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Arachidonic Acid Induces the Migration of MDA-MB-231 Cells by Activating Raft-associated Leukotriene B4 Receptors

Atasi De Chatterjee^{1,3}, Debarshi Roy^{1,3,‡}, Priscilla Guevara^{1,3}, Rituraj Pal^{2,3}, Mahesh Naryan^{2,3}, Sukla Roychowdhury^{1,3}, and Siddhartha Das^{1,3,*}

¹Department of Biological Sciences, Research Center, University of Texas at El Paso, El Paso, TX 79968-0519, USA

²Department of Chemistry, Research Center, University of Texas at El Paso, El Paso, TX 79968-0519, USA

³The Border Biomedical Research Center, University of Texas at El Paso, El Paso, TX 79968-0519, USA

Abstract

Background: The migration of tumor cells is critical in spreading cancers through the lymphatic nodes and circulatory systems. Although arachidonic acid (AA) and its soluble metabolites have been shown to induce the migration of breast and colon cancer cells, the mechanism by which it induces such migration has not been fully understood.

Objective: The effect of AA on migratory responses of the MDA-MB-231 cell line (a triple-negative breast cancer cell) was examined and compared with MCF-7 (estrogen-receptor positive) breast cancer cells to elucidate the mechanism of AA-induced migration.

Methods: Migrations of breast cancer cells were examined with the help of wound-healing assays. AA-induced eicosanoid synthesis was monitored by RP-HPLC. Cellular localizations of lipoxygenase and lipid rafts were assessed by immunoblot and confocal microscopy.

Results: AA treatment stimulated the synthesis of leukotriene B₄ (LTB₄) and HETE-8, but lowered the levels of prostaglandin E₂ (PGE₂), prostaglandin D₂ (PGD₂), and HETE-5 in MDA-MB-231 cells. Further analysis indicated that AA increased the expression of 5-lipoxygenase (5-LOX) in this cell line and inhibiting its expression by small molecule inhibitors lowered the production of LTB₄ and reduced migration. In contrast, MCF-7 cells did not show any appreciable changes in eicosanoid synthesis, 5-LOX expression, or cellular migration.

Conclusion: Our results suggest that AA treatment activates the BLT1 receptor (present in membrane microdomains) and stimulates the synthesis of LTB₄ production, which is likely to be associated with the migration of MDA-MB-231 cells.

*Corresponding author: Department of Biological Sciences, University of Texas at El Paso, 500 W. University Avenue, El Paso, TX 79968-0519/USA, Tel: (915)-747-6896, Fax: (915)-747-5808, sdas@utep.edu.

‡Current address: Mayo Clinic, Rochester, MN.
Baylor College of Medicine, Houston, TX.

Keywords

Breast cancer; MDA-MB-231; MCF-7; eicosanoids; leukotrienes; lipid rafts

Introduction

Breast cancer is a common cancer among women across the globe. Epidemiological studies suggest that the intake of red meat or other high-fat foods that contain arachidonic acid (AA) or other omega 6-polyunsaturated fatty acids (ω 6-PUFA) can increase the risk of various types of cancers, including breast cancer [1–3]. AA is initially incorporated into the plasma membrane and then released by the action of phospholipase A₂s (PLA₂s). Free and unesterified AAs are then utilized by the cells to synthesize various inflammatory eicosanoid molecules such as leukotrienes (LTs), prostaglandins (PGs), thromboxanes (TXs), and hydroxyeicosatetraenoic acids (HETEs) [2]. All of these molecules are involved in the immune response and modulation and, in many cases, excess production of these eicosanoids can lead to cellular proliferation and malignancy [4]. Studies suggest that AA not only promotes the adhesion and migration of endothelial cells, but it can also act as a regulator of wound healing and angiogenesis [5]. The level of prostaglandin E₂ (PGE₂), a major product of cyclooxygenase-2 (COX-2) reaction, is elevated in breast cancer cells and functions as a pro-tumorigenic agent [6,7]. COX-2-dependent PG synthesis has been shown to be involved in maintaining the motility and invasion of breast cancer cells [8]. Inhibitors of COX-2 could block the radiation-induced migration of MDA-MB-231 cells [9], which suggests that the COX-2 pathway is important and involved in the migration of breast cancer cells.

Like COX pathways, lipoxygenase (LOX)-derived eicosanoids have been implicated in tumor formation and malignancy [10], although their role in cancer metastasis is not well understood. LOX enzymes use AA as a substrate (like COX) to produce LTs, HETEs, and hydroperoxyeicosatetraenoic acids (HPETEs). There are several types of LOXs—i.e., 5-LOX, 8-LOX, 12-LOX, and 15-LOX—and they are classified according to their positional specificity (e.g., 5, 8, 12, and 15) toward unsaturation in the AA molecule. One of the important enzymes of the LOX group, 5-LOX, synthesizes HETEs and various LTs, including leukotriene A₄ (LTA₄), leukotriene B₄ (LTB₄), leukotriene C₄ (LTC₄), leukotriene D₄ (LTD₄), and leukotriene E₄ (LTE₄) [10]. Recent reports indicate a direct association of increased 5-LOX activity with the progression of hepatocellular carcinoma, intestinal polyposis, and breast carcinoma [11, 12].

