/MEK/MAPK activation through a series of phosphorylation. MAPK activation stimulates the expression of several genes which are associated with cell proliferation and cancerous signaling [42].

Many new therapeutic approaches are being targeted against these signaling molecules for the prevention of angiogenesis and metastasis. The target genes associated with promoting cancerous events are, C-JUN, ATF-2, SP-1, c-MYC, CREB, c-FOS, STAT, ELK, etc.

The primary mechanism for endogenous fatty-acid syntheses is carried out by an enzyme called fatty-acid synthase (FAS), which is up-regulated during tumor formation and metastasis [43; 44]. It has also been suggested that omega-6 fatty acids (i.e., AA and linoleic acid (LA)) play critical roles in inducing breast tumors [45]. Arachidonic acid has been termed as pro-neoplastic fatty acid [46] because of its role in inflammation, cell proliferation, apoptosis, metastasis, and angiogenesis [47]. AA is associated with membrane phospholipids that originate from LA present in the fatty diet. Incidentally, LA is an unsaturated fatty acid (C18: 2) and can be imported into cells by different fatty-acid binding proteins (FABPs) [48; 49; 50]. The LA, once internalized, undergoes desaturation and elongation to produce dihomo gamma linolenic acid (DGLA) before transforming into AA [51]. The DGLA passes through another desaturation catalyzed by 5-desaturase for the formation of AA. The important factor in this conversion is the availability of microsomal 6-desaturase enzyme [52]. Arachidonic acid synthesized through this reaction is incorporated into the membrane phospholipids and can be released by the action of phospholipase A_2 (PLA_2) [53]. Phospholipase A_2 (PLA_2) is divided into Ca^{2+}-dependent, Ca^{2+}-independent and secretory PLA_2s. The Ca^{2+}-dependent cytoplasmic PLA_2s ($cPLA_2$) and secretory PLA_2s participate in releasing AA from the arachidonoyl-phospholipid in normal and cancer cells, which immediately reacts to endogenous cycloxygenase, lipoxygenase, or cytochrome p450 to produce

eicosanoids [54]. As mentioned above, eicosanids are associated with many different physiological and pathological outcomes, including inflammation, leukocyte infiltration, cell proliferation etc.

Breast cancer cells synthesize excess prostaglandin E_2 (PGE_2) and also over-express the corresponding receptor molecules (i.e., the PG receptors). Prostaglandin (PG) receptors are the members of G-protein-coupled receptors (GPCRs) and are involved in generating downstream signaling pathways that are involved in the angiogenesis and in alterations in cell adhesion, morphology, motility, invasion, and metastases [55]. Currently, there are eight known PG receptors of different cell types, which are PGD_2 receptor (DP2), four subtypes of PGE receptors (i.e., EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP). PGE_2 is the major secretory product of breast tumor epithelial cells and is found in fibroblasts, macrophages, and lymphocytes at the tumor site. Higher levels of PGE_2 are correlated with cancer aggressiveness and metastatic potential [55]. PGE_2 stimulates estrogen biosynthesis and influences the progression of the cancer [56]. It also stimulates expression of the aromatase gene *CYP19* by activating protein kinase A and protein kinase C signaling pathways. *CYP19* is responsible for estrogen biosynthesis in breast cancer affecting breast tumor development and growth, [57]. On the other hand, LT formation corresponds to cellular growth, leukocyte infiltration, and DNA synthesis in cancer cells [58]. Excess LT syntheses and 5-lipoxygenase over-expression have been reported in colorectal cancer and lung cancer. It is interesting that the specific lipooxygenase (LOX) inhibitors have the potential to reduce tumor growth and metastasis [59]. The presence of BLT (B leukotriene receptor) receptors in the

membrane is important for binding with secreted leukotriene B_4 (LTB_4) and for autocrine signaling. LTB_4 mediates the inflammatory responses in the target cells by activating the phosphorylation of NFκB. Studies suggest that BLT1 is present in lipid rafts (LRs) in the membrane, and disruption of LRs can potentially inhibit the pro-inflammatory responses generated by LTB_4 [60]. LRs are the membrane-bound lipid domain responsible for the endocytosis of different molecules and ligands. Rafts are characterized in different species and have been shown to play an important role in cellular metabolism and synthesis. Cancer cells having an increased amount of metabolic rate have elevated raft formation, resulting in active transportation machinery compared with normal epithelial cells. Raft-specific markers such as monosialo ganglioside (GM1) [61], flotilin [62], caveolin [63], mmp-9 [64], uPAR [65], etc., are over-expressed in various cancer cell lines. Figures 8A, 8B, and 8C show the role of lipid rafts in the cancer process by activating various pro-metastatic signaling events [66].

1.5. Recent Developments in Breast Cancer Therapeutics

Therapeutic strategies to treat breast cancer patients depend on the detection and diagnosis. The results obtained from these diagnoses guide the physicians to prescribe a course of anti-estrogen (e.g., tamoxifen) or anti-progesterone (e.g., CDB 4124) or anti-HER2 (e.g., trastuzumab)-based therapies along with adjuvant and sometimes traditional chemotherapeutic compounds (e.g., microtubule disruptors, nucleoside analogues, or radiations). These anti-receptor antibodies or analogues would counteract the elevated levels of the specified receptor proteins, but in the majority of cases the

disease is diagnosed in the late stage, and therefore the treatment cannot rely solely on a specific drug or antibody. Microtubule destabilizing drugs, along with suitable anti-hormonal therapy, are used to produce a more effective therapeutic strategy—e.g., DM-1 (thiol-containing maytansinoid antimicrotubule agent), along with trastuzumab (anti-HER2 antibody). Aromatase inhibitor has also been widely used to inhibit the estrogen synthesis in cases of estrogen-positive breast cancer among post-menopausal women. Examples of aromatase inhibitors are exemestane (aromasin), anastrozole (arimidix), etc., and these drugs are sometimes associated with other chemotherapeutic agents such as tamoxifen or gemcitabane (nucleoside analog) to produce a combinatorial drug treatment against estrogen-sensitive breast cancers.

Recent advances in oncogenic drug discovery led to the use of antibody-based therapy. The best example would be *Herceptin*^{TM}*(*trastuzumab*)* from Genentech, which is an antibody against the HER2 receptors. This antibody binds to the specific receptor and can deactivate the receptor function. Yet, there is always the possibility of developing other factors that are essential for maintaining the carcinogenic status of an individual, such as the dimerization of receptor proteins to escape the therapeutic interventions.

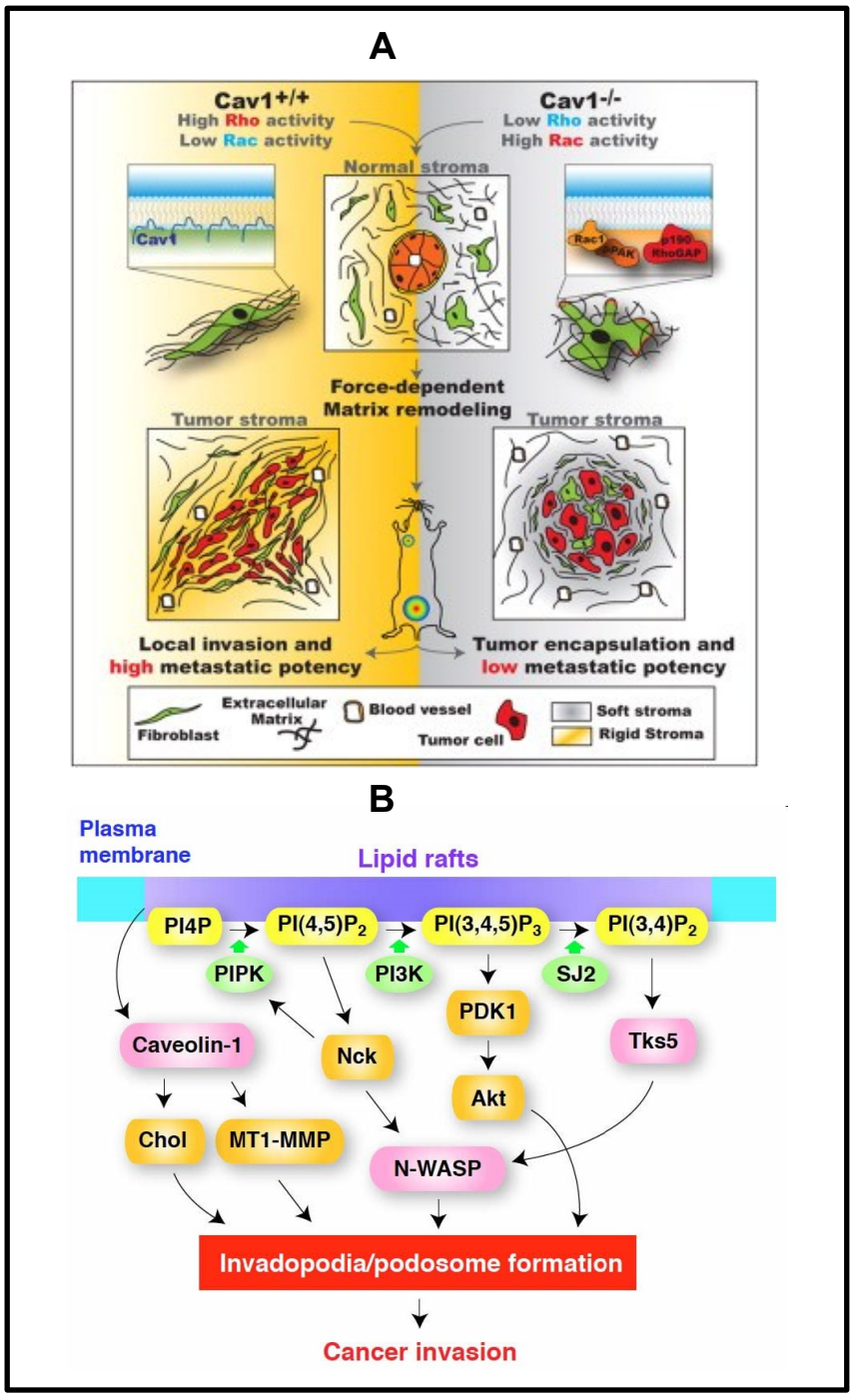


Figure 7

Lipid raft/caveolin and cancer cell signaling

Figure 7. Lipid raft/caveolin and cancer cell signaling

Figure 7A shows the role of a lipid raft protein, caveolin-1 in the initiation of cellular invasion and metastasis mediated by a high Rho activity and a low Rac activity. Caveolin-1 overexpression causes the extracellular matrix remodeling which favors tumor metastasis [66]. Figure 7B demonstrates the various lipid raft molecules, neuronal Wiskitt-Aldrich syndrome protein (N-WASP), Matrix metalloproteinases (MMP), cholesterol and their participation in the formation of invadopodia in cancer metastasis [67].

Table-3

Clinical Trials Recent clinical trials. Some of the major clinical trials in breast cancer are described in Table-3 along with the targeted pathways.

Clinical Trial	Pathway targeted	Drugs
CLEOPATRA	HER2 dimerization and anti-HER2 antibody	Pertuzumab and Trastuzumab
BOLERO-1	mTOR inhibiton, anti-HER2 antibody, anti-microtubule	Everolimus, Trastuzumab and Paclitaxel
BOLERO-2	mTOR inhibition and aromatase inhibition	Everolimus and exemestane
TDM1	Anti-HER2 antibody and antimicrotubule agent	Trastuzumab and mayatansinoid

1.6. FOCUS OF MY RESEARCH

In the present study, I demonstrate that while AA treatment of invasive MDA-MB-231 breast cancer cells inhibits the synthesis of various PGs, it induces the production of LTB_4, HETE-5, and HETE-8 molecules. Results also show that LTB_4 modulates the migration of cancer cells by activating NFκB-linked pro-inflammatory cytokines (IL-6 and IL-8) and caveolae. Nordihydroguaiaretic acid (NDGA), a common lipoxygenase inhibitor is able to decrease the synthesis of LTB_4 and inhibits the NFκB, IL-6, and IL-8 syntheses. Methyl-β cyclodextrin (MBCD), a known LR disruptor, interferes with LR biogenesis and with the migration of MDA-MB-231 cells. Overall, invasive breast cancer cells exhibit increased inflammatory and metastatic migration/invasion upon the addition of exogenous AA. A novel leukotriene (LT)-mediated activation of inflammatory response/migration via the lipid raft microdomain was proposed in the current dissertation.

1.7. HYPOTHESIS

Arachidonic acid promotes metastatic migration and invasion of breast cancer cells by regulating leukotriene-induced inflammatory pathway.

1.8. DISSERTATION GOALS

The overall goals of this work are to understand the arachidonic acid (AA)-induced lipid signaling in invasive and non-invasive breast cancer cells and to identify important lipid mediators that facilitate this process. Epidemiological studies support the idea that the consumption of a high-lipid diet could lead to different physiological malfunctions, which can ultimately produce malignancy. Therefore, an attempt has been made to understand the key factors affected by AA in invasive and non-invasive breast cancer cells by addressing the following questions.

1. Does AA influence the eicosanoid synthesis in invasive and non-invasive breast cancer cells differently, and if so, how does that affect the migration and invasion processes?

2. How does AA-induced eicosanoid synthesis modulate the inflammatory reactions in breast cancer cells?

3. Are there any connections among AA-induced eicosanoid productions, inflammatory reactions, activation of lipid rafts, and metastatic migration/invasion of breast cancer cells?

I hope that addressing these questions, at least in part, should unravel some of the key mechanisms by which bioactive lipid mediators regulate the initiation and progression of breast cancers that affect millions of women worldwide every year.

CHAPTER 2

SPECIFIC AIM 1

TO INVESTIGATE THE MODULATION OF EICOSANOID SYNTHESES BY ARACHIDONIC ACID IN INVASIVE AND NON-INVASIVE BREAST CANCER CELLS.

2.1. INTRODUCTION

The relationship between the high-fat diet and the incidence of cancers is now well established. Diets enriched with polyunsaturated fatty acids (PUFA) are one of the risk factors for breast, colon, and prostate cancers [68; 69; 70]. Among different PUFAs, the omega-6 fatty acids, especially the AA and omega-3 fatty acids (e.g., eicosapentanoeic acid) play pivotal roles for maintaining the cellular homeostasis and inflammation [71]. Omega-6 fatty acids are known to stimulate the inflammatory reactions because they are involved in synthesizing the inflammatory eicosanoid molecules such as PGs, LTs, TXs, and HETE compounds as shown in Figures 9 and 10. The inflammatory eicosanoids include 2- and 4-series molecules (e.g., PGE_2, LTB_4, etc.), whereas 3- and 5-series compounds, which are derived from omega-3 fatty acids, are anti-inflammatory (e.g., PGE_3, LTB_5, etc.).

Dietary PUFAs, once taken up by cells, are incorporated (esterified) into membrane phospholipids followed by the hydrolysis by phospholipase A_2. Free fatty acids (unesterified FFA) that are released from the membrane are then metabolized by two major pathways in the cell to synthesize various eicosanoids by cyclic (cyclooxygenase-mediated) and/or by linear (lipoxygenase-mediated) pathways [72].

2.1.1 Cyclic pathway

Cycloxygenase (COX-1 and COX-2) enzymes use free AA to produce various eicosanoids such as PGs, prostacyclins, and TXs (Figure 9A). The COX-1 is constitutively expressed in mammalian cells at a low level, whereas COX-2 is inducible and is often over-expressed under stimulation. Prostaglandins (PGs) were first isolated

from the semen, and the name signifies that these molecules originate from the prostate gland [73].

PGs are unsaturated carboxylic acids (with a 20-carbon skeleton because they are AA derived) and made of a five-member ring. They are sub-classified based on the nature and position of a specific substituent on the ring. Prostaglandin A (PGA), prostaglandin E (PGE), prostaglandin J (PGJ), prostaglandin F (PGF), and prostaglandin K (PGK) all have keto groups in various locations on the ring, and they are distinguished from one another because of the positioning of double bonds and hydroxyl groups in the molecules [74]. For example, PGF has two hydroxyl groups, while PGK has two keto groups. Prostacyclins (known as PGIs) are characterized by an oxygen bridge between carbons 6 and 9. Another COX-mediated eicosanoid, thromboxane A (TXA), contains an unstable bicyclic oxygenated ring structure, while thromboxane B (TXB) has a stable oxane ring.

PGs are associated with activation of the inflammatory response, production of pain, and fever [75]. Thromboxane stimulates constriction and clotting of platelets [76]. Leukocytes infiltrate to the site of tissue injury to minimize tissue destruction and synthesize PGs. PGE_2 promotes uterine contractions and is applied to induce labor. PGs are also involved in several other organs such as the gastrointestinal tract (they inhibit acid synthesis and increase secretion of protective mucus), and they increase blood flow in the kidneys. PGE_2 secretion is highly regulated in colon cancer, pancreatic cancer, and different metabolic disorders [77; 78] Anti-inflammatory medications such as aspirin block COX enzymes and inhibit the oxygenation of arachidonic acid

conversion to prostaglandins. Blocking the syntheses of PGs relieves the pain and fever.

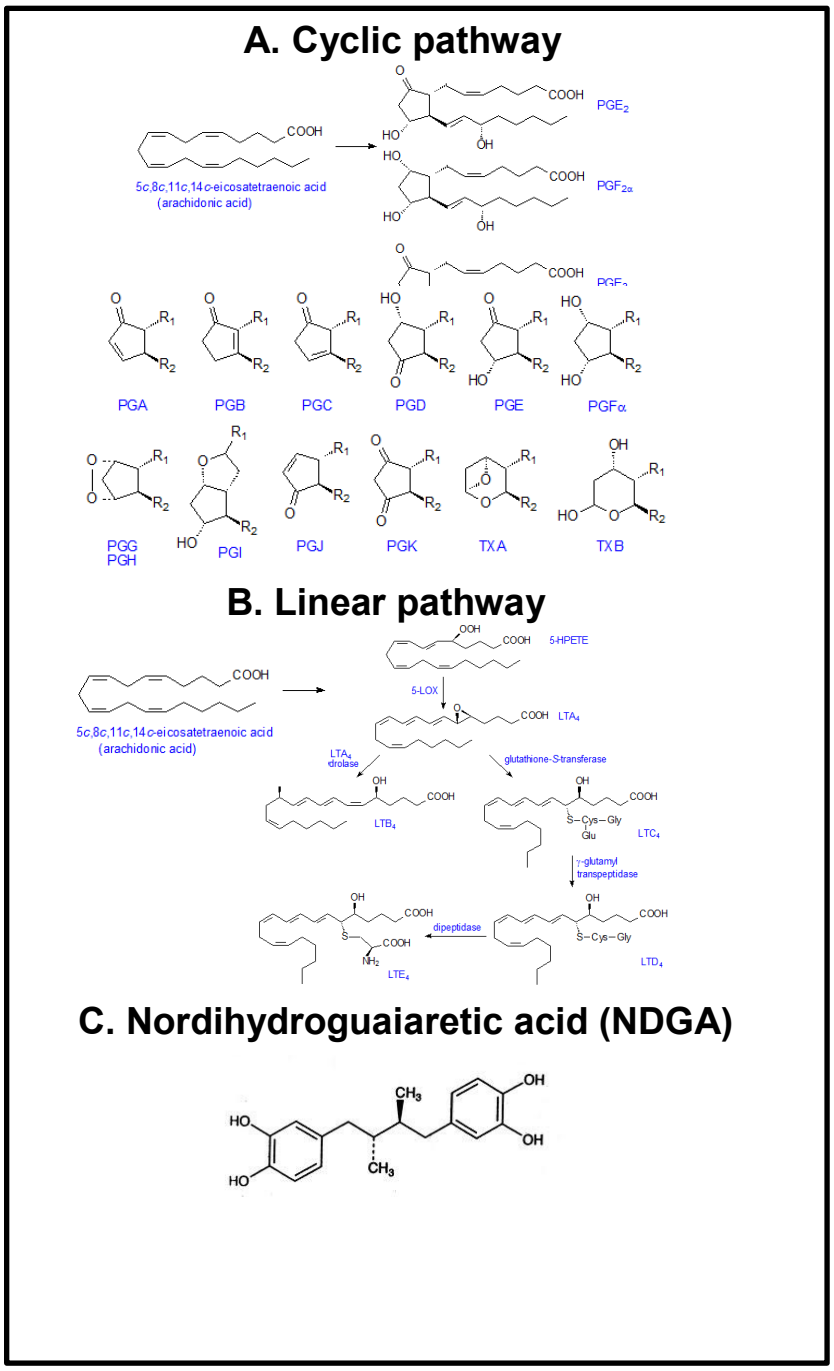


Figure 8

Cyclic and linear pathways of arachidonic acid and eicosapentaenoic acid metabolism in mammalian cells

Figure 8. Cyclic and linear pathways of arachidonic acid and eicosapentaenoic acid metabolism in mammalian cells

Figure 8A is an overview of eicosanoids synthesized from arachidonic acid (omega-6) and eicosapentaenoic acid (omega-3) by the cyclic pathway. The metabolic products from these pathways are, PGE2, PGF2α, PGE3, PG3α, PGA, PGB, PGC, PGD, PGG, PGH, PGJ, PGK, TX-A and TX-B. The metabolic by-products from linear pathway are shown in Figure 8B. The products are LTA_4, LTB_4, LTC_4, LTD_4, LTE_4, 5-HETE, 8-HETE, 12-HETE and15-HETE. Figure 8C depicts the chemical structure of a lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA).