LTB₄, a 4 series of eicosanoids, is synthesized from unstable LTA₄ by the action of LTA₄ hydrolase. This is a powerful bioactive lipid that has been implicated in various physiological functions including inflammation, tumor promotion, and metastasis. LTB₄, which also has a role in anaphylactic reactions, is responsible for different lung cancer pathologies [13], and it can stimulate the proliferation of pancreatic cancer cells [14], suggesting the importance of LTB₄ in promoting lung and pancreatic cancers. In a recent study, high concentrations of exhaled LTB₄ and IL-8 were shown to be associated with

neutrophilic inflammation in the airways of lung cancer patients [13]. In addition, a pro-tumorigenic effect of LTB₄ in melanoma cells has also been reported [15].

There is evidence that AA and its metabolites can promote invasion/migration via the Rho and PI3K/Akt-dependent pathways [16]. Involvement of Stat-5 in AA-mediated migration of MDA-MB-231 cells has also been proposed [17]. Oleic acid has been shown to promote migration through AA-dependent pathways [18].

Despite all of this, it is not known if AA activates the same or a different set of molecules in two different breast cancer cell lines—MCF-7 and MDA-MB-231. Because it has been previously shown that lipid rafts (LRs), or caveolae-based signaling, is important for tumor progression and malignancy [19], we asked whether LR could be involved in regulating AA-induced migration of basal-like cell line MDA-MB-231 and luminal-type MCF-7 cells. These two cell lines (MDA-MB-231 and MCF-7) differ in genome aberrations, transcriptional profiles, invasive properties, and therapeutic targeting [20]. In this paper, we demonstrate the following: (1) AA treatment induces the production of LTB₄ and HETE8 in MDA-MB-231 cells; (2) AA suppresses the production of PGs, LTs, and HETE molecules in MCF-7 cells; (3) LOX inhibitors block the migration of MDA-MB-231 cells in culture and (4) methyl- β cyclodextrin (MBCD), a common LR disruptor, interferes with LR assembly, BLT1 receptor immunostaining, caveolin 1 expression, and the migration of MDA-MB-231 cells.

Materials and Methods

Cell culture and treatments

MDA-MB-231 and MCF-7 cells were cultured in RPMI-1640 and DMEM/Ham's F-12 (Gibco Invitrogen, Carlsbad, CA) media, respectively. 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% CO₂) until they reached ~80%–90% confluency. Confluent cells were treated with trypsin (0.25% Trypsin-EDTA, Hyclone), harvested by centrifugation, plated on a 6-well plate ($\sim 0.5 \times 10^6$ cells per well), and treated with AA (sodium salt, 100 μ M) (Sigma Chemicals, St. Louis) for 24 h with and without prior treatment with the 5-LOX inhibitor, nordihydroguaiaretic acid (NDGA, 5 μ M; Sigma Chemicals), and zileuton (100 μ M; Sigma Chemicals). Control and treated cells were collected, washed, and subject to further analyses as described in the text.

Cell viability assay

The cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium salt (MTS) assay. Briefly, cells were treated with various concentrations of AA or inhibitors (NDGA, zileuton) and the cytotoxic effects were determined following the method described by Malich et al [21].

Analysis of eicosanoids by reverse-phase, high-performance, and liquid chromatography (RP-HPLC)

MDA-MB-231 and MCF-7 cells were grown in tissue-culture flasks (T75 vented cap, BD Biosciences, CA), harvested, and cultured in 6-well plates ($\sim 0.5 \times 10^6$ cells/each well) for 24 h. Fresh media (2 ml) was added to each well and incubated for an additional 24 h in the presence of AA (100 μ M) and NDGA (5 μ M). The media from control and treated wells was collected by centrifugation (12,000 rpm, 10 min), and eicosanoids (secreted into the medium) were extracted using ethyl acetate following the protocol previously described by Franchi et al [22]. Samples were vortexed and centrifuged at 3000 rpm for 10 min, and the top layer containing eicosanoids was transferred to a Teflon-lined vial and dried under N_2 gas. The dried samples were resuspended in 40 μ l of methanol and stored at -20°C for further analysis by HPLC (Waters Inc., Milford, MA). Samples were mixed with acetonitrile (1:1000) and subjected to HPLC following the method of Moraes et al. [23] using a reverse-phase C18 column (Supplecon, St. Louis, MO). Eicosanoids were eluted with the help of trifluoroacetic acid (0.1%)-acetonitrile solvent system (flow rate: 0.4 ml/min) and analyzed by HPLC. Individual eicosanoid peaks were identified by comparison with the respective standards (i.e., PGE₂, PGD₂, LTB₄, HETE5, and HETE8) that were purchased from Cayman Chemical (Ann Arbor, MI). The elution times of the eicosanoids were as follows: PGE₂: 60 min; PGD₂: 64 min; LTB₄: 72 min; HETE5–5: 85 min; and HETE8: 90 min. Eicosanoids from control and treated samples were quantified by calculating the areas covered under each peak using EMPOWER software (Water Inc., Milford, MA).

Immunoblot analysis

Control and treated cells were harvested, washed in cold PBS, lysed by freeze-thaw in a lysis buffer followed by measuring proteins using a Bio-Rad Protein Assay Kit. Approximately 40- μ g of protein was applied to each lane and subjected to electrophoresis in SDS-PAGE (10%), followed by immunoblot analyses on the polyvinylidene fluoride (PVDF) membranes, as previously described by Albert et al [24]. The PVDF membranes were incubated overnight (6–10 $^\circ\text{C}$) with anti-5-LOX antibody or BLT1 receptor polyclonal antibody (rabbit polyclonal, Cayman Chemical, Ann Arbor, MI), anti-caveolin-1 antibody (rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, monoclonal, 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 IgG (Thermo Scientific, Rockford, IL) and/or goat anti-rabbit IgG (KPL Biomedical, Chantilly, VA). An 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).

Confocal microscopy

Cells were grown overnight in 4-well, chambered Lab-TEK II slides containing either an RPMI-1640 or DMEM/F12 medium, and were treated with AA (100 μ M) and/or NDGA (5 μ M) for 24 h, fixed with methanol (100%, chilled) for 5 min at -20°C before blocking with normal goat serum (NGS, 5%; Sigma-Aldrich, St. Louis, MO) for 1 h. As described in the

figure legends, 4% paraformaldehyde-fixed cells were incubated overnight in the 4°C with antibodies. The slides were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or Alexa Fluor 568 conjugated goat anti-rabbit secondary antibody (1:500). The cells were mounted with ProLong® Gold Antifade Reagent with DAPI (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). Each experiment was repeated three times. Cells were randomly selected from each slide using the same resolution, laser power, and detector-gain.

Cell migration assays

Migration of breast cancer cells was examined with the help of wound-healing assays, which is standard procedure in monitoring cell migration [5]. Approximately 0.8×10^6 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, 10%). Cells were allowed to incubate overnight in 5% FBS-supplemented medium. 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- μ L pipette tip [5] and treated individually or in combination with AA (100 μ M), NDGA (5 μ M), zileuton (100 μ M), LTB₄ (100 nM) PGD₂ (10 ng/ml), and methyl- β -cyclodextrin (MBCD, 1 mM) for 24 h. The cell images that had migrated between wounded regions were captured using a Nikon-TMS microscope equipped with a Nikon F-601 camera and then counted. The cells treated with NDGA or AA+NDGA were also supplemented with either PGD₂ or LTB₄.

Lipid rafts and leukotriene B4 receptors

Lipid rafts (LRs) were labeled with an LR labeling kit (Vybrant Alexa Fluor 488) containing cholera toxin subunit B (CTXB) that was purchased from Invitrogen (Carlsbad, CA). Live control and treated cells were labeled with the CTXB (1 μ g/ml) conjugated to Alexa Fluor 488 for 10 min followed by labeling with anti-CTXB antibody (1:200 dilution) for 15 min. The cells were then fixed with 4% paraformaldehyde followed by blocking with NGS. For the identification of leukotriene B4 receptors (BLT1), polyclonal antibody (1:200; rabbit, Cayman Chemical, Ann Arbor, MI) was used followed by Alexa Fluor 568 conjugated goat anti-rabbit secondary antibody (1:500). The cells were mounted with ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA), counterstained with DAPI, and analyzed by an LSM 700 confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

Colocalization analysis

The quantitative evaluation of co-localization between CTXB and BLT1 was performed using ZEN 2009 software. Regions of interest (ROIs) were defined manually within cells to reduce background fluorescence contribution. The degree of colocalization of the two fluorophores was determined using a squared Manders' overlap coefficient (OC) of fluorescent signals (green and red for CTXB and BLT1, respectively), implemented on a pixel-by-pixel basis [23]. According to Manders et al. [25], OC ranges from 0 to 1, whereas 0 indicates no pixels and 1 indicates perfectly colocalized pixels in the selected ROI. The

values for selected ROIs were acquired from images taken from 10–12 cells from different microscope fields, using ZEN 2009 software.

Cytokines assay

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, IFN γ , and TNF α) were measured using a commercially available ELISA assay kit (SA Biosciences, Qiagen, Frederick, MD). The concentrations of different cytokines were quantitated from the supernatants isolated from control and AA-treated MCF-7 and MDA-MB-231 cells following the manufacturer's protocol. Briefly, $\sim 1 \times 10^5$ cells were plated in 12-well plates and treated with AA, AA+NDGA, or NDGA for 24 h, as previously mentioned. The supernatants were removed and centrifuged at 12,000 rpm for 5 min. Concentrations of cytokines in the culture supernatants were analyzed by a commercially available enzyme-linked immunoassay kit (Qiagen) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Bio-Rad) at 450 nm, and the data are represented as absorbance values with proper statistical evaluations.

Measuring the level of phospho-NF κ B

To measure the levels of phospho-NF κ B p65, we used the Multispecies InstantOne ELISA Kit, (Thermo Fisher). Approximately, 0.5×10^6 cells were plated in a 6-well plate and grown overnight for attachment. The cells were incubated for an additional 24 h in the presence of AA (100 μ M), AA (100 μ M) + NDGA (5 μ M), or NDGA (5 μ M). 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 manufacturers' instructions. The samples' absorbance was measured using a microplate reader (Bio-Rad Laboratories) set at 450 nm. Values are expressed as relative optical density (OD).