2.1.2 Linear pathway

LTs and HETE compounds are synthesized by the linear pathway (Figure 9B). Different lipoxygenase enzymes (e.g., 5-LOX, 12-LOX, 15-LOX, etc.) catalyze the oxygenation of free fatty acids to synthesize LTs and HETE compounds [15]. Upon stimulation, the cellular 5-LOX enzyme translocates to nuclear membrane and co-localizes with 5-LOX activating protein (FLAP) and $cPLA_2$. This interaction converts free AA to 5(S)-hydroperoxy-6, -8, -11, -14-eicosatetraenoicacid (5-HPETE). This 5-HPETE can be reduced to form 5-HETE compounds [54]. The 5-LOX enzyme in the presence of FLAP hydrolyzes AA to synthesize LTA_4, which is further hydrolyzed to LTC_4 and then to LTB_4, which is secreted by the cells and causes vasoconstriction, allergic reaction, hypersensitivity, and inflammation [79]. Subsequently, 8-LOX, 12-LOX, or 15-LOX oxidized AA to synthesize 8-, 12-, and 15-HETE compounds, which are also associated with various types of cancer [80; 81]. Anti-asthma medications include 5-LOX inhibitors to inhibit the synthesis of LTs. Figure 9C represents the structure of a commonly used biphenolic LOX inhibitor, nordihydroguaiaretic acid (NDGA).

The effect of LT formation corresponds to cellular growth and DNA synthesis in cancer cells, and LOX inhibitors are reported to reduce tumor growth and metastasis [58; 59]. Recently, it has been demonstrated that LTB_4 is able to induce growth and sub-tumorigenic inocula of melanoma cells [82]. Inhibition of 5-LOX induces apoptosis in prostate cancer cells by down-regulating protein kinase C enzyme [83]. Another contemporary finding by Cheon et al. [84] showed a pro-tumorigenic role of

hematopoietic 5-LOX in inducing colorectal polyposis. Guo et al. [85] showed the connection between oral cancer and elevated 5-LOX activity.

The LOX products from linear pathway of AA metabolism transduce signals by specific membrane-bound receptors [86]. LTB_4 primarily binds to the G-protein-coupled receptors called BLTs—i.e., BLT-1 and BLT-2. BLT-1 is the primary receptor for LTB_4, and inhibiting the activity of these receptors shows a positive regulatory effect for the prevention of carcinogenesis. Ihara et al. [87] found a remarkable expression of BLT-1 in human colon cancer cell lines. BLT-2, the low-affinity LTB_4 receptor, is up-regulated in estrogen receptor negative (ER^-) breast cancer cells and has been noticed to work in tandem with reactive oxygen species (ROS) to generate pro-survival signaling [88]. Ovarian cancer patients showed a high level of expression of LTB_4 and BLT-2 [89]. Another recent work by Hu et al. [90] suggested a feedback loop involving FASN/p-ERK/5-LOX/ and LTB_4 in the growth of metastatic breast cancer cells.

2.2. Materials and Methods

2.2.1. Cell culture and treatments

MDA-MB-231 (Figure 10A) and MCF7 (Figure 10B) were cultured in DMEM/Ham´s F-12 and RPMI-1640 medium (Gibco Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (10% FBS, Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Hyclone, UT). Cells were grown in a humidified atmosphere consisting of 95% air and 5% CO_2 until they reached ~90% confluency. Confluent cells

were treated with trypsin (0.25% Trypsin-EDTA, Hyclone), harvested by centrifugation, and plated in either 96-well plates (~20,000 cells per well) or 6-well plates (0.5 X 10^6 cells per well) to perform either MTS assay or Western blot analyses. To evaluate the toxic effect of AA, cells were treated with various concentrations of AA (Sigma-Aldrich, St. Louis, MO) (10–300 μM) for 24 h.

To perform eicosanoid extraction, cells were plated in a 6-well plate (~0.5 X 10^6 cells per well) and were grown overnight. Fresh media (2 ml) was added to each well and incubated for an additional 24 h in the presence of AA (100 μM), NDGA (10 μM), or AA+ NDGA (100 μM + 10 μM).

2.2.2. Dose response

MCF7 and MDA-MB-231 cells were plated (~20,000) in 96-well plates and grown overnight. The cells were treated with different concentrations of AA (e.g., 10 μM, 25 μM, 50 μM, 100 μM, 200 μM, and 300 μM) for 24 h to find a concentration that did not exhibit any toxic effects and that was safe for conducting the in-depth investigations described in this and other chapters of my dissertation. Hydrogen peroxide (H_2O_2) was used as a positive control. A dye containing methyl-tetrazolium salt (MTS) was added to each well of a 96-well plate and incubated for an additional 30 min at 37 ^oC. The absorbance of the well plate was measured in a microplate reader at 590 nm.

2.2.3. Apoptosis assay

I also evaluated the effect of AA on inducing the apoptosis of MDA-MB-231 and MCF-7 cells. For this, cells were cultured in 6-well plates for 24 h and treated with various concentrations of AA (10–300 μM). Whole cell lysates were prepared and were subjected to perform the immunoblot analysis to measure the activation of polyadenyl ribose polymerase (PARP). Anti-PARP antibody (Cell Signaling Technology, Danvers, MA) was used to detect the expression of full-length (~110 KD) and cleaved PARP (~89 KD) protein.

2.2.4. Analyses of eicosanoid by reverse–phase, high-performance liquid chromatography (RP-HPLC)

MDA-MB-231 and MCF7 cells were grown in 6-well plates until they reached 90% confluency before treating with AA in the presence or absence of NDGA for 24 h. Cells were separated from the medium, and eicosanoids were extracted using ethyl acetate extraction as described before [91]. The samples were vortexed and centrifuged at 3,000 rpm for 10 min. The top layer containing ethyl acetate was transferred to a Teflon-lined vial and dried under N_2 gas. The dried samples were resuspended in 40 μl of methanol and stored at -20 ^{o}C for further analysis by HPLC (Waters, Inc., Milford, MA). Different eicosanoid standards were analyzed before assessing the eicosanoids from individual samples. The elution times of the eicosanoids were as follows: PGE2, 60 min; PGD2, 64 min; LTB_4, 72 min; HETE5, 85 min, and HETE8, 90 min.

Samples were mixed with acetonitrile (1:1,000) and subjected to HPLC following the method of Moraes et al. [92]. Eicosanoids were eluted with the help of a trifluoroacetic acid (0.1%)-acetonitrile solvent system (flow rate: 0.4 ml/min). Individual eicosanoids were quantified by calculating the areas covered under each peak using EMPOWER software (Waters, Inc., Milford, MA).

2.2.5. Statistical analyses

All results were expressed as the means ±SD. Statistical analysis was performed using Microsoft Excel (2007) software, and the student's t tests were carried out to assess the mean differences between the treatment and control. Values were considered to be statistically significant when $p < 0.05$.*

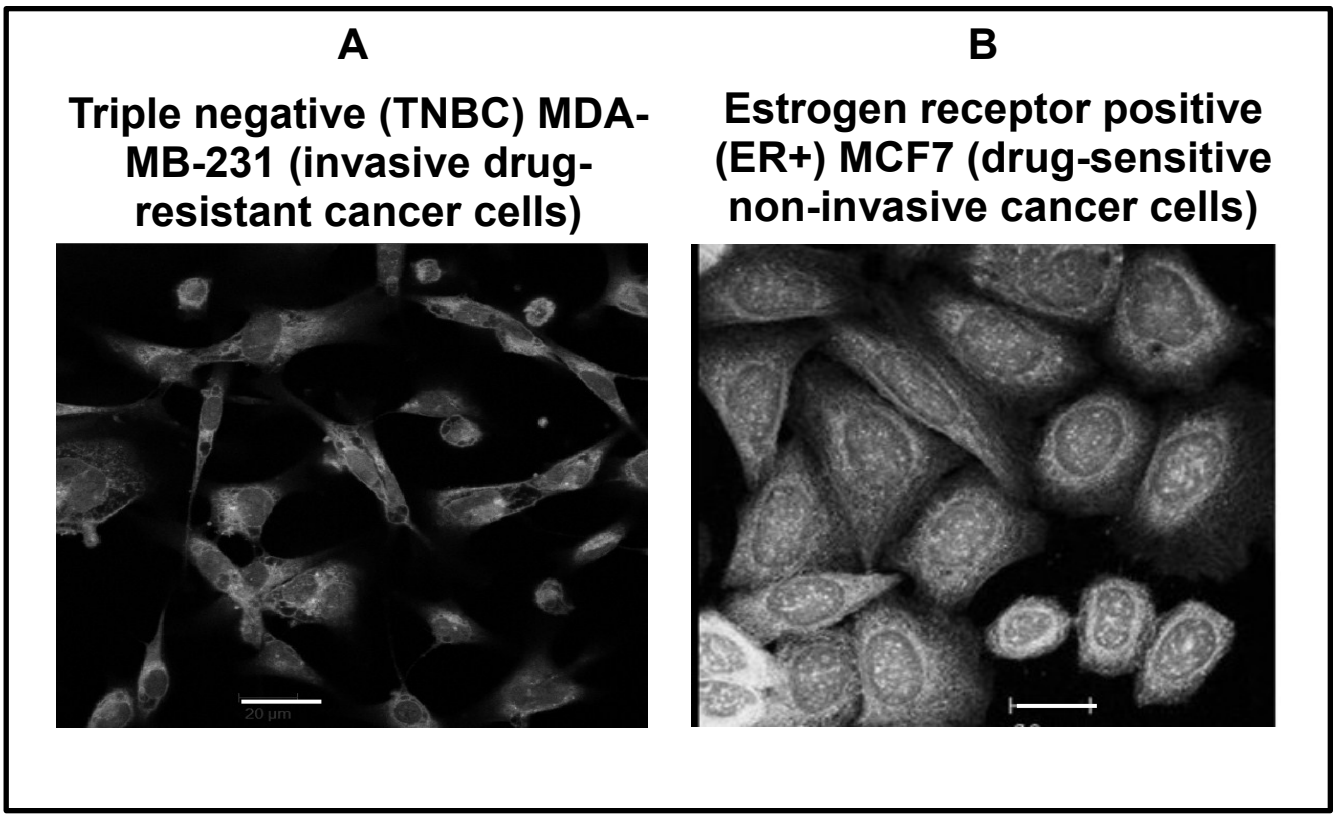


Figure 9

Breast cancer cell images

Figure 9. Breast cancer cell images

Figure 9A and 9B represent the cellular images of MDA-MB-231 and MCF7 cells respectively. MDA-MB-231 is a triple negative, invasive and fibrous-like breast cancer cells, whereas MCF7 is estrogen receptor positive and minimal invasive luminal epithelial cells. Bar: 20μM

2.3. RESULTS

2.3.1. AA and cellular toxicity

Arachidonic acid (AA) is a polyunsaturated fatty acid and a major component of red meat, vegetable oil, etc. We performed a dose-response assay to evaluate the toxic effect of AA on two cancer cell lines, MDA-MB-231 and MCF-7. The cells were briefly treated with various concentrations of AA (i.e., 10, 25, 40, 100, 200, and 300 μM) for 24 h and subjected to MTS assay to determine the cytotoxic effects of AA [93]. I found that the IC_{50} (i.e., the half-maximal inhibitory concentration) of AA was ~200 μM in both MDA-MB-231 and MCF7 cells (Figures 11A and 11B). However, 100 μM AA was used in the current study, which was much lower than the concentration at IC_{50}, and no cytotoxicity was observed at this concentration of AA.

Because AA is known to induce apoptosis, and because of the reason that 100 μM AA was used in this experiment, I asked what doses of AA could induce the apoptosis in these two breast-cancer cells. Polyadenyl ribose polymerase (PARP) assay was performed to identify the dose at which AA can induce the apoptosis in MDA-MB-231 and MCF-7 cells. Full-length PARP protein (PARP-I, ~116 KD) is constitutively expressed in a majority of mammalian cells, and during apoptosis activated caspase-3 cleaves PARP into a relatively smaller fragment (~89 KD). The result, shown in Figure 11C, revealed that a 200-μM concentration of AA can induce the cleavage of PARP-1 with caspase-3, which is a determinant of the activation of an apoptosis cascade. These results suggested that AA at 100-μM concentration was safe for conducting the experiments described in this and the following chapters. Furthermore, other

laboratories also used 100 μM AA for studying the signaling events in cancer and other cells [94; 95].

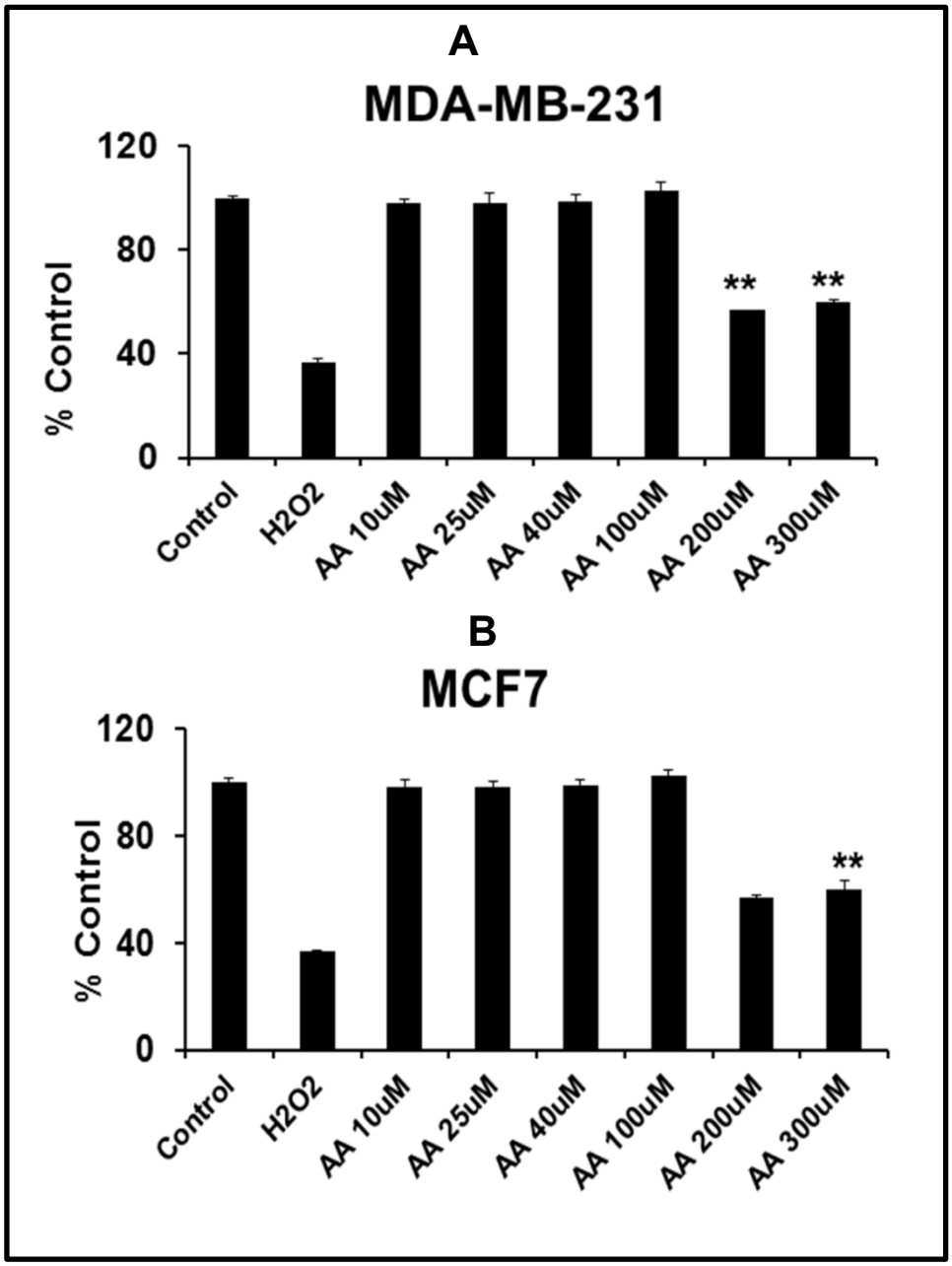


Figure 10

Dose response assay

Figure 10. Dose-response assay

MDA-MB-231 and MCF7 cells were cultured in 96-well plates and treated with various concentrations of arachidonic acid (indicated in the figure) for 24 h and the viability was tested by 3-(4,5-dimethyl thiazol-2yl)-5-(3-carboxymethhoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. MTS compound is bio-reduced by viable cells into a reddish brown colored formazan product. The optical density (OD) of formazan product is measured at an absorbance of 490nm. The OD value is directly proportional to the number of viable cells.