Statistical analyses

To calculate differences between the treatment and the control sets, one-way analyses of variance (ANOVAs) followed by the Holm-Šídák methods were performed using Sigma Plot (version 12) software. Student's *t* tests were performed for the results shown in Fig. S1, and a *P* value of < 0.05 was considered statistically significant [26]. The results obtained from HPLC data (Fig. 1) and Fig. S2 were expressed as the mean $\pm$ SE, and statistical analysis was performed using Microsoft Excel (2007) software. The student's *t* tests between the treatment and control groups were carried out using the GraphPad Software. Results were considered to be statistically significant when *p* values were less than 0.05 (< 0.05).

Results

Arachidonic acid induces LTB $_4$ and HETE8 production in MDA-MB-231 cells

The incorporation of AA to membrane phospholipids is critical for arachidonoyl-phospholipid (AA-PL) synthesis, which in turn releases free AA and serves as a precursor of eicosanoid molecules [27]. However, it is not clear how AA and its metabolites regulate the invasiveness of breast cancer cells. Therefore, in an effort to understand the possible role of AA-induced eicosanoid production, we evaluated how AA modulates eicosanoid synthesis

in both invasive (MDA-MB-231) and mildly invasive (MCF-7) breast cancer cells. First, a dose-response assay was conducted to evaluate cell viability in the presence of AA. The cells were briefly treated with various concentrations of AA (10, 25, 40, 100, 200, and 300 μ M) for 24 h and then subjected to an MTS assay to evaluate the cytotoxic effects of AA exposure [21]. We found that the IC_{50} of AA was $\sim$ 200 μ M in both MDA-MB-231 and MCF-7 cells (Fig. S1). Therefore, the experiments in this present study were conducted using 100 μ M of AA, a concentration at which the cells remain viable. It has been previously reported that at a 100- μ M concentration, AA does not exhibit any toxic effects on cancer cells [28, 29].

In the current study, using a C18 reverse-phase column attached to an HPLC, eicosanoids were resolved and identified by comparing their positions with standard eicosanoids, and quantified by measuring the areas under each peak using the integration software EMPOWER 3 (Waters Corporation, Milford, MA). The results (Figs. 1A and 1B) indicate that the eicosanoid profiles in MDA-MB-231 and MCF-7 cells are very different from each other. While the basal levels of the 5-LOX products—i.e., HETE₅ and HETE₈—are higher in MCF-7 cells than in MDA-MB-231 cells, the amount of PGE₂ is several-fold higher in MDA-MB-231 cells (Figs. 1A and 1B). Figure 1C shows the quantitative assessments of different lipid mediators (in the two cell lines) and how AA affects their syntheses. Notably, AA (100 μ M) suppresses the synthesis of all eicosanoids in MCF-7 cells, but in MDA-MB-231 cells, such treatments down-regulate PGE₂ and PGD₂ and stimulate the syntheses of LTB₄ ($\sim$ 4 fold) and HETE₈ ($\sim$ 3–4 fold). To further understand if excess LTB₄ production by MDA-MB-231 cells is linked to the LOX pathway, nordihydroguaiaretic acid (NDGA), a 5-LOX inhibitor [30], was tested. Figure 1D shows that AA induced-LTB₄ production in MDA-MB-231 could be blocked by NDGA (5 μ M). Interestingly, NDGA by itself also inhibited endogenous LTB₄ synthesis. Additionally, we found that NDGA inhibited HETE₈ production as well (data not shown). As LTB₄ has been shown to be involved in simulating the growth and proliferation of various forms of cancer [14, 15, 30]—and we found that AA stimulates the production of LTB₄ (Figs. 1C and D)—we mainly focused on the role of LTB₄ in inducing the production of inflammatory cytokines and promoting the migration of MDA-MB-231 cells in the current investigation.

Arachidonic acid stimulates the expression of 5-LOX in MDA-MB-231 cells

5-LOX is an essential enzyme of the AA pathway, and it is involved in the synthesis of LTB₄ and various cysteinyl-LTs including LTC₄, LTD₄, and LTE₄ [10]. Because AA treatment increases the production of LTB₄ in MDA-MB-231 cells, we examined whether AA stimulates the expression of the 5-LOX in MDA-MB-231 cells. To address this, AA-treated cells were lysed, and total protein preparations were analyzed by immunoblot. Results show that the expression of 5-LOX ($\sim$ 79kD) in MDA-MB-231 cells increases ($\sim$ 25%) with AA treatment and are inhibited by a LOX inhibitor, nordihydroguaiaretic acid (NDGA) (Figs. 2A and 2B). It is also apparent that the inhibition of 5-LOX by NDGA can be reversed in part by AA. Unlike MDA-MB-231, MCF-7 cells do not show any appreciable changes by AA or NDGA. Confocal microscopy results (Figs. 3A and 3B) show that although both cells express the 5-LOX, only MDA-MB-231 responded to AA treatment significantly as evidenced by increased immunostaining with the LOX antibody. Figure 3A shows that 5-

LOX in MDA-MB-231 cells is mostly localized in the cytoplasm (Image a, red). Upon stimulation by AA, the expression of 5-LOX increases ~2 fold compared to the control and extends throughout the cytoplasm (Image b). As shown in Images c and d (Fig. 3A), NDGA reduces the AA-stimulated 5-LOX reactivity by ~2 fold. On the other hand, 5-LOX expression increases slightly by AA treatment in the MCF-7 cells. Interestingly, NDGA shows a minimal effect on 5-LOX reactivity in MCF-7 cells (Images e–h, Fig. 3A). Figure 3B shows the quantitative analysis of immunofluorescence images. Although NDGA is known to inhibit the arachidonic acid-lipoxygenase pathway and thereby reduces inflammatory reactions, it also functions as an antioxidant agent and has been shown to block the protein trafficking from the ER to Golgi, which cause redistributions of Golgi proteins into the ER and affects the intracellular levels of calcium (reviewed by Lü et al. 2010 ref. 32). Therefore, it is likely that NDGA directly or indirectly interferes with the synthesis and trafficking of 5-LOX and thereby reduces the expression of this enzyme as shown in Fig. 3.

Inhibitors of LOX enzyme inhibit the migration of MDA-MB-231 cells

The migratory behavior of cancer cells is associated with the cellular interactions of the extracellular matrix as well as signals that promote cell motility [33–35]. Therefore, we tested whether AA-induced LOX-5 activation and LTB₄ synthesis is linked to the migration of MDA-MB-231 cells. In addition to NDGA, we also included zileuton in this experiment. Zileuton is a known inhibitor of 5-LOX and suppresses PG biosynthesis by blocking the release of AA in macrophages and other cells [36]. Briefly, MDA-MB-231 cells were placed in a 6-well plate and incubated overnight as described in the Materials and Methods section. The monolayers were wounded and thereafter cultured for 24 h in the presence of two LOX inhibitors—i.e., NDGA and zileuton. The dose-dependent response of NDGA and zileuton in the migration of MDA-MB-231 and MCF-7 is shown in Fig. S2. These two inhibitors were used individually or in combination with AA, LTB₄, and PGD₂. PGD₂, produced by COX-2, was used to compare and evaluate the effect of LTB₄ produced by the 5-LOX. Briefly, 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. The monolayers were wounded by scratching with a sterile 10-μL pipette tip [5] and treated individually or in combination with AA (100 μM), NDGA (5 μM), zileuton (100 μM), and LTB₄ (100 nM), and PGD₂ (10 ng/ml) for 24 h, followed by a count of the cells that had migrated in the scratched areas. Figure 4A shows the number of migratory cells (MDA-MB-231) across the wounded region after 24 h of incubation in control and AA-treated plates. While the AA treatment increased (Image b), NDGA and zileuton blocked the migration significantly (Images d and f). NDGA and zileuton were also effective in reducing the AA-induced migration of MDA-MB-231 (Images e and g). Although AA treatment in addition to LTB₄ (Image h) promoted the migration to a large extent, PGD₂ (Image i) supplement, along with AA treatment, showed a significant reduction in migration. As expected, MCF-7 cells showed little or no migration across the wounded region under these experimental settings (Fig. 4B). Values shown in Table-1 represent the effects of AA, inhibitors, and LTB₄ and PGD₂ on the migration of MDA-MB-231 and MCF-7 cells.

The disruption of lipid rafts interferes with the migration of MDA-MB-231 cells

LTB₄ or other cysteinyl LTs synthesized by LOX pathways are secreted from the cells and act via G protein-coupled BLT receptors [35]. It has been reported that BLT receptors (i.e., BLT-1 and BLT-2) are located within the LRs of human neutrophils and that disruption of LRs suppresses LTB₄ syntheses [37]. Since AA stimulated LTB₄ production in MDA-MB-231 cells (Fig. 1C), and the inhibitors of the LOX enzyme block cellular migration (Fig. 4), we asked whether LTB₄-mediated cellular migration occurs through the LRs. Earlier reports have suggested that the integrity of raft microdomains could be critical for the survival of triple-negative breast cancer cells, as well as for the development of malignancy [38, 39]. Therefore, we performed migration assays to examine whether the disassembly of raft microdomains interferes with the mobility of MDA-MB-231 cells. For this, we used methyl- β -cyclodextrin (MBCD), a commercially available LR disruptor that eliminates cholesterol from membranes and raft domains [38]. Briefly, the migrations of MDA-MB-231 and MCF-7 cells across the scratched wound were carried out in the presence and absence of MBCD (1 mM) as previously described. Results show that MBCD reduces the cellular migration in MDA-MB-231 cells significantly (Figs. 5A and 5B).

Because LRs and caveolin-1 are required for invadopodia formation and extracellular matrix degradation by human breast cancer cells [40], and since MBCD blocks the migration of MDA-MB-231 cells (Fig. 5), we asked if caveolin-1 (a marker of LRs) expression is also altered by AA treatment. Anti-caveolin-1 antibody was used to evaluate the expression of caveolin-1 by immunoblot and confocal microscopy analysis (Fig. 6). Results show that the expression of caveolin-1 is increased slightly by AA treatment and that NDGA reduces the caveolin-1 expression (Figs. 6A and 6B). Figure 6C shows the caveolin-1 expression (green) in MDA-MB-231 cells, which is predominantly concentrated in the plasma membrane (Image a, Fig. 6C). While AA treatment increased the intensity of staining significantly both in membrane and cytoplasm (~3 fold, Image b), NDGA reduced the expression (Image c). The NDGA-mediated reduction of caveolin-1 expression could be reversed partially by co-treatment with AA (Image d). The quantitative analysis of immunostaining is shown in Fig. 6D. In a separate experiment, we tested the reactivity of anti-caveolae antibody on MCF-7 cells. The cells showed weak reactivity against caveolin-1 antibody, and AA-treatment did not change the staining pattern (not shown).