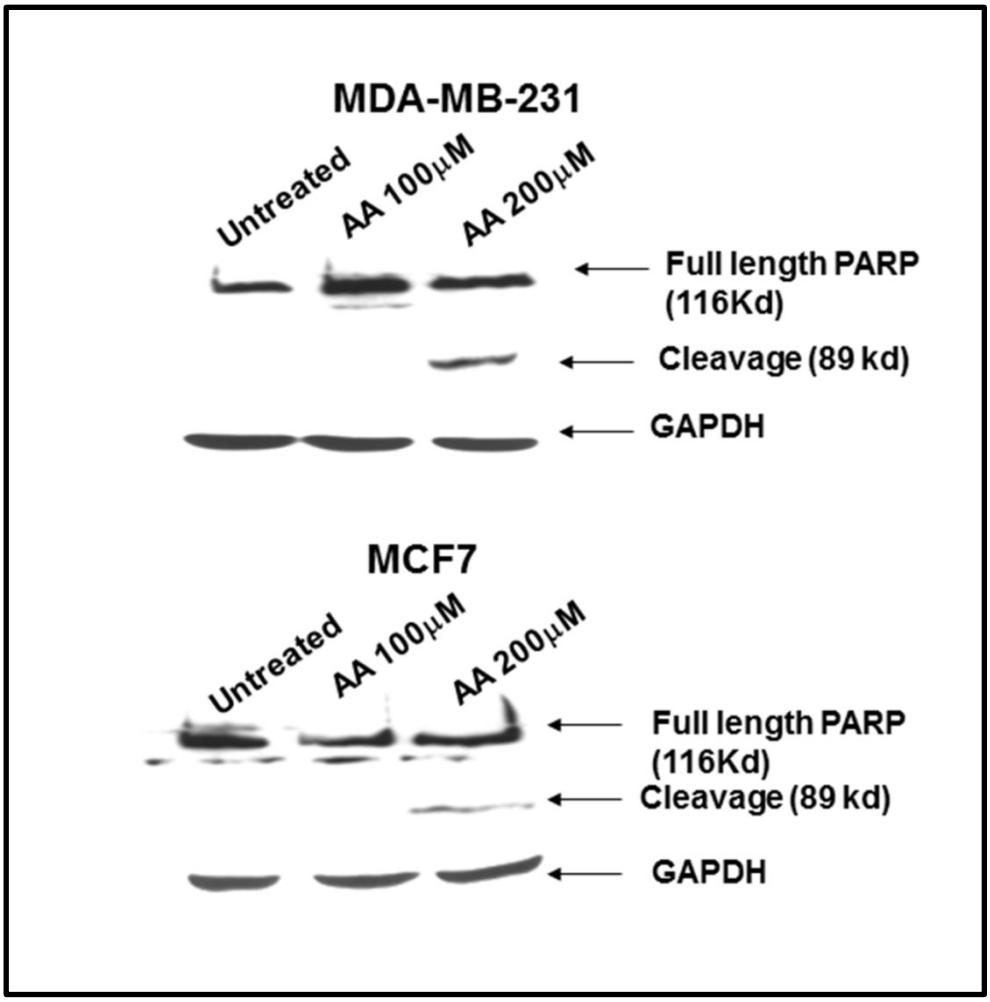


Figure 11

PARP assay

Figure 11. Poly ADP-ribose polymerase (PARP) assay to monitor apoptosis

MDA-MB-231 and MCF7 cells were cultured in 6-well plates and treated with various concentrations of arachidonic acid (AA) for 24 h, cell lysates were prepared and PARP cleavage assay was carried out by immunoblot analysis to assess the apoptosis. The generation of cleaved ~89kD PARP from ~116 kD precursor protein by 200 μM AA treatment is clearly visible.

2.3.3. Effect of AA on eicosanoid syntheses

Even though the incorporation and release of AA from membrane phospholipids are critical for the production of various eicosanoid molecules, it is not clear how exogenously administered AA modulates eicosanoid synthesis in invasive and non-invasive breast cancer cells. To address this, invasive breast cancer MDA-MB-231 and non-invasive MCF7 cells were tested for eicosanoid synthesis under the influence of AA.

The syntheses of various eicosanoids by MDA-MB-231 and MCF7 cells (control and AA treated) were analyzed by reverse phase HPLC, and the areas under each eicosanoid peak were quantified using the integration software EMPOWER 3 (Waters, Inc., Milford, MA). HPLC profiles of different eicosanoids in the control cells are demonstrated in Figures 12A and 12B. The quantitative assessment in Figure 12C shows that MDA-MB-231 synthesizes a higher level of PGE_2 and PGD_2 than MCF7. In contrast, the basal levels of LOX products—i.e, LTB_4, 5-HETE, and 8-HETE—are higher in MCF7 than MDA-MB-231. Interestingly, the AA treatment (100 μM) suppressed the synthesis of all eicosanoids in MCF-7 cells, but in MDA-MB-231 it down-regulated PGE_2 and PGD_2 and stimulated the syntheses of LTB_4 and 8-HETE. Figure 12D shows that AA induces a ~4-fold increase in LTB_4 production in MDA-MB-231, and this could be blocked by NDGA (10 μM), an inhibitor of LT synthesis.

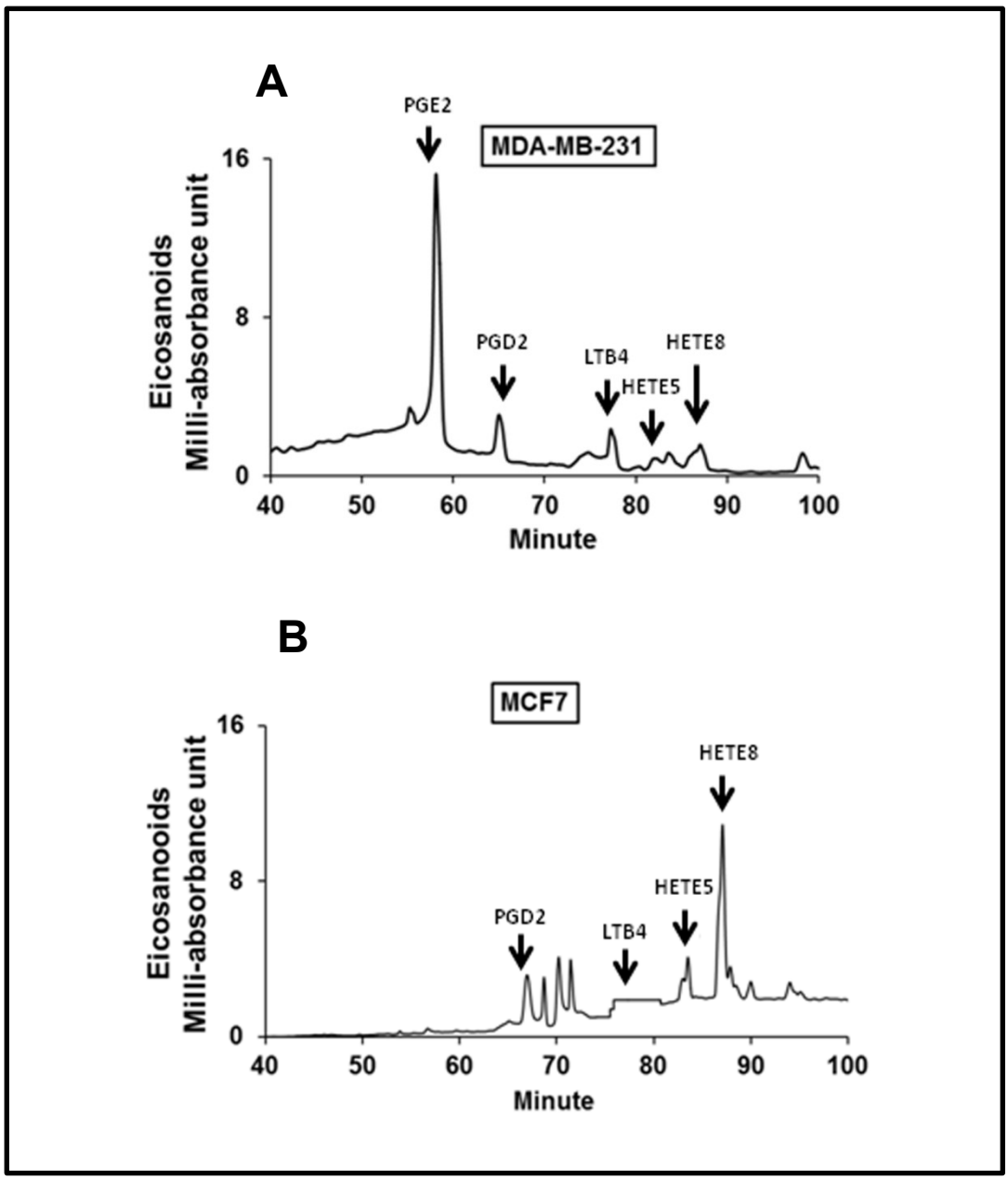


Figure 12

Classes of eicosanoids synthesized by MDA-MB-231 and MCF7 cells

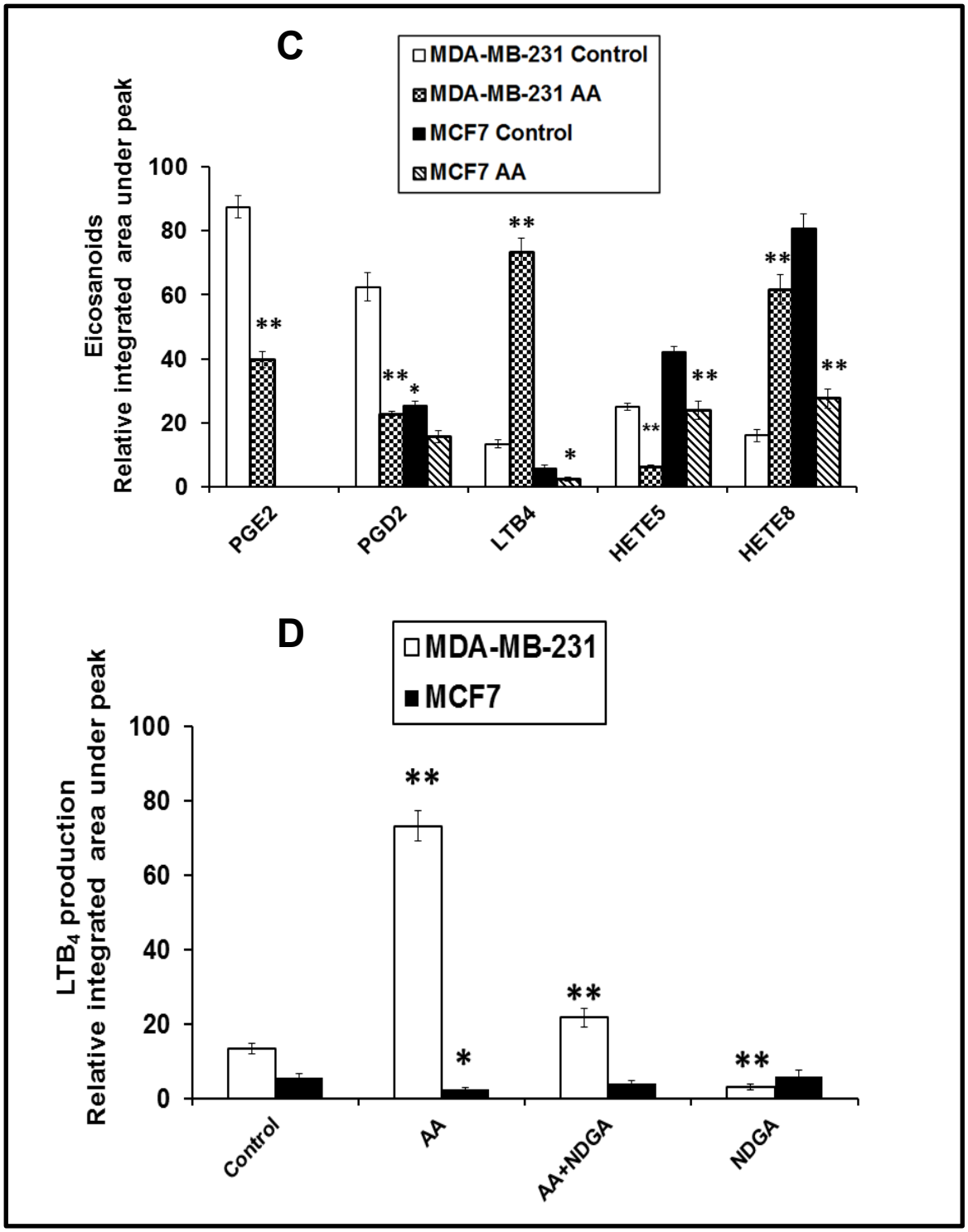

C
MDA-MB-231 Control
MDA-MB-231 AA
MCF7 Control
MCF7 AA
Eicosanoids
Relative integrated area under peak
100
80
60
40
20
0
**
**
*
**
*
**
**
**
PGE2
PGD2
LTB4
HETE5
HETE8
D
MDA-MB-231
MCF7
LTB4 production
Relative integrated area under peak
100
80
60
40
20
0
**
*
**
**
Control
AA
AA+NDGA
NDGA

Figure 12

Figure 12. Classes of eicosanoids synthesized by MDA-MB-231 and MCF7 cells

MDA-MB-231 and MCF7 breast cancer cells were cultured in a growth medium supplemented with fetal bovine serum (FBS, 10%) and cultured until the cells reached confluency (~70–80%). Fresh medium was added to the confluent monolayer and treated with 100 μM AA, 10 μM NDGA (a 5-LOX inhibitor), and 100 μM AA+ 10 μM NDGA for 24 h as described in the Materials and Methods section of this chapter. Secreted eicosanoids were extracted from the media and analyzed by high-performance liquid chromatography (HPLC). Figures 12A and 12B show the HPLC profiles of eicosanoids from MDA-MB-231 and MCF7 cells, respectively. Figure 12C demonstrates the effects of AA (100 μM) on their syntheses. Figure 12D represents the LTB_4 levels in the two cell lines after AA and NDGA treatment. All experiments were repeated at least three times, and the data are given as means ± SE; *$p < 0.01$, ** $p < 0.001$. PGE_2, prostaglandin E_2; PGD_2, prostaglandin D_2; LTB_4, leukotriene B_4; HETE, hydroxyeicosatetraenoic acid.

2.4 CONCLUSION

The goal of Chapter 2 was to provide understanding of how AA, which is a substrate of eicosanoids, modulates the activities of COX-2 and LOX enzymes. I found that AA at 100 μM inhibits the synthesis of COX-2 products but activates two LOX products (LTB4 and 8-HETE) in MDA-MB-231 cells. In contrast, AA inhibits the synthesis of both COX-2 and LOX products in MCF-7 cells. Therefore, I hypothesize that the increased synthesis of LTB_4 and 8-HETE in MDA-MB-231 cells under the influence of AA could be an indication of the changes of the catalytic function of LOXs that allows these groups of enzymes to become hyperactive in invasive breast cancer cells. It is also possible that AA at 100 μM inhibits the catalytic function of COX-2 by an "excess-substrate-inhibition" mechanism that allows the cells to utilize higher concentrations of AA by activated LOX pathways to avoid the toxic effect. The importance of 5-LOX in breast cancer malignancy (as predicted in the current study) can be further supported by the reports from other cancers. For example, elevated activity of 5-LOX is associated with the progression of hepatocellular carcinoma [96], and it also promotes polyposis formation in the intestine [84]. Earlier studies indicated that COX-2 plays an essential role in the development of various forms of cancer and that the mutation in COX-2 genes promotes colorectal carcinogenesis [54]. Interestingly, my results, along with the reports from other laboratories [84; 96], strongly supports the idea that LOX pathway is equally important and involved in the growth and malignancy of breast cells. In fact, a recent study by Wen et al. [97] suggested the role of LTs in breast cancer development and reported an increased activity of 5-LOX with concomitant reduction of COX-2. In

Chapters 3 and 4, I have investigated the pathways by which AA and LTB_4 induce inflammatory reactions and metastatic invasion of MDA-MB-231 breast cancer cells.

CHAPTER 3

SPECIFIC AIM 2

TO EXAMINE WHETHER AA AFFECTS THE PRO-INFLAMMATORY PATHWAYS, CELLULAR MIGRATION, AND INVASION IN CULTURED BREAST CANCER CELLS.

3.1. Introduction

Inflammation, migration, and invasion of cancer cells are associated with the activity of extracellular-matrix ligands and signals necessary for locomotion [66; 98]. Migration facilitates the cancer cells in invading the surrounding tissue and colonizing a new site. Thus, migration and invasion by a cancer cell represent an important step in the spread of the disease across tissues and lymphatic nodes, which are often referred to as metastasis. The mechanisms of cell migration involve complex interactions among various proteins and signaling molecules, activation of focal adhesion kinase (FAK), actin polymerization, lammellopodia formation, and the involvement of extracellular matrix as well as integrin molecules [7; 99]. Reports suggest that various environmental factors, carcinogens, hormones, and fatty acids, including AA, can influence the migration of various cells, including those of breast cancer [8; 9]. AA can activate the nuclear factor kappa beta (NFκB)-mediated inflammatory pathway, which subsequently can degrade the extracellular matrices and promote angiogenesis (Figures 13A and 13B). Studies also suggest that AA promotes the adhesion and migration of endothelial cells and can act as a regulator of wound healing and angiogenesis [8; 100]. The level of PGE_2, a major product of COX-2 reaction, is elevated in breast cancer cells and functions as a pro-tumorigenic agent [101; 102]. COX-2-dependent PG synthesis has been shown to be involved in maintaining the motility and invasion of breast cancer cells [103; 104], and the inhibitor of COX-2 could block the radiation-induced migration of MDA-MB-231 cells [105], suggesting that the COX-2 pathway is important and is involved in the invasion and metastatic migration of breast cancer cells. Like COX

pathways, LOX-derived eicosanoids are also involved in tumor formation and malignancy [106; 107], although many of their roles in inducing migration and metastasis of breast cancer cells are not well understood. In Specific Aim 1 (Chapter 2), I demonstrated that while AA stimulates the production of LTB_4, it inhibits the synthesis of PGs and other eicosanoids in MDA-MB-231 cells. 5-LOX is a member of the LOX family of enzymes that synthesizes LTA_4, LTB_4, LTC_4, LTD_4, and LTE_4 [108]. LTB_4, a 4 series of eicosanoids, is synthesized from unstable LTA_4 by the action of LTA_4 hydrolase. LTB_4 is a powerful bioactive lipid that is involved in various physiological and pathological functions, stimulates the cells via autocrine and paracrine signaling, and acts through the LTB_4 receptor (known as the BLT receptor). LTB_4 has a role in the anaphylectic reactions and is responsible for different lung cancer pathologies [109], and it can stimulate the proliferation of pancreatic cancer cells [110]. In a recent study, a high concentration of exhaled LTB_4 and IL-8 were shown to be associated with the neutrophilic inflammation in the airways of lung cancer patients [109], and a pro-tumorigenic effect of LTB_4 in melanoma cells has also been reported [82]. Recently, Guo et al. have shown that the activation of the 5-LOX pathway is induced in ethanol-induced oral cancer [85]. Likewise, the inhibition of 5-LOX induces apoptosis in prostate cancer cells by down-regulating the activity of protein kinase C [83]. Another recent finding by Cheon et al. [84] suggested a pro-tumorigenic role for hematopoietic 5-LOX in the immune microenvironment and recommended 5-LOX inhibition for the treatment of colorectal polyposis and cancer. A direct association between increased 5-LOX activity and the progression of hepatocellular carcinoma and intestinal polyposis strongly suggests that it plays a role in inflammation, tumor promotion, and metastasis [84; 96].

The goal of Specific Aim 2 is to delineate the inflammatory pathways that are stimulated by excess LTB_4 and how they are linked to the migration and invasion of MDA-MB-231 cells.