Arachidonic acid promotes the colocalization of BLT1 with lipid rafts

Because AA treatment increases the expressions of LTB₄ and LOX (Figs. 1–3), and the BLT1 receptor is located in LRs [37], we asked whether AA stimulates the expression of BLT1 and increases its colocalization with LRs. To investigate this, BLT receptors of MDA-MB-231 and MCF-7 cells were immunostained with an antibody against the BLT1 receptor (red) and LRs were identified by labeling with cholera toxin subunit B (CTXB, green). Images a–f (Fig. 7A, red) show immunostaining with the BLT1 antibody (red), images a'–f' (green) depict the labeling with CTXB (recognizes LRs), and images a''–f'' (yellow continuous or punctate appearances) demonstrate the colocalization of BLT1 with CTXB with DAPI overlay. The AA treatment in MDA-MB-231 increases the labeling intensity significantly for the BLT1 antibody (~3 fold, image b) and CTXB staining (~2 fold, image b'). The merged image shows bright and well-defined colocalization of the two antibodies

(Image b''). However, the changes of intensity in the AA-treated MCF-7 cells are low and not significant. MBCD has a profound effect in reducing the colocalization of BLT1 and CTX B (Image c'', MDA-MB-231 and f'', MCF-7 cells). The mean intensity/unit areas of CTXB and BLT1 for the MDA-MB-231 and MCF-7 cells are shown in Fig. 7B. These results support the idea that AA not only activates the BLT receptor located in the LRs of MDA-MB-231 cells, it also stimulates the synthesis of LTB₄ and cellular migration. To evaluate the degree of colocalization among CTXB and BLT1 along the cell membrane, a part of the cell membrane was outlined using a white contour (not shown in the figure) as a region of interest (ROI) and the co-localization scattergram (using Zeiss ZEN 2009 software) is shown in Fig. 7C, in which green (CTXB) and red (BLT1) signals are assigned to the x and y axes, respectively. Each pixel is represented as a dot, and pixels with well co-localized signals appear as a scattered diagonal line [41, 42]. The average Manders' overlap coefficients for MCF-7 cells (0.41 ± 0.013) and MDA MB 231 cells (0.90 ± 0.045) suggest strong colocalization between CTXB and BLT1 along the cell membrane of MDA MB 231. Almost ~90% of MDA MB 231 cells show strong colocalization of CTXB and BLT1 (Manders' overlap coefficients 0.9 or above).

Arachidonic acid elevates the levels of NF κ B, IL-6 and IL-8 in MDA-MB-231 cells

Some of the key molecules that are shown to be associated with cancer inflammation include various transcription factors and pro-inflammatory cytokines [43]. The transcriptional factor NF κ B, a potential tumor promoter, is considered to be an important modulator of innate immunity and inflammation [44]. For example, NF κ B is responsible for the activation of IL-6 and IL-8, which are two pro-inflammatory cytokines in tumor cells [45, 46]. Because phospho-NF κ B p65 is known to be linked to the expression of cytokines, and AA activates 5-LOX expression (Figs. 2 and 3), we asked whether AA also modulates the levels of phosphorylated NF κ B and pro-inflammatory cytokines. Figure 8A shows that AA stimulates (~2 fold) the production of phospho-NF κ B p65 (ser 536) and NDGA blocks this stimulation. Compared to MDA-MB-231 cells, however, MCF-7 cells exhibit a low level of phospho-NF κ B expression and remains unaltered by AA or NDGA. Since this transcription factor is linked to the expression of various cytokines, we tested whether AA modulates a variety of interleukins and other inflammatory molecules. 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, IFN γ , and TNF α) were measured using a commercially available ELISA assay kit, as mentioned in the Materials and Methods section (data not shown). We observed that, with the exception of IL-6 and IL-8, the majority of cytokines that we measured were either very low or could not be detected (data not shown). Our results show that MDA-MB-231 cells produce higher levels of IL-6 and IL-8 (~2 and ~5 fold, respectively) than MCF-7 cells. Furthermore, in MDA-MB-231 cells, AA increases the production of IL-6 and IL-8 (Figs. 8B and 8C). Interestingly, the induction of IL-6 by AA is higher (~3 fold) than that of IL-8. Following AA treatment, NDGA prevented the increase of both cytokines. In addition, NDGA alone caused the reduction of IL-6 and IL-8 significantly, which could be because NDGA inhibits the metabolism of endogenous LOX products and cytokines. These experiments suggest that AA-induced 5-LOX activation and LTB₄ production are linked to IL-6 and IL-8 synthesis.

Discussion

Understanding the mechanism of the metastatic migration of breast cancer cells is a matter of great interest. Reports indicate that while unsaturated free fatty acids like oleic acid (OA) and linoleic acid (LA) stimulate cellular proliferation and migration of MDA-MB-231 cells, saturated fatty acids inhibit cell proliferation and apoptosis [47–49]. The activation of CCR10, a member of the chemokine receptor family, was reported to stimulate the migration of breast cancer cells through the ERK1/2/MMP-7 signaling pathway, and that EGF promotes the migration of MDA-MB-231 cells by modulating the functions of phosphatidylinositol kinase and phospholipase C [50]. In the current study, we investigated the mechanism of AA-dependent cellular migration of MDA-MB-231 cells. Since AA is the precursor of eicosanoid molecules, we tested the hypothesis that altering the synthesis of eicosanoids could be linked to the migration of breast cancer cells.