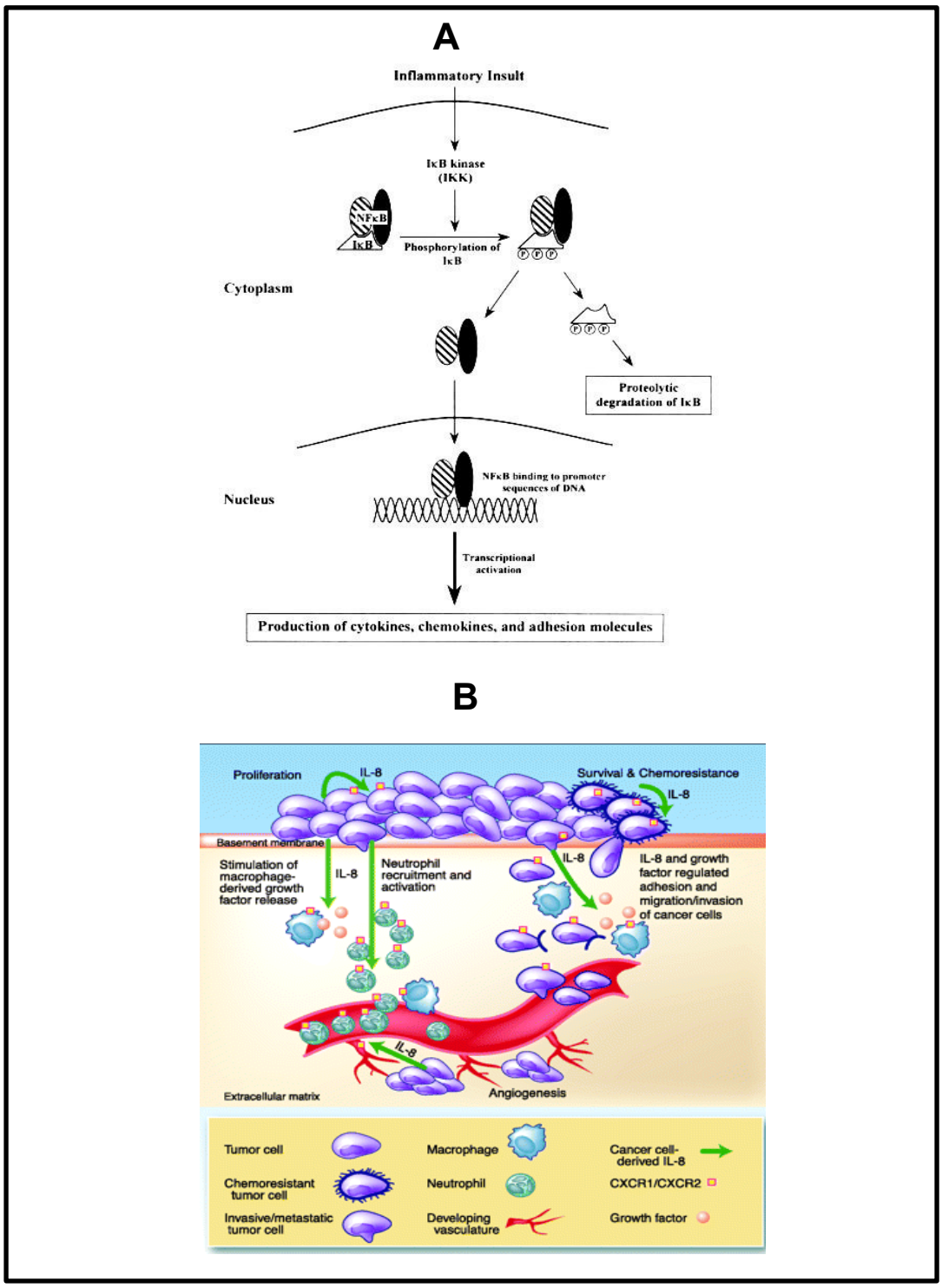


Figure 13

Inflammation and tumor microenviornment

Figure 13. The Inflammation and microenvironment of tumor cells

Figure 13A illustrates the Ikβ and NFκB-mediated inflammatory responses in cancer cells. Cellular inflammation causes the activation of NFκB which subsequently activates the transcription of cytokines, chemokines and adhesion molecules which all together promotes the process of tumorigenesis [111]. Figure13B suggests the IL-8 mediated neutrophil recruitment, stimulation of macrophage derived growth factors and adhesion, migration and invasion by cancer cells [112].

3.2. MATERIALS AND METHODS

3.2.1. Cell culture and treatments

MDA-MB-231 and MCF7 were cultured in RPMI-1640 and DMEM/Ham´s F-12 medium (Gibco Invitrogen, Carlsbad, CA). Both RPMI and DMEM were supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Hyclone, Logan, UT). Breast cancer cells were grown in a humidified atmosphere (95% air and 5% CO_2) until they reached ~90% confluency. Confluent cells were treated with trypsin (0.25% Trypsin-EDTA; Hyclone), harvested by centrifugation, plated on a 6-well plate (~0.5 X 10^6 cells per well) and treated with AA (sodium salt, 100 μM; Sigma) for 24 h with or without prior treatment with the LOX-5 inhibitor nordihydroguaiaretic acid (NDGA, 10 μM; Sigma). Control and treated cells were collected, washed, and subjected to further analyses as described in the text.

3.2.2. Immunoblot analyses

Control and treated cells were harvested, washed in cold PBS, and lysed by freeze-thaw in a lysis buffer, and the protein concentrations were measured with the help of a protein assay kit manufactured by Bio-Rad (Hercules, CA). An equal amount of protein (~40 μg) was applied to each lane and subjected to electrophoresis in SDS-PAGE (10%) followed by immunoblot analyses onto polyvinylidene fluoride (PVDF) membranes as described previously by Albert et al. [113]. The PVDF membranes were incubated overnight (6–10 ^oC) with anti-LOX-5 antibody (polyclonal; Gibco Invitrogen, Carlsbad, CA) or anti-GAPDH (monoclonal; Glyceraldehyde 3-phosphate

dehydrogenase; Cell Signaling, Danvers, MA). The membranes were washed with 0.05% Tween-20 in Tris-Buffered saline (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse and/or goat anti-rabbit IgG (KPL Biomedical, Chantilly, VA). The enhanced chemi-luminescence (ECL) technique was used for the detection of protein bands. The intensities of protein bands were measured using Labworks software (UVP Lab Products, Upland, CA).

3.2.3 Confocal microscopy

MDA-MB-231 and MCF7 cells were grown in 4-well, chambered Lab-TEK II slides overnight containing either RPMI-1640 or DMEM/F12 media. The cells were treated with AA and/or NDGA and were fixed with methanol (100%, chilled) for 5 min at -20 ^{o}C before being blocked with normal goat serum (NGS, 5%; Sigma) for 1 h. Methanol-fixed cells were incubated overnight in the cold room with anti-5-LOX (1:200; polyclonal, Cayman Chemical, Ann Arbor, MI) 1% NGS. The slides were washed three times and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, mounted with ProLong® Gold antifade reagent mixed with DAPI (Gibco Invitrogen, Carlsbad, CA). The confocal images were captured with the help of an LSM 700 Zeiss confocal microscope and analyzed using Zen 2009 software (Carl Zeiss, Thornwood, NY) as described earlier. Each experiment was repeated three times. Cells were randomly selected from each slide using the same resolution, laser power, and detector-gain. Student's t tests were used to assess differences (means) between the treatment and control*;* $p < 0.05$ was considered significant.

3.2.4 Phospho-NFκB assay

To measure the levels of phospho-NFkB (p65), ~0.5 x 10^6 cells per well were plated in 6-well plates and cultured overnight. Cells were incubated for an additional 24 h in the presence of AA (100 μM), AA (100 μM) + NDGA (10 μM), or NDGA (10 μM). Cell lysates were prepared using the lysis buffer provided with the kit, and equal concentrations of proteins were loaded in an antibody-coated, 96-well plate following the instructions provided by the manufacturer. The absorbance of the samples was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) set at 450 nm. Values are expressed as relative optical density (OD) ± SD.

3.2.5. Analysis of IL-6 and IL-8 production

Approximately 1 x 10^5 cells were cultured in 12-well plates for the determination of IL-6 and IL-8 levels. Cells were treated with AA, AA + NDGA, or NDGA for 24 h as mentioned above, and the supernatants were removed and centrifuged at 12,000 rpm for 5 min. The concentrations of IL-6 and IL-8 in the culture supernatants were analyzed by a commercially available enzyme-linked immunoassay kit (SA Biosciences, Qiagen, MD) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Bio-Rad Laboratories, Hercules, CA) at 450 nm, a standard curve was generated by plotting the optical density in y-axis against the amount (ng) of the cytokines in the x-axis (i.e., OD vs ng/ml), and the amounts of interleukins present in the samples were determined in ng/ml/10^5 cells ± SD.

3.2.6. Cell migration and invasion assays

Migration of breast cancer cells was examined with the help of wound-healing assays, which are standard procedure in monitoring cell migration and angiogenesis [8]. For migration assays, ~0.5 X 10^6 cells were plated in each well of a 6-well plate and incubated overnight in a medium (RPMI-1640 or DMEM) containing Fetal Bovine Serum (FBS, 5%). It was observed that 5% FBS (instead of 0 or 10%) was optimal to monitor the effect of AA-induced migration of MDA-MB-231 cells. The monolayers were wounded by scratching with a sterile 10-μL pipette tip [8], and they were treated with AA (100 μM), AA (100 μM) + NDGA (10 μM), NDGA (10 μM), or methyl-β-cyclodextrin (MBCD, 1 mM) for 24 h. The images of the cells that had migrated between wounded regions were captured using a Nikon TMS microscope equipped with a Nikon F-601 camera, and they were counted. Each experiment was repeated for three times (n=3) with multiple technical replicates.

Cell invasion assays, which represent the growth and metastatic migrations of cancer cells, were carried out in Transwell chambers [114]. Briefly, cells were cultured in 12-well plates (~3 x 10^5/well) for 24 h in the presence of AA, NDGA, and MBCD as described above. Cells were harvested and suspended in 500 μl medium containing 5% FBS and added to the upper layer of Transwell chambers. The bottom wells were filled with 1 ml of culture medium containing 10% FBS (to promote cell growth and invasion through the matrigel of Transwell chambers). The cells that had migrated to the lower chamber after 24 h of incubation were examined and photographed using a Nikon TMS microscope equipped with a Nikon F-601 camera. Migrated cells were also stained with

calcein (Invitrogen), a fluorescence dye that labels the viable cells. Both cell counts and fluorescence intensities were taken into account to measure the invasiveness [115].

3.2.6. Statistical analyses

All results were expressed as the means ± SD. Statistical analysis was performed using Microsoft Excel (2007) software, and student's t tests were carried out to assess the differences (means) between the treatment and control. Values were considered to be statistically significant when $p < 0.05$.*

3.3. RESULTS

3.3.1. Arachidonic acid stimulates 5-LOX expression in MDA-MB-231 cells

The 5-LOX is an essential enzyme of the AA pathway and is involved in the synthesis of LTB4 and various cysteinyl-LTs, including LTC_4, LTD_4, and LTE_4. Because AA treatment increases the production LTB4 in MDA-MB-231 cells, we asked if AA stimulates the expression of this enzyme in these triple-negative breast cancer cells. To address this, AA-treated cells were analyzed by immunoblot (Figures 14A and 14B) and confocal microscopy (Figures 14C and 14D) using anti-5-LOX antibody. Figure 14A shows that while both cells express 5-LOX protein, only MDA-MB-231 responded to AA treatment as far as the stimulation of 5-LOX is concerned. AA treatment induced the synthesis of 5-LOX by ~2–fold, as evidenced by the densitometry analysis shown in Figure14B, and this increased expression could be reduced by NDGA. In contrast, the level of 5-LOX in MCF7 remained unaltered by AA and NDGA treatment. In fact, NDGA alone stimulated the expression of 5-LOX slightly in MCF7 cells (Figures 14A and 14B).

The expression of 5-LOX was also monitored in MDA-MB-231 and MCF7 cells by immunofluorescence microscopy. The results indicate that anti-5-LOX antibody reacts with cellular LOX in both cells (as evidenced by green fluorescence), although the fluorescence intensity in MDA-MB-231 is higher (~2-fold) than MCF7 (Figures 14C and 14D). Figure 14C shows that 5-LOX in spindle-shaped MDA-MB-231 cells are mostly localized in the cytoplasm (photograph a). Upon stimulation by AA, the expression of 5-LOX increases ~2-fold compared with the control, and a greater part of 5-LOX translocates from cytoplasm to elongated extensions (photograph b). As shown in

photograph c (Figure 14C), NDGA reduces the AA-stimulated 5-LOX reactivity effectively (~4-fold). On the other hand, in MCF7, which exhibits dome-shaped epithelial-like morphologies, 5-LOX expression increases slightly and is concentrated mainly in the nucleus. Interestingly, NDGA shows minimal effects on 5-LOX reactivity in MCF7 cells (Figures 14C and 14D).

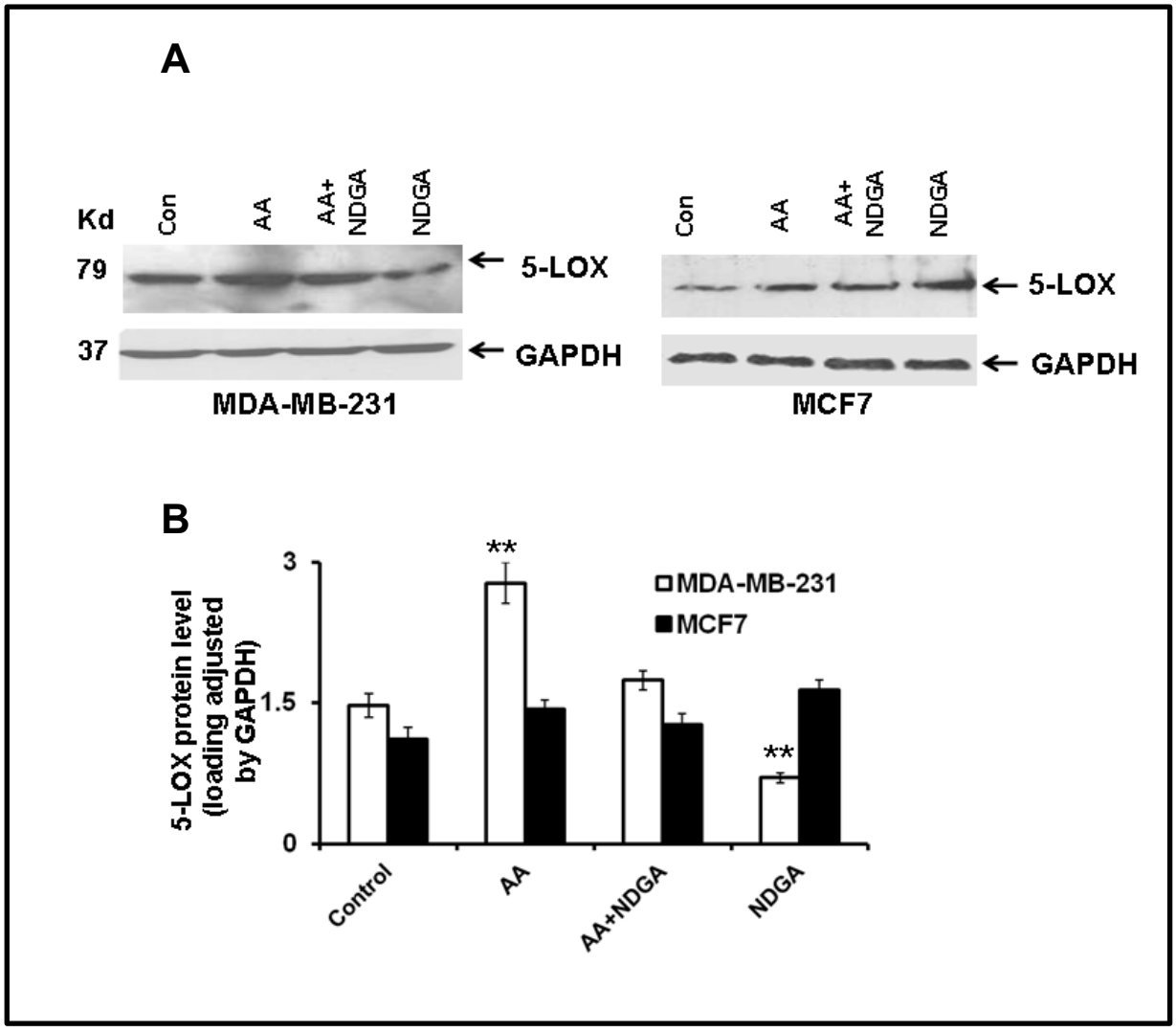


Figure 14

5-LOX expression in MDA-MB-231 and MCF7 cells

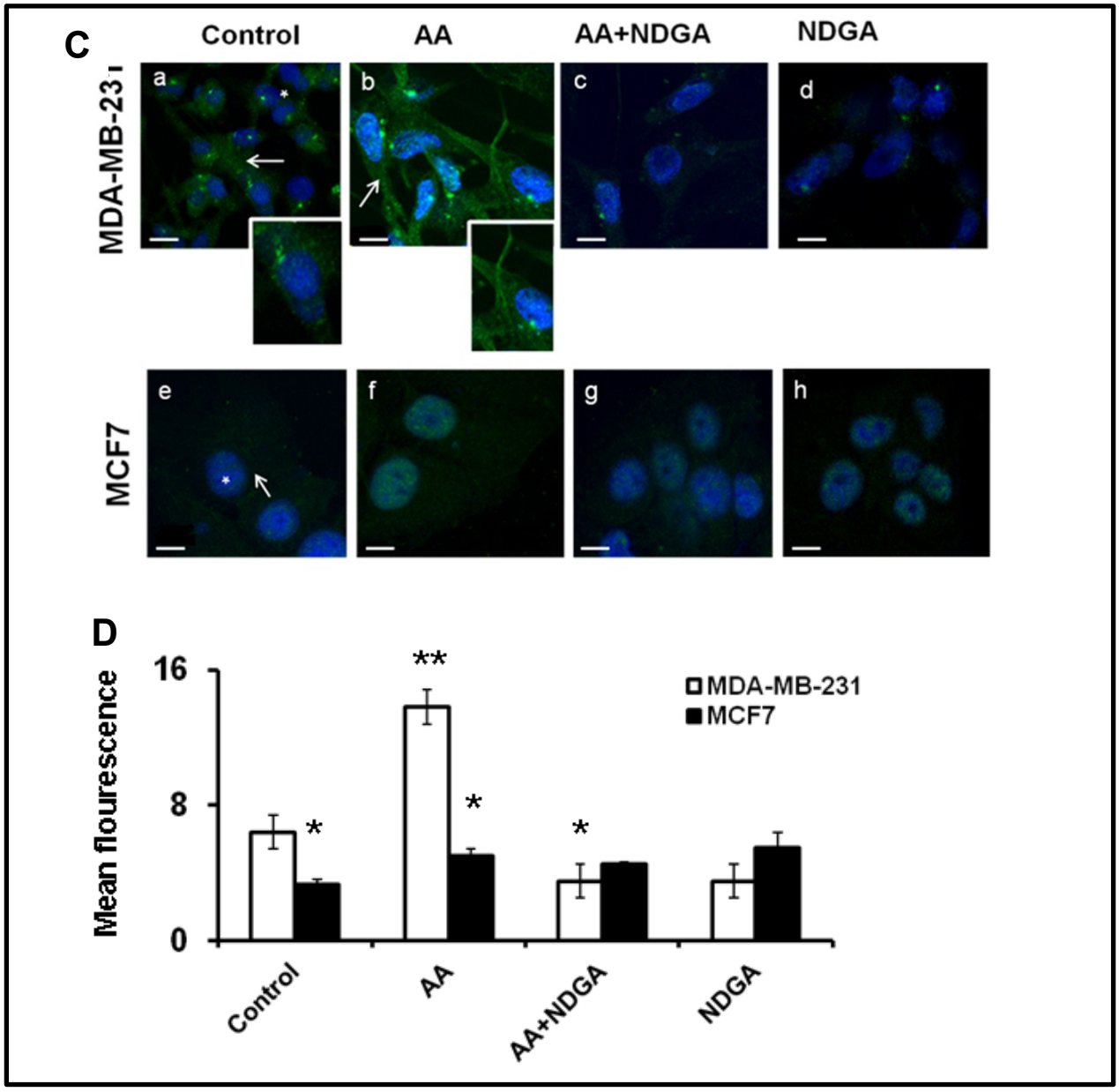

C
Control
AA
AA+NDGA
NDGA
MDA-MB-231
MCF7
a
b
c
d
e
f
g
h
D
Mean flourescence
16
8
0
**
*
*
*
MDA-MB-231
MCF7
Control
AA
AA+NDGA
NDGA

Figure 14

Figure 14. Arachidonic acid stimulates the expression of 5-lipoxygenase (5-LOX) activity in MDA-MB-231 cells