We used two well-studied breast cancer cell lines, MDA-MB-231 and MCF-7, to evaluate the effect of AA on eicosanoid-dependent cellular migrations. These two functionally distinct cell lines differ from each other in morphologic, genomic, transcriptomic, migratory, and therapeutic responses properties [20]. Although both cells have epithelial-like structures, MDA-MB-231 cells are spindle-shaped with an invasive phenotype while MCF-7 exhibits dome-like morphologies. Genomic analyses have revealed the presence of substantial genomic heterogeneity/abnormalities and a transcriptional profile identified luminal and basal-like clustered genes in both MDA-MB-231 and MCF-7 cells. Reports also have suggested that these two breast cancer cells differ in their glycosphingolipid (GSL) compositions [51]. Glycolipids (GLs) and GSLs (i.e., gangliosides) are involved in cell growth, cellular recognition, and metastasis. Although the amount of total GLs is higher in MCF-7 cells, gangliosides are higher in MDA-MB-231 cell lines. Among all gangliosides, the most striking difference is found in the case of GM3 (mono-sialo dihexosyl-ganglioside). GM3, which regulates the growth factor function, is ~18 fold higher in MDA-MB-231 [51].

Our results show that AA treatment suppresses the syntheses of PGs in MDA-MB-231 cells and stimulates the syntheses of LOX products (e.g., LTB₄ and HETE8). Furthermore, AA stimulates cellular migration, which is blocked by LOX inhibitors (NDGA and zileuton), thus suggesting that the LOX pathway is equally important and activated during the migration of breast cancer cells. The increased synthesis of LTB₄ in MDA-MB-231 cells under the influence of AA could be an indication of LOX's catalytic function changes, which enable these groups of enzymes to become hyperactive. In our study, the involvement of LOX, especially the 5-LOX, in stimulating the LTB₄ synthesis was evidenced by the fact that the expression of 5-LOX activity is up-regulated by AA treatment and that NDGA, a LOX inhibitor, lowers this increased expression (Figs. 2 and 3). Zileuton, another 5-LOX inhibitor, also reduces the migration of triple-negative breast cancer cells (Fig. 4, Table-1). BLT1, the LTB₄ receptor, resides in lipid rafts, and thus assembly and disassembly of lipid rafts alter the receptor-specific LTB₄ secretion, which results in migrating MDA-MB-231 cells in culture. The importance of 5-LOX in breast cancer malignancy can be further supported by reports on other cancers. For example, elevated activity of 5-LOX is associated with the progression of hepatocellular carcinoma [12] and also promotes polyposis

formation in the intestine [11]. These reports are consistent with findings described in this study.

It has been previously proposed that malignant transformation is associated with the secretion of inflammatory molecules, including tumor-necrosis factor, interleukins, and chemokines. Among the interleukins, IL-1 was found to be correlated with gastric carcinoma [52], IL-6 with hematological malignancies [53], and IL-8 with melanomas [54]. In glioblastoma, the activation of the transcription factor NF κ B was shown to be connected with the expression of IL-8 [55]. Our results showing the AA-induced increased expression of IL-6 and IL-8 productions (Figs. 8B and 8C) clearly demonstrate that these two pro-inflammatory cytokines could act as contributing factors to the development and progression of invasive breast cancers and that the LOX pathway could play an important role in this process.

In regards to LRs, it has been previously reported that the disassembly of membrane microdomains interferes with survival, invadopodia formation, and ECM (extracellular matrix) degradation by MDA-MB-231 cells [38, 56]. In our study, we found that the disruption of raft domains by MBCD interferes with the migration of MDA-MB-231 cells (Fig. 5) most likely through binding with cholesterol, one of the major constituents of LRs. This observation further supports the idea that the cholesterol-dependent assembly of LRs is important for AA-mediated LOX activation, LTB₄ production, and the migration of MDA-MB-231 cells. LTB₄, which is synthesized from AA by the action of 5-LOX, is released from cells and re-internalized by BLT1 receptors present in the LR.

LTB₄ subsequently stimulates the cells by activating the transcription factor NF κ B [57] that up-regulates the expression of cytokines such as IL-6 and IL-8. The increased production of these inflammatory cytokines is either directly or indirectly linked to the migration by MDA-MB-231 cells. Others have previously reported that AA causes an increase in the migration of MDA-MB-231 cells by activating focal adhesion kinase (FAK) [18]. Thus, it is possible that AA induces the migration of invasive breast cancer cells by activating FAK via LTB₄ and IL-6/IL-8 cytokines. Wen et al. [30] have reported that AA induces angiogenesis of mammary tumors by activating LOX and mTOR pathways but not COX-2, further supporting our hypothesis that LOX is important for breast cancer metastasis. Currently, we are in the process of generating 5-LOX knockout MDA-MB-231 cells, as well as variants that expressing hemagglutinin-tagged 5-LOX. This research enables us to conduct an in-depth investigation to determine the precise role of the LOX pathway in breast cancer cell movement.