MDA-MB-231 and MCF7 cells were cultivated in 6-well plates or 4-well chamber slides containing growth medium for 24h and treated with either AA or AA+NDGA or NDGA as described in Figure 12 before immunoblot and confocal microscopy analyses. Figure 14A demonstrates the expression ~79 KD 5-LOX in MDA-MB-231 and MCF7 in the presence of AA (100 μM) or NDGA (10 μM) or AA+NDGA combination. Figure 14B shows the densitometric analysis of each band shown in Figure 14A. Glyceraldehyde-3-phosphodehydrogenase (GAPDH, ~37kD) was used as a loading control. Figure 14C exhibits the cellular localization of 5-LOX in MDA-MB-231 and MCF7 cells in the presence of AA and/or NDGA. For confocal analyses, the cells were fixed and labeled with anti-5-LOX polyclonal antibody, and the images were captured with the help of a Carl Zeiss Laser Scanning Confocal Microscope as described in the text. photograph a: MDA-MB-231, control; photograph b: AA-treatment; photograph c: treatment with AA+NDGA; photograph d: NDGA treatment; photograph e: MCF7control; photograph f: AA treatment; photograph g: treatment with AA+NDGA, and photograph h: the treatment with NDGA. Arrow denotes cytoplasm and asterisk indicates the nucleus. Bar: 10 μM. The quantitative assessments of mean fluorescence intensity of 5-LOX in MDA-MB-231 and MCF7 cells are shown in Figure 14D. All data represent the average of three independent experiments. N = 3; * $p < 0.01$, ** $p < 0.001$

3.3.2 Arachidonic acid elevates the levels of NFκB, IL-6 and IL-8 in MDA-MB-231 cells

Neoplasm is often preceded by inflammatory reactions before the characteristics of malignancy are observed. Some of the key molecules that are shown to be associated with cancer inflammation include various transcription factors and pro-inflammatory cytokines [116]. The transcriptional factor NFκB, which is also a potential tumor promoter, is considered to be an important modulator of innate immunity and inflammation [117]. For example, NFκB is responsible for the activation of IL-6 and IL-8, two pro-inflammatory cytokines in tumor cells [118; 119]. Because phospho-NFκB p65 (RelA) is known to be linked to the expression of cytokine genes, and because AA activates 5-LOX expression (Figure 14), we asked whether AA also modulates the expression of phosphorylated NFκB and pro-inflammatory cytokines. Figure 15A shows that AA stimulates (~2-fold) the production of phospho-NFκB p65 and (ser 536) and that NDGA blocks this stimulation. Compared with MDA-MB-231, however, MCF7 exhibits a low level of phospho-NFκB expression and remains unaltered by AA or NDGA. Since this transcription factor is linked to the expression of various cytokine genes, we inquired as to whether interleukins and other inflammatory molecules are modulated by AA. Various pro- and anti-inflammatory cytokines (i.e., IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFNγ, and TNFα) were measured using commercially available, specific assay kits as mentioned in the Materials and Methods section. We observed that except for IL-6 and IL-8 the majority of cytokines were either very low or could not be detected (not shown). The results show that MDA-MB-231 cells produce higher levels of IL-6 and IL-8 (~2- and ~5-fold, respectively) compared with MCF7, and that AA

increases the production of these two interleukins further (Figures 15B and 15C). Interestingly, the induction of IL-6 by AA is more dramatic than it is for IL-8. As expected, NDGA is effective in lowering the levels of both interleukins that were stimulated by AA. NDGA by itself also showed some reduction of IL-6 and IL-8, and this could be due to the fact that NDGA inhibits the metabolism of endogenous AA and interferes with cytokine production. These experiments suggest that AA-induced 5-LOX activation and LTB_4 production are linked to NFκB IL-6 and IL-8 synthesis.

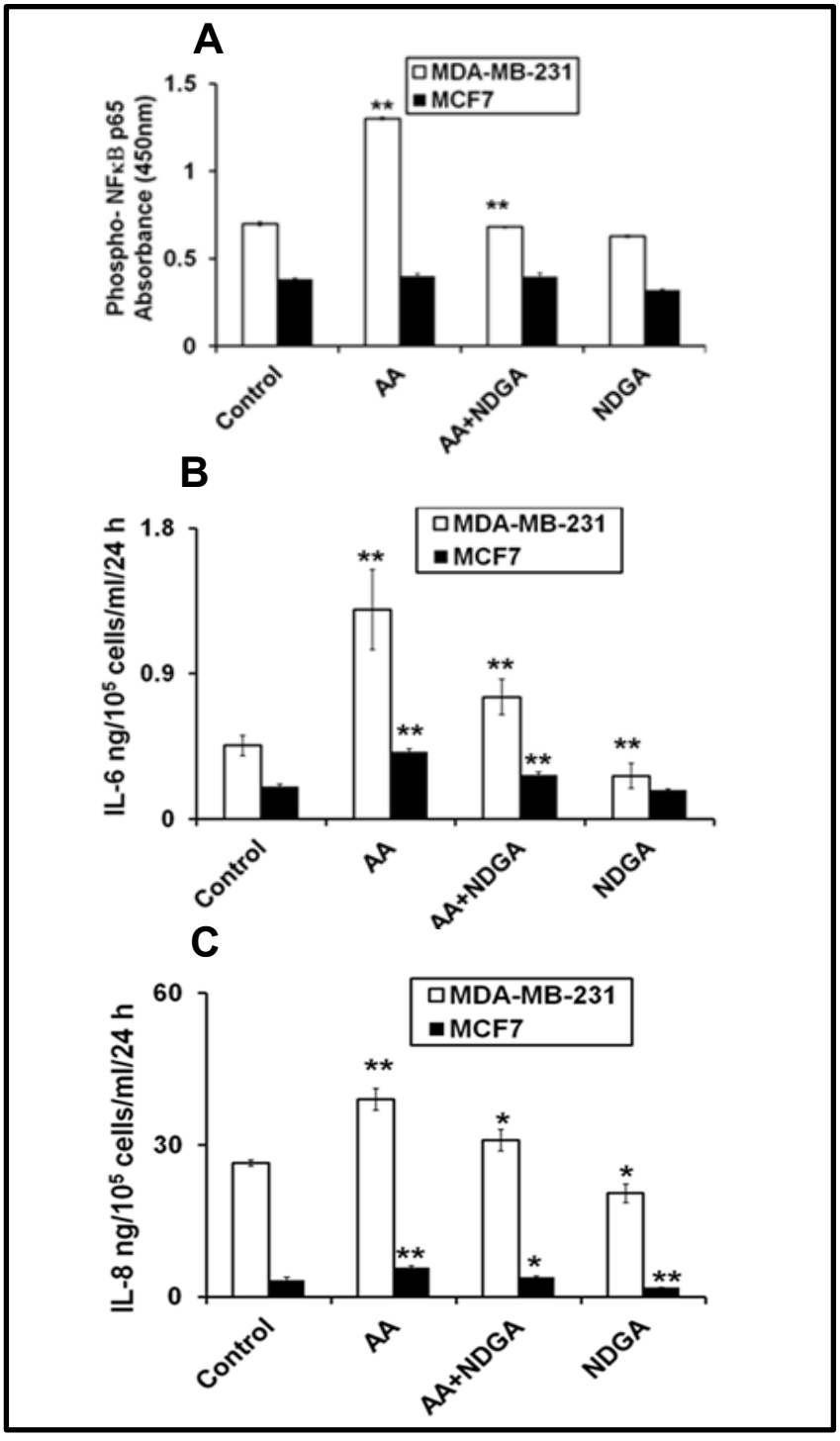


Figure 15

Arachidonic acid stimulates the production of phospho-NFκB (p65), IL-6 and IL-8

Figure 15. Arachidonic acid stimulates the production of phospho- NFκB (p65), IL-6 and IL-8

MDA-MB-231 and MCF7 cells were cultured and treated with AA, AA+NDGA, or NDGA before measuring the phospho-NFκB (Figure15A), IL-6 (Figure15B) and IL-8 (Figure 15C) activities using commercially available assay kits as described in the methods and materials. Data represent the average of three independent experiments. *p < 0.01,** p < 0.001.

3.3.3 Arachidonic acid-induced LTB_4 production is associated with the migration and invasion of MDA-MB-231 cells

Metastatic migration of cancer cells is associated with the activity of extracellular-matrix ligands and signals necessary for locomotion [98; 99]. Migration promotes the cancer cells in invading the healthy tissue to form tumors. Therefore, we tested the possibility that AA-induced LOX-5 activation and LTB_4 synthesis facilitate the migration and invasion of MDA-MB-231 cells. Briefly, MDA-MB-231 or MCF7 cells (MCF7 was used as a negative control) were placed in a 6-well plate and incubated overnight as described in Materials and Methods. The monolayers were wounded by scratching and were cultured for 24 h in the presence and absence AA and/or NDGA before the counting of the migratory cells. Figure 16A compares the migration of MDA-MB-231 cells across the wounded region at 0 and 24 h of incubation in the culture plate (photographs a and b). Photograph c (Figure 16A) shows that the number of migratory cells in AA-treated plates increased ~2-fold and that NDGA blocked this increased migration significantly (photographs d and e, Figure 16A). As expected, MCF7 cells showed little or no migration across the wounded region (photographs f–j). Figure 16B measures the number of migrated cells in the referred wounded region shown in Figure 16A. Side by side with the migration assay, we also examined the invasion of matrigel by MDA-MB-231 cells and asked whether this invasion could be influenced by AA. Invasion capability was monitored in Transwell invasion chambers as described in Materials and Methods. Cells were loaded into the upper chambers, incubated for 24 h, and the viable cells that moved to the lower chambers were assessed by counting the cells with the help of a microscope and testing their viabilities by labeling with calcein. Figure 17A

demonstrates the number of MDA-MB-231cells (~80 cells/microscopic field) that migrated to the lower chambers by crossing the matrigel, and this number increased in the presence of AA by ~1.7-fold. NDGA blocked this invasion when treated with and without AA. Data shown in Figure 17B further support the cell-count results (Figure 17A) and indicate that the invading MDA-MB-231 cells are viable and that the number of viable cells that migrated into lower chambers after AA treatment increased by ~1.5-fold. As expected, NDGA reduced the migration of MDA-MB-231 cells into the lower chambers (Figure 17B).

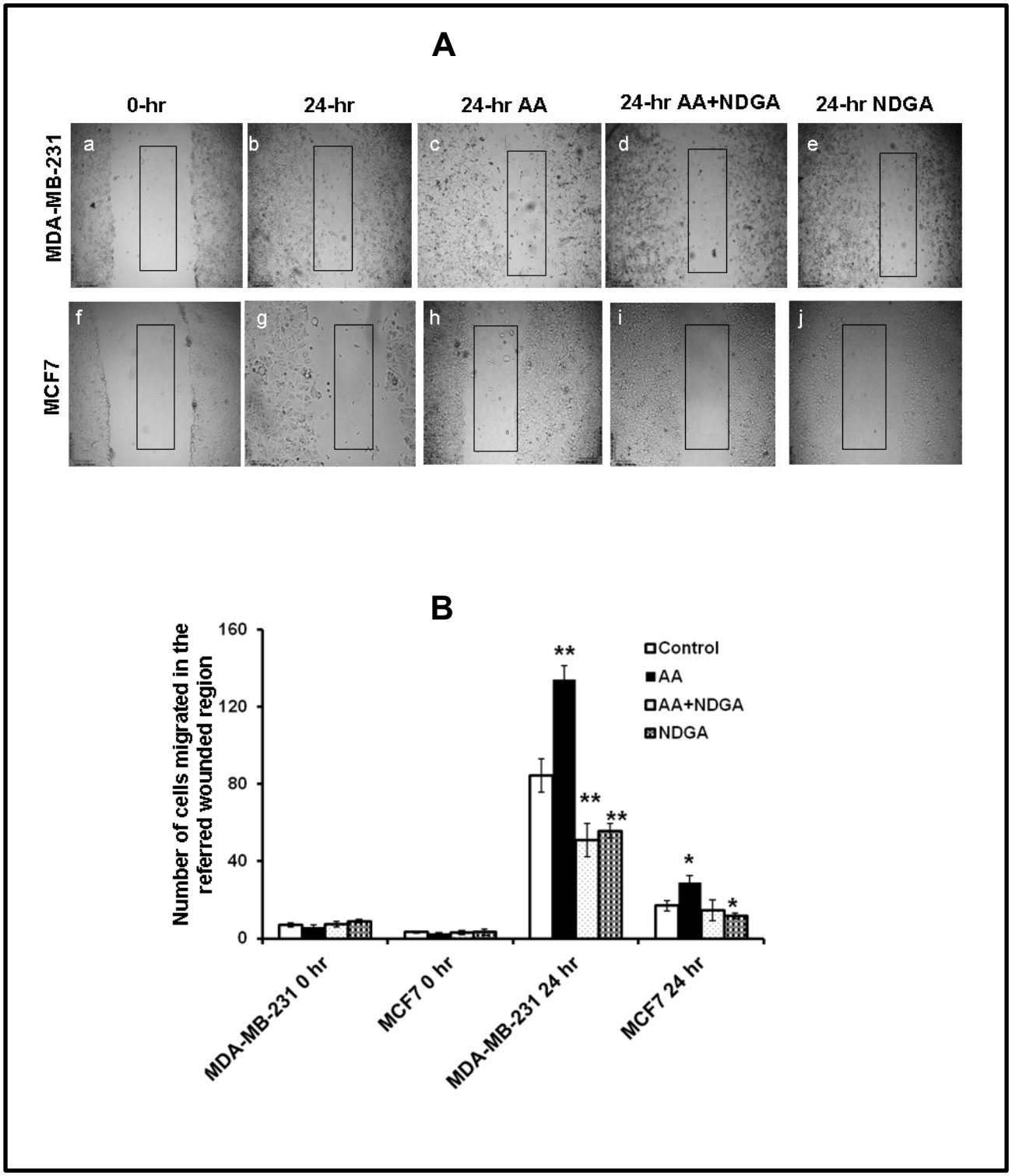


Figure 16

The migration of MDA-MB-231 cells are induced by arachidonic acid and blocked by NDGA

Figure 16. The migration of MDA-MB-231 cells are induced by arachidonic acid and blocked by NDGA

Approximately 1 X 10^6 cells were grown in a 6-well plate in growth medium supplemented with 5% FBS. The cells (~70–80% confluent monolayer) were scratch-wounded by a 10-μM pipette tip and treated for 24 h with AA (100 μM), AA (100 μM) + NDGA (10 μM) or NDGA (10 μM). The photographs were captured at 0 h and 24 h time points as marked in the photographs. Figure 16A: photograph a: MDA-MB-231 at 0 h wound-scratch; photograph b: 24 h; photograph c: migration at 24 h in the presence of AA (100 μM); photograph d: AA+NDGA (100 μM + 10 μM); photograph e: NDGA (10 μM); photograph f: MCF7- at 0 h wound-scratch; photograph g; at 24 h wound-scratch; photograph h: treatment with AA (100 μM); photograph i: treatment with AA+NDGA (100 μM + 10 μM); and photograph j: NDGA-treatment (10 μM). The cells migrated in the wound-region were counted and are represented in Fig. 16B. All experiments were repeated at least three times, and the data are given as means ± SE; *$p < 0.01$, ** $p < 0.001$.

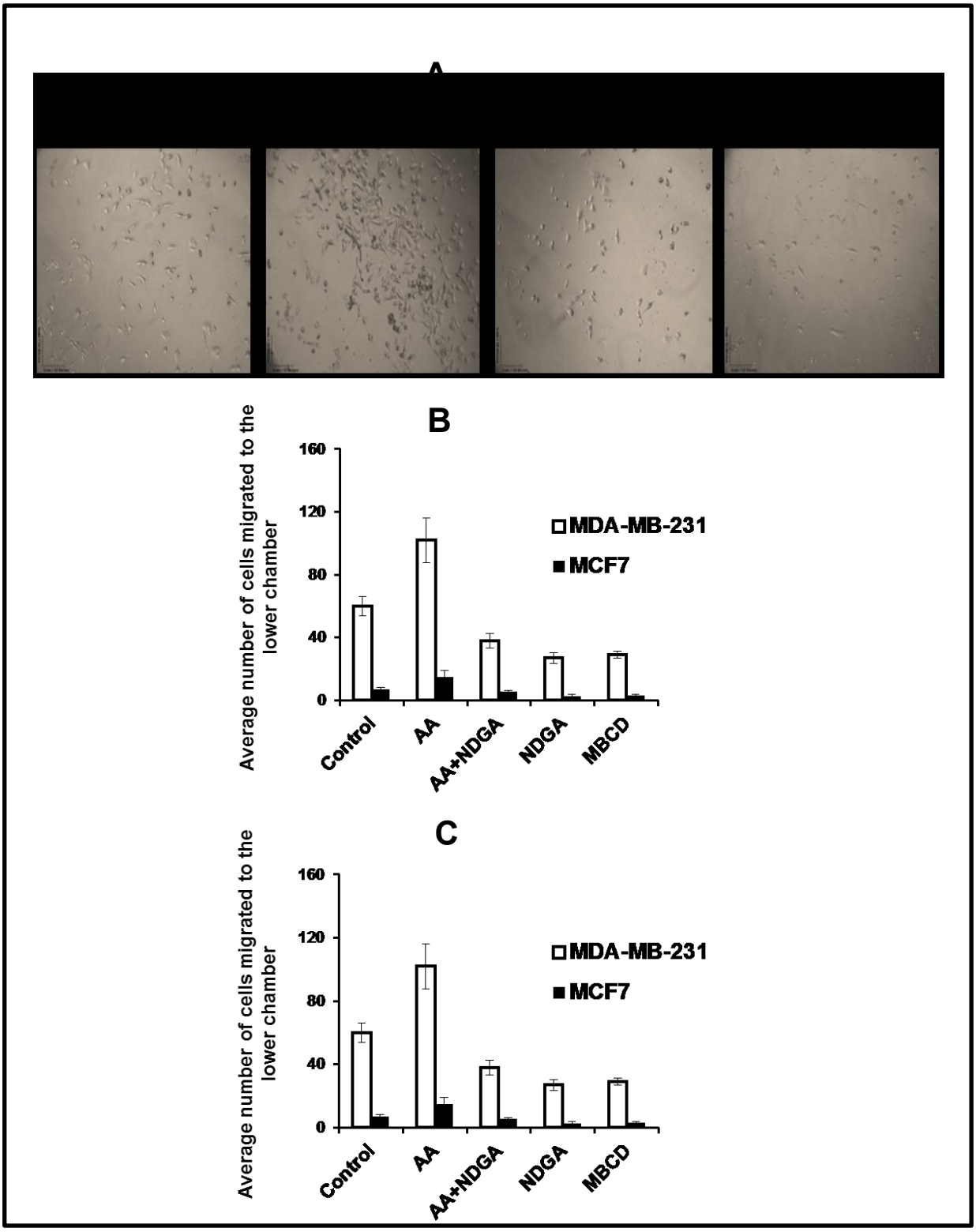


Figure 17

Arachidonic acid-stimulates the invasiveness of MDA-MB-231 cells

Figure 17. Arachidonic acid-stimulates the invasiveness of MDA-MB-231 cells

Cells were cultured in a 12-well plate and were treated with AA (100 μM), NDGA (10 μM) as mentioned before. Cells were then harvested and suspended in 500 μl medium containing 5% serum and loaded on to the upper wells of a Transwell Chamber. The bottom wells were filled with 1 ml of complete culture medium containing 10% serum. The photographs of invading breast cancer cells in 24-h in the lower wells were captured (Figure 17A) and counted from four different microscopic fields. Figure 17B represents the average number of cells counted. The invaded cells in the lower wells were stained with 2μM of calcein and incubated for 30 min at room temperature. The fluorescence of the viable cells was measured by fluorescence plate reader at an excitation and emission of 490 and 520. Figure17C represents the mean fluorescence of the viable wells. The results shown here are the average of two individual experiments which were carried out in duplicate.

3.4. CONCLUSION

The results depicted in Specific Aim 1 indicate that AA treatment suppresses the syntheses of PGs in MDA-MB-231 and stimulates the syntheses of LOX products—e.g., LTB_4 and 8-HETE—suggesting that the LOX pathway is equally important and activated during malignancy. The increased synthesis of LTB_4 in MDA-MB-231 cells under the influence of AA could be an indication of the changes in the catalytic function of LOXs that allow these groups of enzymes to become hyperactive in breast cancer. Thus, in Specific Aim 2, I examined the effect of AA and NDGA (LOX inhibitor) on the secretion of the pro-inflammatory cytokines IL-6 and IL-8, which showed a significant up-regulation after AA treatment, whereas NDGA was able to inhibit the AA-induced IL-6/IL-8 productions in MDA-MB-231 cells. It is interesting that invasive MDA-MB-231 cells exhibited higher levels of IL-6 and IL-8 than MCF7, suggesting a positive role for these two cytokines in the inflammation invasion of breast cancer cells. I also made an effort to investigate the stimulation of phospho-NFkB p65 (ser 536), which is known to regulate the transcription of IL genes. I found that invasive cells (MDA-MB-231) show a higher level of phosphorylation of NFkB compared with the non-invasive cells (MCF-7). AA treatment shows the increase of NFkB phosphorylation, and NDGA inhibits this process. Again, this finding shows, by modulating NFkB phosphorylation, the pro-inflammatory role of AA in breast cancer cells. These findings suggest that AA-mediated activation of pro-inflammatory signaling in invasive breast cancer cells occurs via the 5-LOX-mediated LTB_4 synthesis and subsequent activation of NFkB and cytokine secretion.

Migration and invasion represent an important step in the metastasis and are often referred to in trying to understand cellular aggression. After elucidating the role of 5-LOX in the initiation of signaling cascade, I next demonstrated that AA promotes cellular migration and invasion via LTB_4. Increased production of IL-6 and IL-8 is either directly or indirectly linked to migration and invasion by MDA-MB-231 cells. Earlier, AA was reported [9] to increase the migration of MDA-MB-231 cells by activating focal adhesion kinase (FAK), although the actual mechanism was not proposed. Thus, it can be speculated that AA induces the migration of invasive breast cancer cells by activating FAK via LTB_4 and IL-6/IL-8 cytokines through a mechanism that is yet to be elucidated.

CHAPTER 4

SPECIFIC AIM 3

TO UNDERSTAND WHETHER LIPID RAFT IS INVOLVED IN THE LEUKOTRIENE B_4- MEDIATED INFLAMMATION

4.1. INTRODUCTION

Lipid rafts (LRs) are cholesterol and sphingolipid-rich membrane microdomains responsible for generating intracellular signals downstream [120]. In addition to sphingolipids and cholesterol, mammalian LRs also contain saturated fatty acids, cell-surface proteins, receptors, phospholipids, and glycosyl-phosphatidylinositol (GPI), as demonstrated in Figure 1A. Various membranous proteins, such as caveolin, flotilin, matrix metalloproteinase (MMP), etc., are compartmentalized in the raft domain and play significant roles in the cell signaling process [121; 122; 123]. Cancer cells having an increased amount of metabolic rates exhibit elevated LR formation, resulting in active transportation machinery compared with the normal epithelial cells. LRs are reported to promote the invasiveness and migration of cancer cells by facilitating invadopodia and lamellopodia, although the actual mechanism is not yet clear [124].

Caveolae are subtypes of LRs, and they are composed of caveolins, an integral membrane protein (Figure 1B). Caveolins make flask-shaped invaginations in the membrane, called caveolae [125; 126]. The caveolin family consists of three different isoforms in mammals—caveolin-1, -2, and -3 with caveolin-1 and -2 being expressed in most of the tissues in mammals, whereas caveolin-3 is only expressed in muscle cells [127]. Recently, Goetz et al. [66] reported that cellular metastasis in melanoma is associated with the over-expression of caveolin in the fibroblasts. LR-mediated endocytosis was shown to play a critical role in the regulation of cancerous cell growth, signal transduction, apoptosis, adhesion, cell motility, and tumor metastasis [66; 128]. LR markers like caveolin, flotilin, MMP-9, mono-sialoganglioside (GM1), etc., are found to be over-expressed and play a major role in tumor cell survival, aggression, and

metastatic potential [129; 130; 131]. Antibodies against different LR markers are used for identifying cellular LRs. GM1 is the most commonly used marker among various lipid components of rafts; it is detected using the GM1-binding molecule cholera toxin subunit B (CTxB).

LRs can be fractionated as detergent-resistant membrane (DRM) fractions by treating with nonionic detergents (Triton X-100). Rafts enriched with cholesterol and sphingolipid are insoluble in detergent, and they float in the low-density area during gradient centrifugation [132]. Interestingly, it has been postulated earlier that the LTB_4 receptor-1 (BLT-1) is located within the LRs of activated human neutrophils and that disruption of LRs suppresses LTB_4 syntheses [60]. Thus, to elucidate whether AA-mediated signaling occurs through the LRs, we labeled LRs using anti-caveolin-1 or Ctx B antibody. We hypothesized that LRs play an important role in the LTB_4-mediated signaling. We also addressed the question of whether LRs play any significant role in cellular migration and invasion in breast cancer cells.

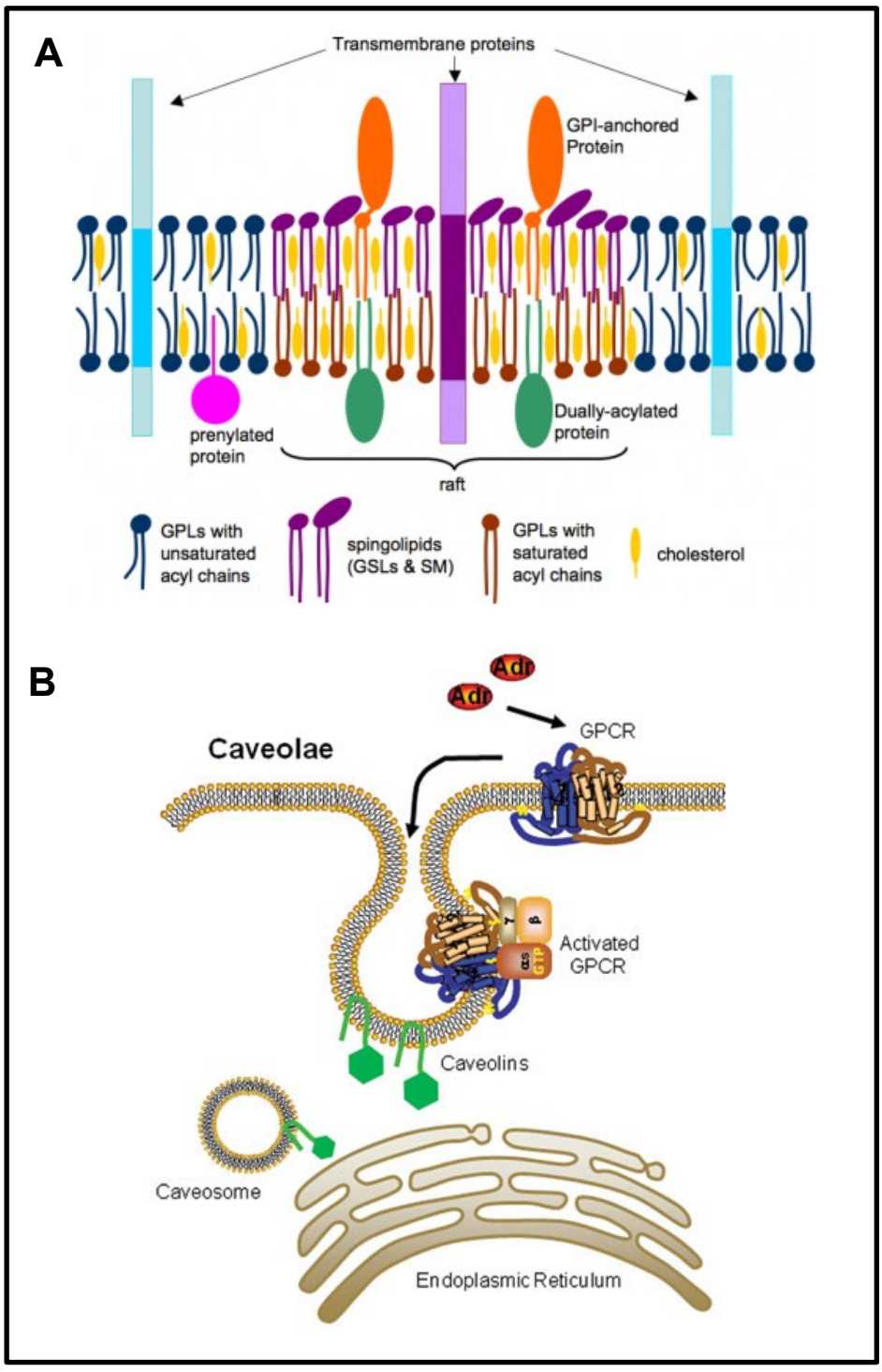


Figure 18

Simplified model of lipid raft

Figure 18. A simplified model of lipid rafts

Lipid rafts are membranous lipid microdomains, which is primarily consisting of several lipid molecules such as sphingolipid, cholesterol, gangliosides, GPI anchored molecules and transmembrane proteins (Figure18A) [133]. Figure18B demonstrates the formation of a flask shaped specialized lipid raft called caveolae [134].

4.2 MATERIALS AND METHODS

4.2.1. Cell culture and treatments

MDA-MB-231 and MCF7 cells were cultured in tissue culture flasks as previously mentioned. Upon ~90% confluence, the cells were trypsinized and harvested. Then the cells were plated in either a 6-well plate (~0.5 X 10^6 cells per well), 12-well plates, or 4-well chamber slides to perform the immunoblot experiment, the cholesterol assay, and confocal microscopy, respectively. The cells were treated with AA (sodium salt, 100 μM; Sigma) for 24 h with or without prior treatment with the LOX-5 inhibitor nordihydroguaiaretic acid (NDGA, 10 μM; Sigma), and the cells were also treated with the lipid raft disruptor Methyl-β– cyclodextrin (MBCD, 1mM; Sigma). Control and treated cells were collected, washed, and subjected to further analyses as described in the text.

4.2.2. Immunoblot analyses

Control and treated cells were harvested, lysed, and subjected to electrophoresis in SDS-PAGE (10%), followed by immunoblot analyses on (polyvinylidene difluoride) PVDF membranes as described in the previous chapter. The PVDF membranes were incubated overnight (6–10^{o}C) with anti-Caveolin-1 antibody (monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA) anti-Flotilin-1 antibody (monoclonal, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-GAPDH (monoclonal, Glyceraldehyde 3-phosphate dehydrogenase, Cell Signaling, Danvers, MA). The membranes were washed with 0.05% Tween-20 in Tris-Buffered saline (TBST) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse and/or goat anti-rabbit IgG (KPL Biomedical, Chantilly, VA). The enhanced chemi-luminescence (ECL) technique

was used for the detection of protein bands. The intensities of protein bands were measured using Labworks software (UVP Lab Products, Upland, CA).

4.2.3. Confocal microscopy

MDA-MB-231 and MCF7 cells were grown overnight in 4-well chambered Lab-TEK II slides containing either DMEM/F12 media or RPMI-1640. The cells were treated with AA and/or NDGA and were fixed with methanol (100%, chilled) for 5 min at -20 ^{o}C before blocking with normal goat serum (NGS, 5%; Sigma) for 1 h. Methanol-fixed cells were incubated overnight in the cold room with anti-caveolin-1 antibody (1:200; polyclonal, Santa Cruz Biotechnology) or anti-CTXB antibody (1:200; monoclonal, Gibco Invitrogen, Carlsbad, CA) in 1% NGS. The slides were washed three times and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or anti-mouse antibody, mounted with ProLong® Gold antifade reagent mixed with DAPI (Gibco Invitrogen). The confocal images were captured with the help of an LSM 700 Zeiss confocal microscope and analyzed using Zen 2009 software (Carl Zeiss, Thornwood, NY). Each experiment was repeated three times, then cells were randomly selected from each slide, and the same resolution, laser power, and detector-gain were used. The student's t tests were used to assess differences (means) between the treatment and control. $P < 0.05$ was considered significant.

4.2.4. Cholesterol assay

Cellular cholesterol content was quantitated using Amplex Red Cholesterol assay kit (Gibco Invitrogen) according to the manufacturer's instructions. Briefly, cells (~1x10^{5}

cells/well) were placed in 12-well plates, incubated for 24 h, and treated as mentioned previously. Cell lysates were prepared from each well, and equal concentrations of proteins were used for this flourescence-based assay. The samples were measured with a fluorescence microplate reader (Bio-Rad, Hercules, CA) using excitation of 530 nm and emission of 590 nm.

4.2.5. Migration and invasion assay

Cells were plated at a density of ~0.5 x 10^6 in each well of a 6-well plate in the presence of media (RPMI-1640 or DMEM) containing 5% serum for 24 h. The monolayers were wounded by scratching with a 10-μL pipette tip. Images were captured using a Nikon TMS microscope equipped with a Nikon F-601 camera. Cells that had migrated between wounded regions were counted at three different observations (n = 3), and cell-invasion assays were carried out in Transwell chambers. Briefly, cells were cultured in 12-well plates (~3 x 10^5/well) for 24 h in the presence of MBCD as described above. Cells were harvested, suspended in 500 μl of medium containing 5% FBS, and added to the upper layer of Transwell chambers. The bottom wells were filled with 1 ml of culture medium containing 10% FBS (to promote cell growth and invasion through the matrigel of Transwell chambers). The cells that were migrated to the lower chamber after 24 h of incubation were examined and photographed by a Nikon TMS microscope equipped with a Nikon F-601 camera. Migrated cells were also stained with calcein (Invitrogen, Carlsbad, CA), a fluorescent dye that labels the viable cells. Both cell counts and fluorescence intensities were taken into account to measure the invasiveness.

4.3. RESULTS

4.3.1. Arachidonic acid-induced LR/caveolae syntheses

LTB_4 or other cysteinyl-LTs synthesized from AA by LOX pathways are secreted from the cells and act via G-protein-coupled BLT receptors. It has been postulated that BLT receptors (i.e., BLT-1 and BLT-2) are located within the LRs of human neutrophils and that disruption of LRs suppresses LTB_4 syntheses [60]. Because AA-stimulated LTB_4 production in MDA-MB-231 cells and LTB_4 was found to be associated with cell migration and invasion, we asked whether if LTB_4-mediated cell movement occurs through the LRs/caveolae.

First, LRs in both MDA-MB-231 and MCF7 cells were identified by labeling with anti-caveolin-1 antibody (Figure 19C) and an Alexa flour-conjugated cholera toxin subunit B (CTXB) LR labeling kit (Figure 20). Our initial investigation indicated that MDA-MB-231 and MCF7 cells react differently to anti-caveolin-1 antibody and CTX B labeling reagent. We noted that MDA-MB-231 cells, while they could be labeled with anti-caveolin-1 antibody (Figure 19C), showed little or no reactivity with CTX B labeling reagent (Figure 20). On the other hand, MCF7 reacted with CTXB (Figure 20) but not with anti-caveolin antibody (Figure 19C), suggesting that caveolae (a subset of LRs) are more predominant in MDA-MB-231 cells than MCF7. We found that AA treatment increased the anti-caveolin-1 antibody labeling of MDA-MB-231 cells by ~2-fold (Figure 19D) and that NDGA blocked the labeling significantly (photographs a–d, Figures 19C and 19D). The expression of caveolin-1 protein (an important marker of caveolae) by MDA-MB-231 is shown in Figure 19A. As expected, MCF7 cells did not demonstrate any caveolin-1 protein expression (data not shown). While AA increased the expression of caveolin-1

protein, NDGA inhibited its expression (Figure 19A). The densitometric analyses of Figure 2A are illustrated in Figure 2B. Flotilin-1, another important determinant of LR composition, showed a high level of expression in MDA-MB-231 cells, which increased further (~2-fold) by AA treatment. In contrast, MCF7 cells exhibited low Flotilin-1 expression, which was increased ~3-fold by AA treatment (Figure 21).

Because cholesterol is an important component of the LR and caveolae, and because AA stimulated the caveolae expression as judged by labeling with anti-caveolin antibody followed by confocal microscopy, we measured the cholesterol level in MDA-MB-231 and MCF7 cells to explore whether cholesterol synthesis is also affected by AA treatment. Total cellular cholesterol was measured with the help of a commercially available Amplex Red assay kit following the strategy described in the Materials and Methods section. The results demonstrate (Figure 22) that the cholesterol level in MDA-MB-231 cells is slightly higher than MCF7 and is stimulated by AA treatment (~0.4-fold). AA-induced excess cholesterol synthesis was inhibited by NDGA and by methyl-β-cyclodextrin (MBCD), a common LR disruptor (Figure 22).

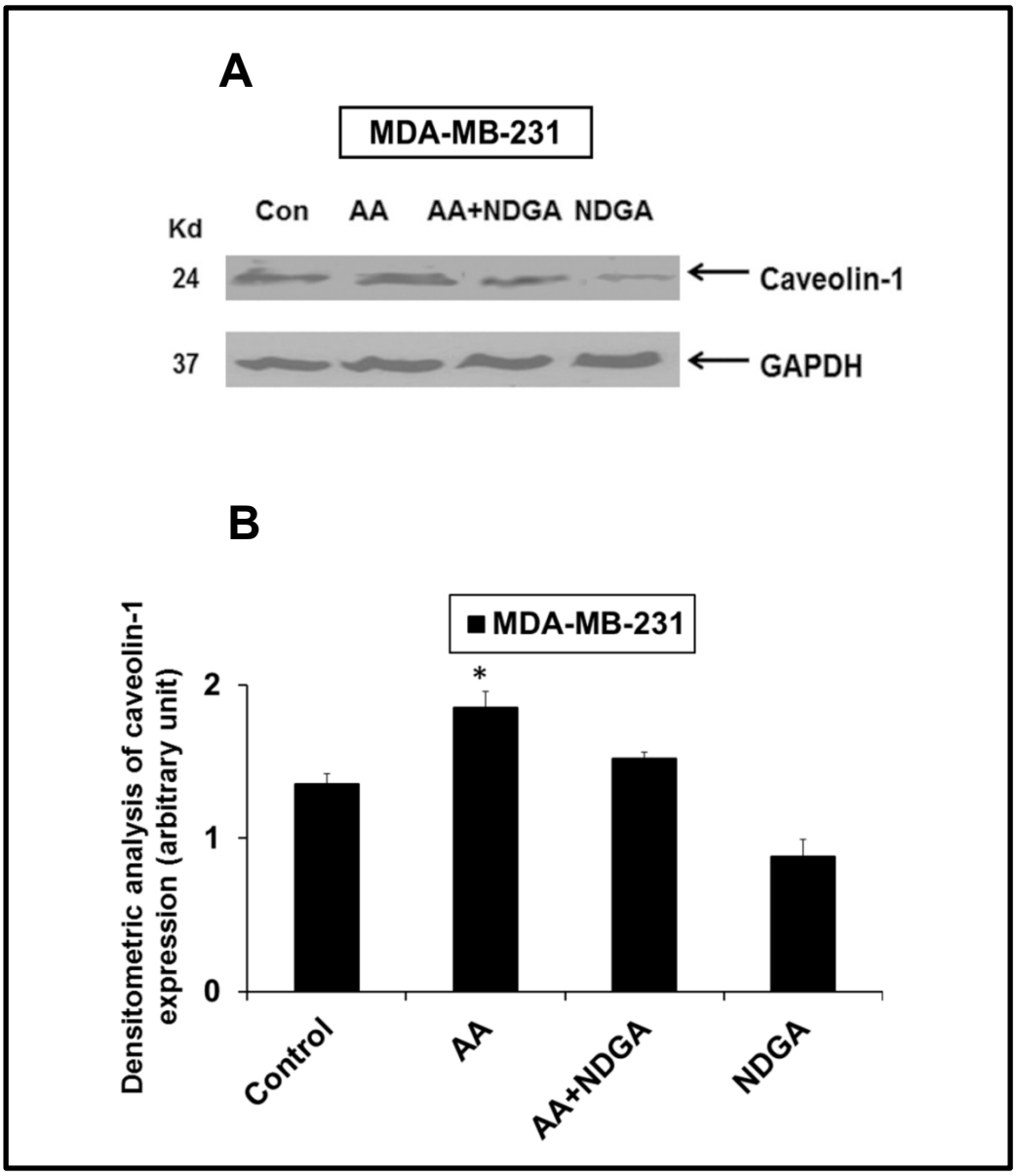


Figure 19

Arachidonic acid induces the caveolin-1 expression in MDA-MB-231 cells

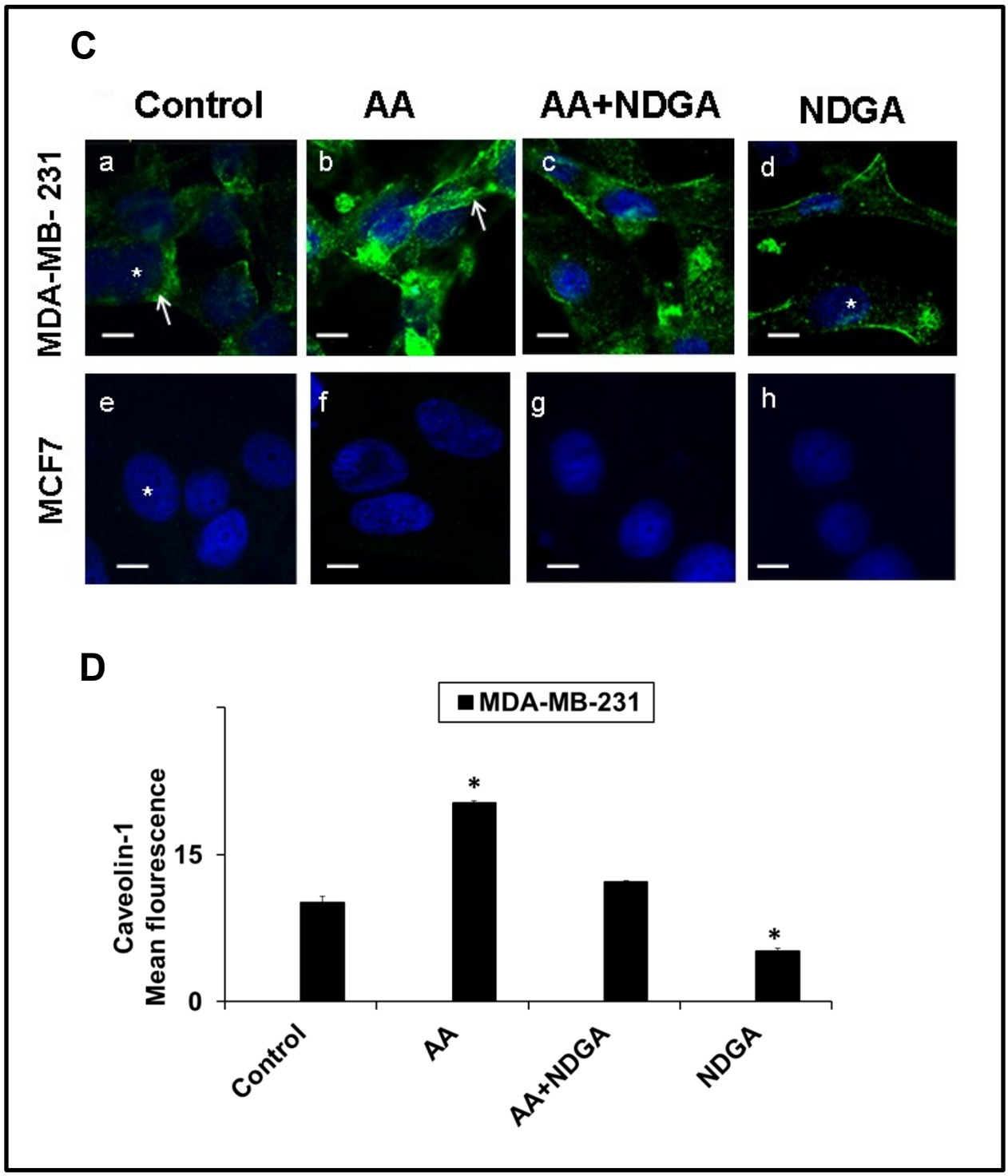

C
Control
AA
AA+NDGA
NDGA
MDA-MB- 231
MCF7
a
b
c
d
e
f
g
h
D
MDA-MB-231
Caveolin-1
Mean flourescence
15
0
Control
AA
AA+NDGA
NDGA

Figure 19

Figure 19. Arachidonic acid induces the caveolin-1 expression in MDA-MB-231 cells

MDA-MB-231 and MCF7 cells were grown in 6-well plates or 4-well chambered slides and treated with AA (100 μM), AA (100 μM) +NDGA (10 μM), or NDGA (10 μM) for 24 h and subjected to immunoblot and immunoconfocal microscopy. Fig. 19A represents the synthesis of caveolae as determined by labeling with anti-caveolin-1antibody. Photograph a: MDA-MB-231, control; photograph b: treatment with AA (100 μM); photograph c: AA (100 μM) +NDGA (10 μM) treatment; photograph d: NDGA (10 μM) treatment. Arrow denotes membrane, and asterisk indicates the nucleus. Bar: 10 μM. The fluorescence intensities of control and treated cells were measured by Zeiss ZEN confocal software and are shown in Fig. 19B. The expressions of caveolin-1 in control and treated cells are shown in Fig. 19C. Figure 19D represents the densitometric analyses of caveolin expression. All experiments were repeated at least three times, and the data are given as means ± SE, * $p < 0.001$.

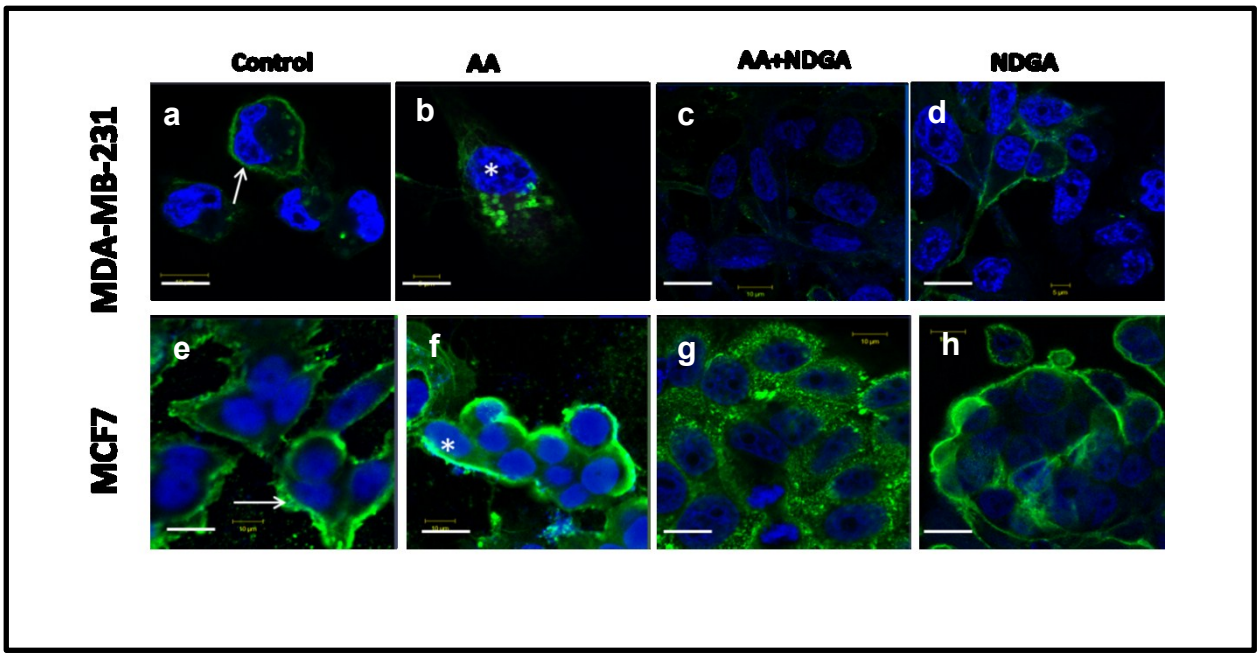


Figure 20

Labeling of MDA-MB-231 and MCF7 cells with anti-cholera toxin antibody

Figure 20. Labeling of MDA-MB-231 and MCF7 cells with anti-cholera toxin antibody

MDA-MB-231 (Figure 20A) and MCF7 (Figure 20B) cells were grown in 4-well chamber slides (LABTEK II) and then treated with AA and /or NDGA as mentioned previously. The cells were fixed and labeled with anti-Ctx-B antibody (FITC-conjugated) to detect mono-sialo ganglioside (GM1) located in the lipid rafts. Nucleus was stained with the DAPI. Photograph a: MDA-MB-231, control; photograph b: AA-treatment; photograph c: treatment with AA+NDGA; photograph d: NDGA treatment; photograph e: MCF7control; photograph f: AA treatment; photograph g: treatment with AA+NDGA, and photograph h: the treatment with NDGA. Arrow denotes cell membrane and asterisk indicates the nucleus. Bar: 10 μM.

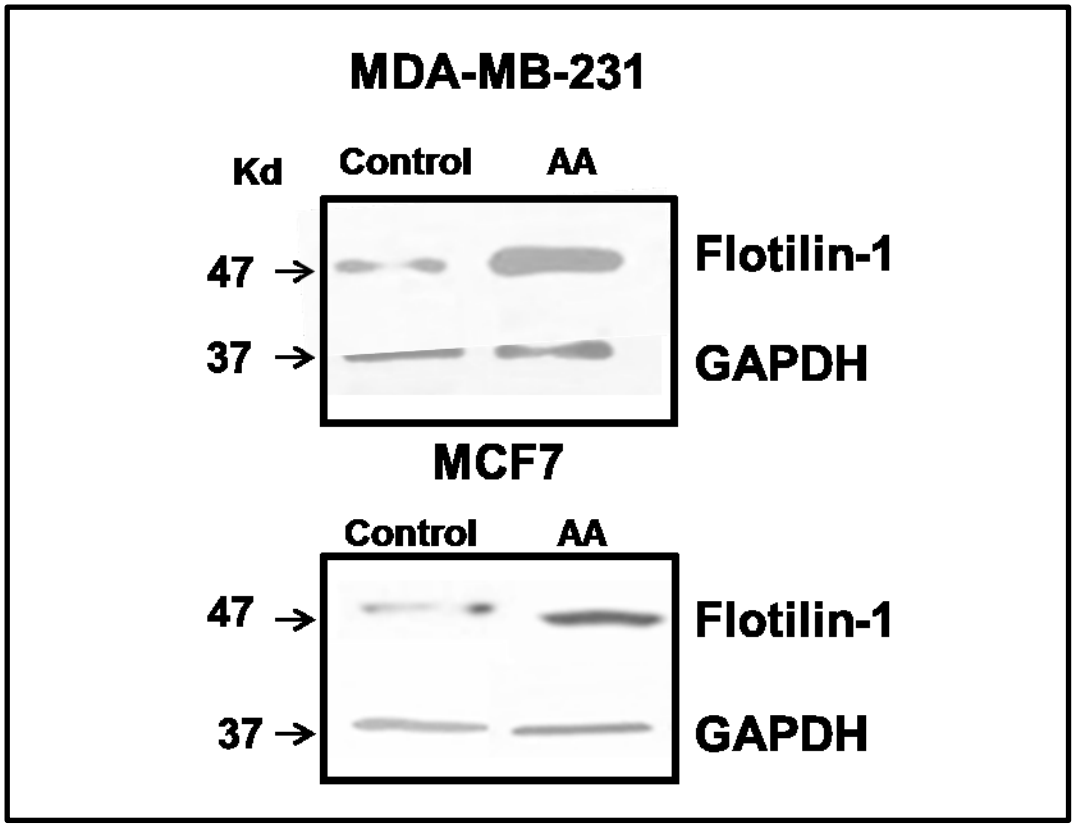


Figure 21

Flotilin-1 expression in MDA-MB-231 and MCF7cells

Figure 21. The expression of flotilin-1 in MDA-MB-231 and MCF7 cells

Control and arachidonic acid treated MDA-MB-231 and MCF7 cells were harvested, lysed and analyzed by immunoblot analysis to detect the protein expression of flotilin-1. GAPDH was used as a loading control.

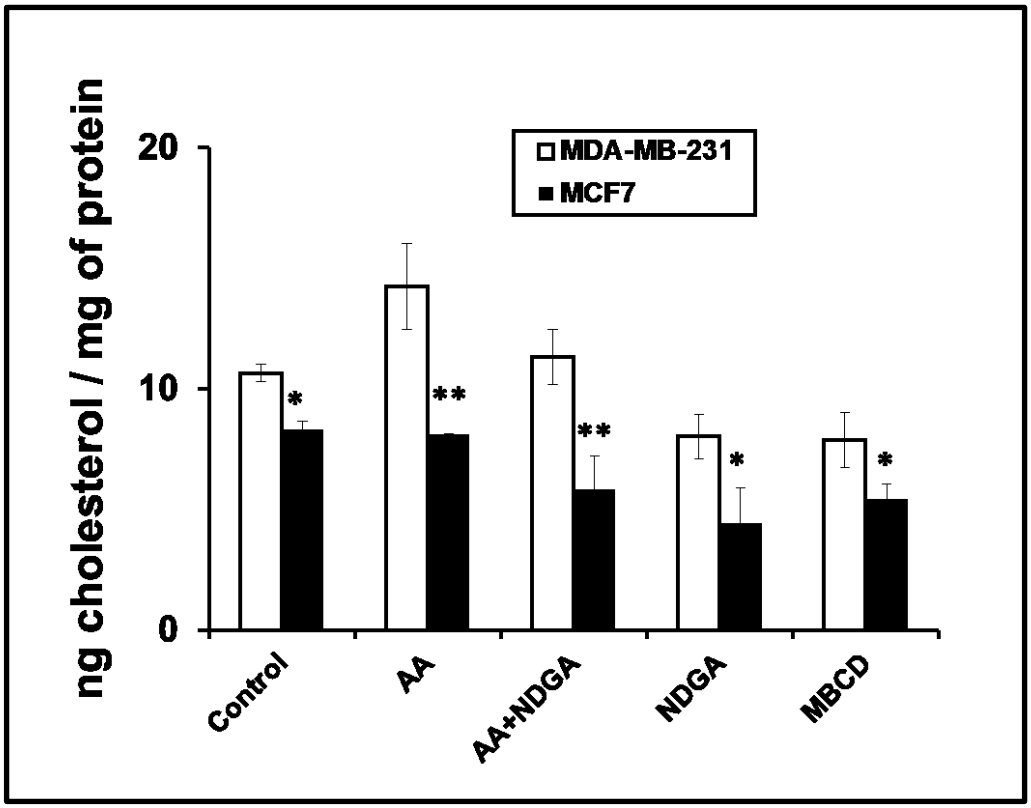


Figure 22

Assay of total cellular cholesterol

Figure 22. Assay of total cellular cholesterol

Approximately 1 X 10^5 cells were grown in a 12-well plate for overnight and were treated with AA (100 μM), AA (100 μM) +NDGA (10 μM), NDGA or MBCD (methyl beta cyclodextrin; a common lipid-raft inhibitor) for 24 h. The cell lysates were used to analyze cholesterol levels using the Amplex Red cholesterol assay kit as mentioned in the Materials and Methods section of this chapter. Data represent the average of three different experiments. *$p<0.01$, ** $p<0.001$.

4.3.2. Lipid rafts in MDA-MB-231 are linked to cellular migration and invasion

The migration and invasion assays were performed to examine whether blocking of the caveolae synthesis interferes with the mobility of MDA-MB-231. As shown, MBCD reduced cellular migration in MDA-MB-231 cells by ~2-fold (photograph b, Figure 23A). Figure 23B shows the quantitative values of migration assay. Likewise, the invasion assay demonstrated that MBCD reduced the number of migratory cells across the matrigel by ~2- to 3-fold (Figure 24B and 24C), which further suggests that LTB_4-mediated invasion of MDA-MB-231 cells involves cholesterol-regulated caveolae biosynthesis.

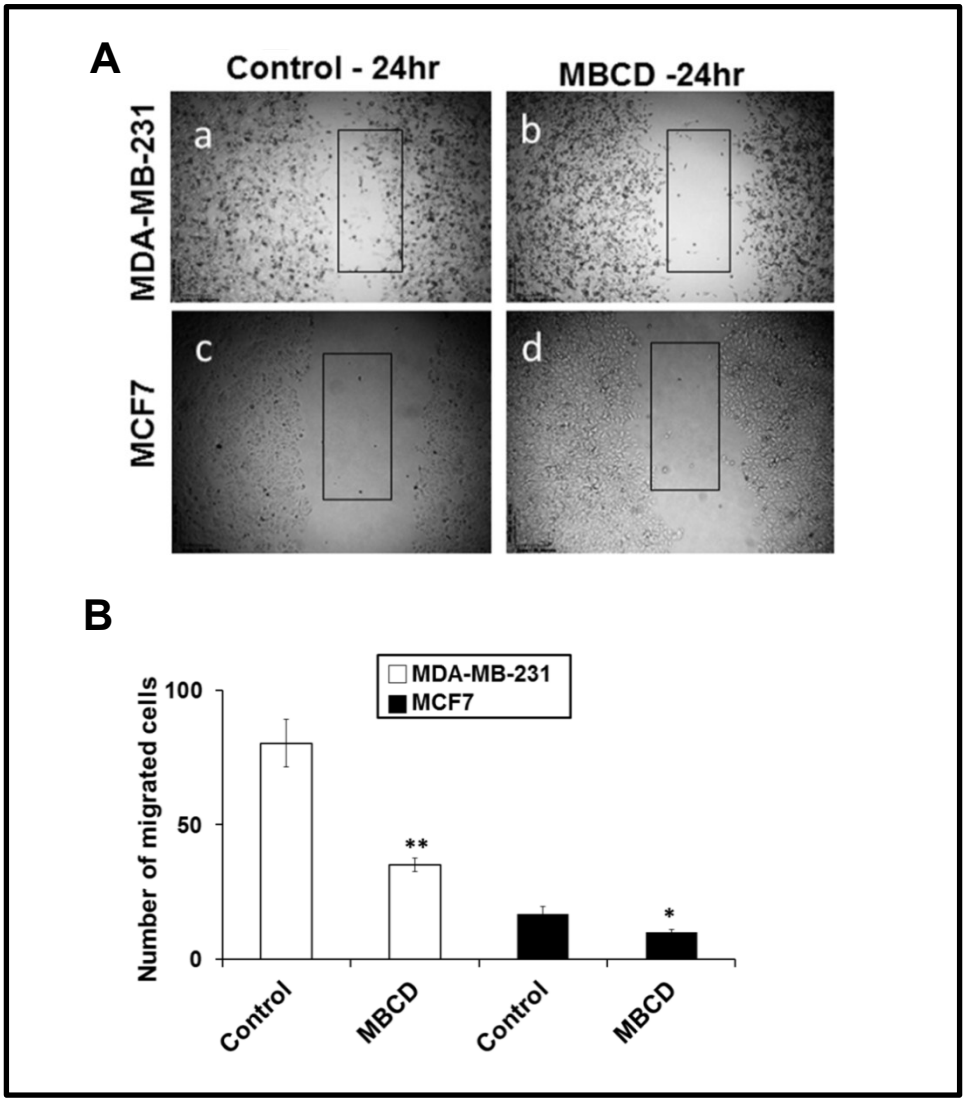


Figure 23

The inhibitor of lipid rafts blocks the migration of MDA-MB-231 cells

Figure 23. The inhibitor of lipid rafts blocks the migration of MDA-MB-231 cells

Approximately 1 X 10^6 cells were grown in a 6-well plate in growth medium supplemented with 5% FBS. The cells (~70–80% confluent monolayer) were scratch-wounded by a 10-μM pipette tip and were treated with 1mM MBCD. Figure 23A demonstrates the effect of MBCD (1 mM) on the migration of MDA-MB-231 (photographs a and b) and MCF7 (photographs c and d) cells. Figure 23B is the graphical representation of the results of migration experiments shown Figure 23A. The experiments were repeated three times, and the data are given as means ± SE; *p < 0.01, ** p < 0.001.

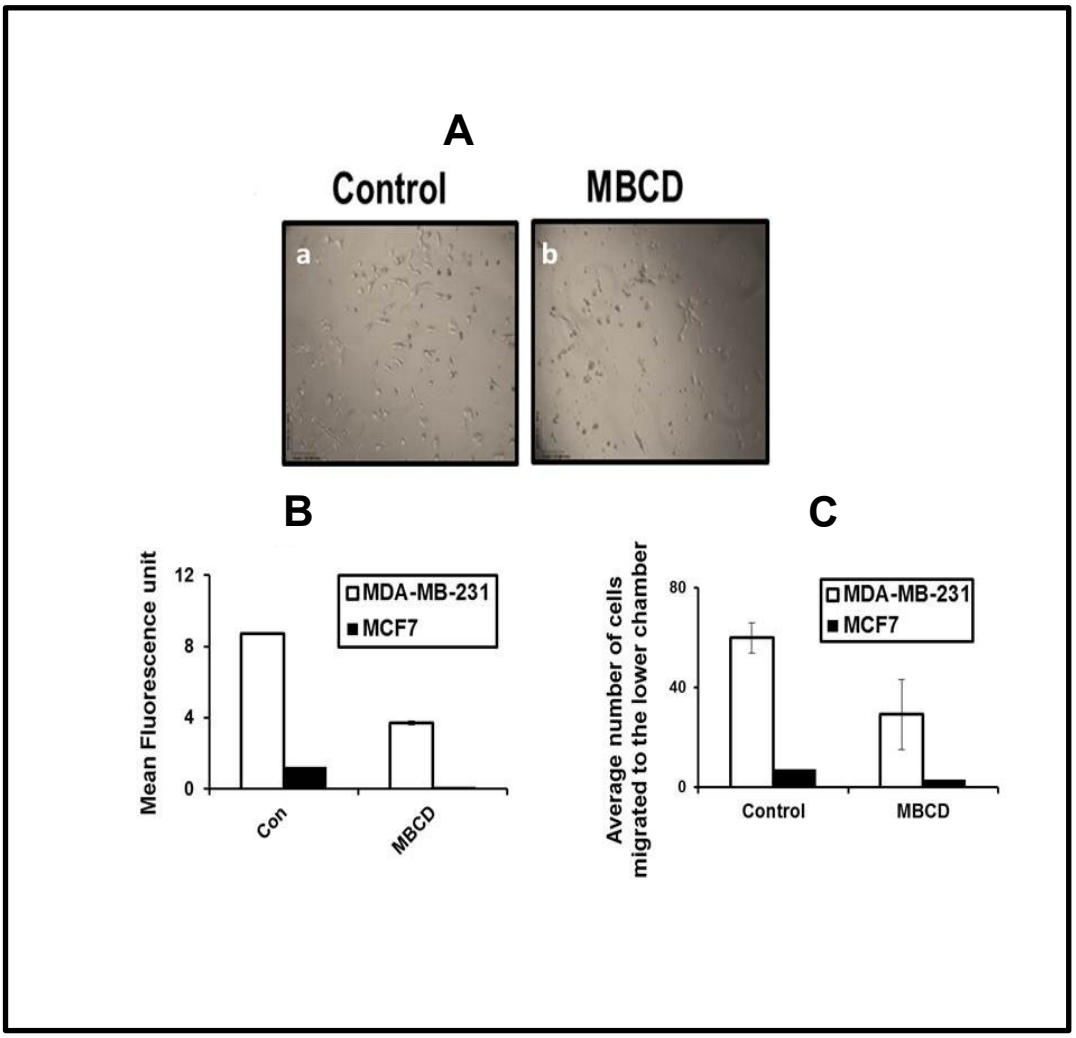


Figure 24

MBCD inhibits the invasion by MDA-MB-231 cells

Figure 24. MBCD inhibits the invasion by MDA-MB-231 cells

Cells were cultured in a 12-well plate and were treated with MBCD (1mM) for 24 h. Cells were harvested and resuspended in 500 μl medium containing 5% serum and applied on the upper wells of a Transwell chamber as mentioned before. The bottom wells were filled with 1 ml of complete culture medium containing 10% serum. The photographs of invading breast cancer cells in 24-h in the lower wells were captured (Figure 24A) and counted from four different microscopic fields. Figure 24B represents the average number of cells counted. The invaded cells in the lower wells were stained with 2μM of calcein and incubated for 30 min at room temperature. The fluorescence of the viable cells was measured by fluorescence plate reader at an excitation and emission of 490 and 520. Figure 24C represents the mean fluorescence of the viable wells. The results shown here are the average of two individual experiments which were carried out in duplicate.

4.4. CONCLUSION

Because LRs play critical roles in activating signal cascades in cancer cells [16], and because LR is known to house BLT receptors [15], we thought it would be interesting to investigate whether the proper assembly of LRs/caveolae is important for AA-induced migration of cancer cells. This is important because we observed that LR/caveolae syntheses in MDA-MB-231 cells is stimulated by AA, which could be due to the binding of LTB_4 with BLT receptor located in the membrane caveolae. Because cholesterol is one of the major constituents of LRs and MBCD binds to cholesterol—resulting in disruption of the LRs—I questioned whether LRs have any significant role in the cellular migration and invasion. It was observed that the disruption of LR/caveolae by MBCD interferes with the migration and invasion of MDA-MB-231 cells (Figures 23 and 24). This observation further supports our hypothesis that the assembly and location of BLT receptor within the caveolae are important for transducing LTB_4-mediated signaling for migration and invasion. As shown in Figure 3, AA also stimulates the GM1 labeling in MCF7 cells, which could be due to the fact that AA is internalized via LRs and interacts with various molecules and receptors other than BLTs to generate downstream signaling for growth and malignancy.

Thus, the results described in this chapter provide new evidence that AA promotes metastatic signaling in invasive breast cancer cells by activating 5-LOX-mediated cytokine secretion and the formation of caveolae.

CHAPTER 5

DISCUSSION

OVERALL PERSPECTIVE, PROPOSED MODEL, AND FUTURE DIRECTIONS.

5.1. Overall Perspective

Breast cancer is one of the major causes of cancer-related death in the United States. The current treatments include endocrine therapies and targeted molecular therapies to treat patients diagnosed with cancers in the breast tissues. However, in many instances, patients develop drug-resistance (or even multi-drug-resistant) breast cancers that could be the result of mutations of target molecules and/or receptors as well as the over-expression of the ATP-binding cassette subfamily G member 2 (ABCG2) [135]. Therefore, it is imperative to take a new look at the strategy of target identification and to develop new anti-cancer agents that are effective, that are low in toxicity, and do not develop the MDR (multiple drug resistance) phenomenon.

Elucidating the signaling pathways associated with the tumor progression in different subtypes of breast cancer is necessary to develop new therapeutics. As far as signaling molecules and receptors are concerned, invasive breast cancers are often characterized by activated receptor tyrosine kinase-mediated PI3K/Akt/mTOR or Ras/MEK/MAPK signaling, both of which are associated with activation of oncogenes. Understandably, in recent years the therapeutic strategies were developed using a combination of small-molecule inhibitors against PI3K, mTOR, or MAPK (e.g., everolimus, BEZ235, etc.) along with anti-receptor (anti-HER2, anti-EGFR, etc.) antibodies or hormonal analogues (e.g., tamoxifane, raloxifene, etc.) to treat the incidence of invasive breast cancers. Carracedo et al. [136] showed MAPK activation as a result of mTOR inhibition in breast cancer cells, which suggests that targeting any single pathway in the cancer processes may not be an effective strategy to limit the

tumorigenesis anymore. On the other hand, a large number of ER^+ cancer patients develop resistance against the most widely used breast cancer drug, tamoxifen, due to the activation of the Akt molecule by epigenetic changes [137; 138] HER-2 positive metastatic breast cancer patients are successfully treated with anti-HER-2 antibody and trastuzumab, but a long-term use of trastuzumab leads to the drug resistance tumorigenesis [139; 140]. To tackle this problem, new anti-cancer small-molecule inhibitors have been designed and tested clinically. This group of drugs includes: (1) tyrosine inhibitor or lapatinib [141], (2) PARP1 inhibitor or olaparib [142], (3) mTOR inhibitor rapamycin and its analogues [41; 113], and (4) sheddase inhibitors [143]. mTORC1 and mTORC2 are also indicated as promising therapeutic candidates because to their important roles in the activation of various oncogenes [144].

While the HER-2 and ER^+ cancers are already having potential therapeutic targets, the question arises as to what the target and strategy should be in the future for treating patients with triple-negative breast cancers or TNBCs? Interestingly, recent reports suggest that there are numerous possible therapeutic targets in TNBCs: serine-threonine protein kinase (Chk1) [145], HLA-DR antigens-associated invariant chain or CD74 (CD74) [146], signal transducer and activator of transcription-1 **(**Stat 1) [146], lysyl oxidase [147], etc. A detailed study by Lehmann et al. [6] reported that different genetic subtypes such as BL1 and BL2 (cell-cycle- and cell-division-specific genes), IM (genes connected with immune signal transduction), M and MSL (genes involved in cell motility, cell differentiation, and ECM receptor interactions), and LAR (other hormone-regulated pathways such as Androgen receptor signaling), etc., could also be potential targets for developing new therapies against TNBCs. Ueno et al. [148] mentioned the

EGFR over-expression in TNBC and a successful inhibition of cell growth by using erlotinib, an EGFR tyrosine kinase inhibitor, which could be another approach to developing therapy against this highly metastatic breast cancer.

Inflammation-mediated signaling pathway and the transformation of normal cells to malignancy has been long debated, but still very little is known about the association of inflammatory molecules and their role in tumor formation. Over-expression of COX and LOX and their metabolites has been shown to enhance carcinogen metabolism, tumor-cell proliferation, and metastatic potential in colon, lung, and breast cancer patients, which justifies the use of anti-COX or anti-LOX drugs for treatment. Although the association between PUFA metabolism, COX/LOX over-expression, and the secretion of inflammatory eicosanoids has been referred to in relation to different cancers, there is still a knowledge gap about how the bioactive lipid mediators (i.e., the products of COX and LOX pathways) interact with various signaling and inflammatory molecules and promote malignancy in breast tissues. Incidentally, a recent report by Rokavec et al. [149] on invasive breast cancers indicated an emerging role of inflammatory cytokine IL-6, which suppresses the microRNA-200C (necessary for restricting the inflammation) and triggers the MEK/MAPK and IKK/NFkB pathway for consistent signaling in malignancy. IL-8, another major inflammatory cytokine, is a novel marker for tumor aggressiveness in receptor-negative breast cancers [150]. Secretion of these cytokines in the tumor micro-environment regulates the self-renewal pathways of cancer cells and activates the pro-carcinogenic PI3K/Stat3/NFkB signaling [151].

In this dissertation, I observed that the invasive TNBC cells MDA-MB-231 respond to AA very differently than do ER^{+}-MCF-7 cells, which could be due to their geneotypic and

phenotypic makeup and other intrinsic metabolic functions. The innovativeness of my dissertation comes from the observation that LTB_4, a product of 5-LOX, is synthesized under the influence of excess AA (100 μM) and plays a significant role in activating inflammatory molecules and determining the metastatic migration and invasion. This could be considered an important paradigm shift from the previous concept that COX-2 and prostaglandins are the most important pathways involved in the development of breast cancer [152]. While I am not in a position to refute the important role of COX-2, I propose that the role of LOX pathway is equally important. In fact, the role of 5-LOX in the formation of colonic polyps [84] and malignancy in breast cancer [97] supports the findings in my research. Future research from our laboratory and laboratories around the world should validate whether 5-LOX and related pathways could be considered as novel targets for developing anti-breast-cancer drugs.

5. 2. PROPOSED MODEL

Based on my results, I have proposed a hypothetical model (Figure 26) describing the overall pathway that leads to the migration and invasion of MDA-MB-231 cells. As indicated, exogenous AA is internalized and incorporated into membrane phospholipids, followed by release from the membrane by the action of cytoplasmic phospholipase-A_2 ($cPLA_2$) into the cytoplasm [54]. Free AA is then converted into LTB_4 by activated 5-LOX and is subsequently released from the cells. Secreted 5-LOX then activates the cell by binding with BLT receptor (via autocrine and paracrine signaling) located in the caveloae/LR. LTB_4 subsequently stimulates the cells by activating the transcription

factor NFκB [153] that up-regulates the expression of cytokine genes such as IL-6 and IL-8. The increased production of these inflammatory cytokines is either directly or indirectly linked to the migration and invasion by MDA-MB-231 cells. Earlier, AA was reported to increase the migration of MDA-MB-231 cells by activating focal adhesion kinase (FAK) [9], but the actual mechanism was not proposed. Thus, it is possible that AA induces the migration of invasive breast cancer cells by activating FAK via LTB_4 and IL-6/IL-8 cytokines. Recently, Wen et al. [97] have reported that AA treatment enhances the incidence of mammary cell tumorigenesis by activating LOX and m-TOR pathways, further supporting the idea that LOX is equally important as COX-2 and should be considered as a potential target for developing new chemotherapeutic agents to reduce the incidence of breast cancer metastasis.

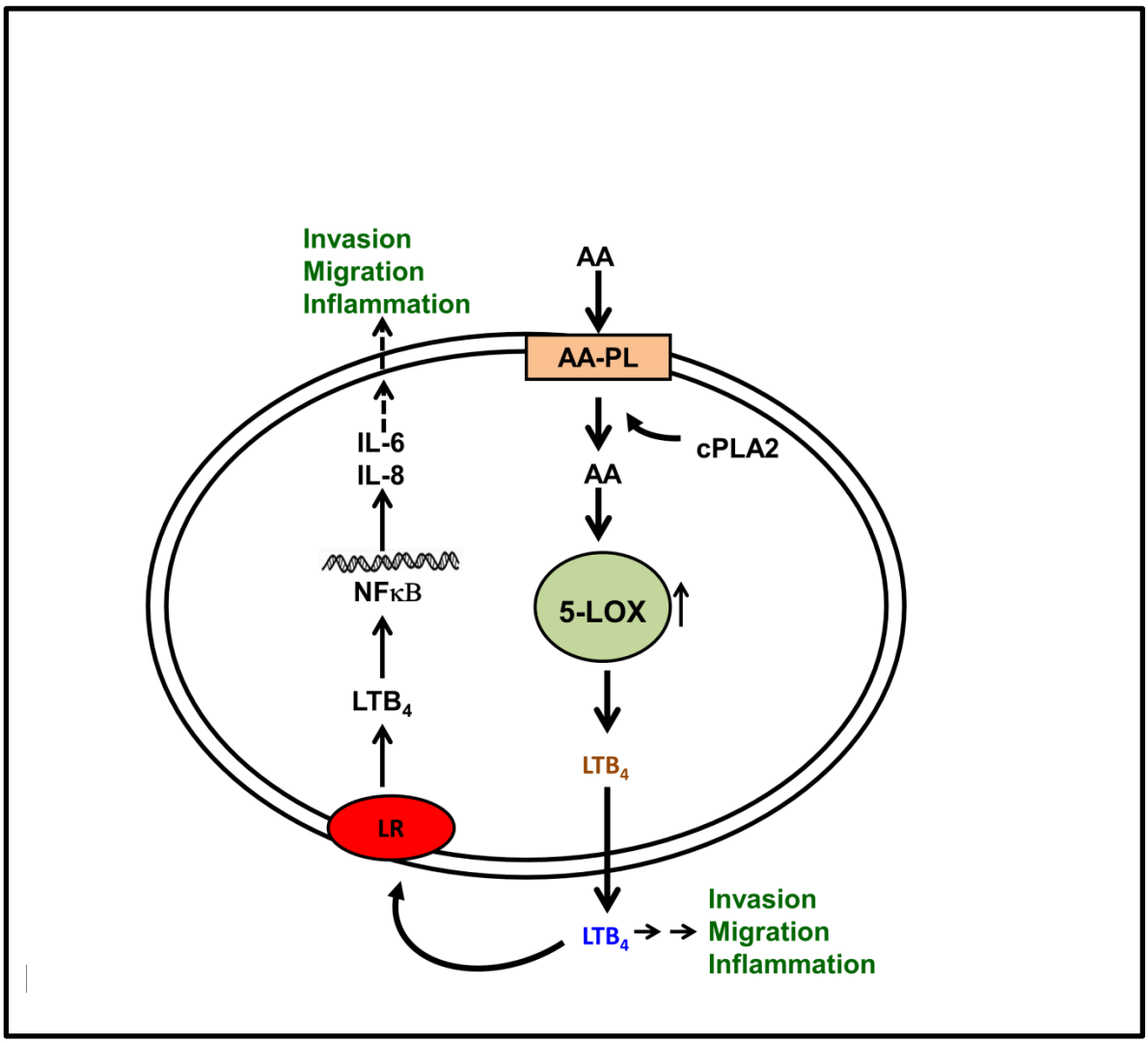


Figure 25

Proposed Model

Figure 25. Integrated results proposing the arachidonic acid stimulated pathway in MDA-MB-231 cells

The model proposes that exogenous AA is taken up by caveolae-mediated endocytic or non-endocytic (simple diffusion or via fatty acid transporters) mechanisms and incorporated into membrane phospholipids to produce AA-PL. AA-PL is then hydrolyzed by $cPLA_2$ and releases free AA that stimulates the activity of 5-LOX. Activated 5-LOX converts AA to LTB_4. The secreted LTB_4 regulate the migration and invasion by a mechanism yet to be elucidated. LTB_4 which is secreted by the cell probably re-enters via LR/caveolae and activates NFκB to produce phospho-NFκB. Phosphorylation of NFκB upregulates the expression of IL-6 and IL-8 genes, which produce the inflammatory reactions, migrations and invasions by MDA-MB-231 cells.

5. 3. FUTURE DIRECTIONS

Currently, our laboratory is in the process in generating 5-LOX knockout and over-expressed MDA-MB-231 cells, which will be used to conduct an in-depth investigation of the mechanism of cell invasion and malignancy in breast cancer. SiRNA-mediated knocking down of 5-LOX in invasive breast cancer cells to determine whether the LOX pathway is crucial for maintaining the cancerous properties should also be pursued. Determining the possible link between AA-induced LTB_4 production and lamelopodia/invadopodia formation is another important area needing to be investigated. Cancer cells maintain their invasiveness by forming lamelopodia/ invadopodia using membrane-bound molecules located in the lipid rafts/caveolae. Therefore, it would be interesting to determine if the proper assembly and disassembly of lipid rafts are essential for LTB_4-mediated activation of MDA-MB-231 cells.

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