Conclusion

Our studies show that AA-induced migration of MDA-MB-231 cells could be linked to the stimulation of LOX enzyme and LTB₄ synthesis. Free AA served as a precursor of eicosanoid molecules including LTB₄, which is secreted and reabsorbed by cells via BLT1 receptors located in lipid rafts. In the case of MCF-7 cells, however, it appears that AA treatment does not influence the LTB₄ production and migration in culture. However, in the current study, only the total LOX enzyme from the whole cell lysate was used. Future

experiments will include active LOX enzymes of cytoplasmic or nuclear origin [58]. An attempt will also be made to overexpress LOX in MCF-7 cells and determine if excess LOX accelerates the migration. The broader aspect of our results could be attributed to the inflammatory and metastatic migration of basal and luminal tumors induced by a fatty diet containing AA and other PUFAs. We propose that LTB₄ synthesis could be targeted for designing novel chemotherapeutic agents to treat breast cancers in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AA	arachidonic acid
BLT	B leukotriene receptor
COX	cyclooxygenase
HETE	hydroxyeicosatetraenoic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
GSL	(glycosphingolipid)
IL	interleukin
LA	linoleic acid
LT	leukotriene
LR	lipid raft
LOX	lipoxygenase
MBCD	methyl-beta-cyclodextrin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium salt
NDGA	nordihydroguaiaretic acid
NFκB	nuclear factor kappa beta

NGS	normal goat serum
OA	oleic acid
OC	overlap coefficient
PLA₂	phospholipase A ₂
PG	prostaglandin
PUFA	polyunsaturated fatty acid
ROI	region of interest
TX	thromboxanes
zil	zileuton

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Fig. (1). Arachidonic acid influences the secretion of eicosanoids by MDA-MB-231 and MCF-7 cells.

Panels A and B show the HPLC profiles of eicosanoids in MDA-MB-231 and MCF-7 cells without any treatment. Panel C demonstrates the effects of AA (100 μ M) on the syntheses of PGD2, PGE2, LTB4, HETE5, and HETE8 by MDA-MB-231 and MCF-7 cells. For statistical analysis, control samples were compared with AA treatments of respective cells (i.e., MDA-MB-231 and MCF-7). Panel D represents the LTB4 levels in the two cell lines after AA, AA+NDGA, and NDGA treatments. For significance calculation, control MDA-MB-231 was compared with AA-treated MDA-MB-231 cells (blue) and control MCF-7 (magenta) was matched with AA-treatment. Experiments (panels C and D) were repeated at least three times in triplicate. Results were given as mean $\pm$ SE and for the significance calculations GraphPad software was employed. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$. AA, arachidonic acid, NDGA, norhydroguaiaretic acid; PGE2, prostaglandin E2; PGD2, prostaglandin D2; LTB4, leukotriene B4; HETE, hydroxyeicosatetraenoic acid. Results shown in Panel C were analyzed by Student's t-tests. For panel D, the p values were analyzed using One-way ANOVA test (Holm-Šidák methods). ** $p < 0.01$; *** $p < 0.001$, NS = Not significant.

Fig. (2). Arachidonic acid stimulates 5-lipoxygenase expression in MDA-MB-231 cells. Panel A: the expression 5-LOX (~79 KD) by MDA-MB-231 and MCF-7 cells in the presence of AA (100 μ M), NDGA (5 μ M), or a mixture of AA+NDGA. GAPDH was used as the loading control. Panel B shows the densitometric analysis of each band shown in panel A. All data represent the average (mean $\pm$ SE) of the three independent experiments. Statistical significance was calculated using One-way ANOVA test followed by the Holm-Šídák methods (panel B). ** $p < 0.01$; NS = Not significant.

Fig. (3). Arachidonic acid increases the expression of 5-lipoxygenase enzyme.

Panel A: Image a: MDA-MB-231, control; Image b: AA-treatment; Image c: treatment with AA+NDGA; Image d: NDGA treatment; Image e: MCF-7, control; Image f: AA treatment; Image g: treatment with AA+NDGA; and Image h: treatment with NDGA. The enlarged images of the white box (a, b, c, and d) show the distinct localization of 5-LOX in the cytoplasm (a', b', c', and d' respectively). The arrow denotes the cytoplasm, and the asterisk indicates the nucleus. Bar: 10 μm. **Panel B:** The quantitative assessments of the mean fluorescence intensities of 5-LOX in MDA-MB-231 (magenta) and MCF-7 (blue). All data represent the average of the three independent experiments. The significance was analyzed using One-way ANOVA test (Holm-Šidák methods). ** p < 0.01; NS = Not significant.

Fig. (4). Arachidonic acid induces the migration of MDA-MB-231 cells.

The cells (~70–80% confluent monolayer) were scratch-wounded using a 10- μ M pipette tip, and treated for 24 h with AA (100 μ M), AA (100 μ M) + NDGA (5 μ M), or NDGA (5 μ M). For MDA-MB-231 and MCF-7, respectively: Image a, a': at 0 h wound-scratch; Image b, b': at 24 h; Image c, c': AA (100 μ M); Image d, d': NDGA (5 μ M); Image e, e': AA+NDGA (100 μ M + 5 μ M); Image f, f': Zileuton (100 μ M); Image g, g': AA+Zil (100 μ M + 100 μ M); Image h, h': AA+LTB4 (100 μ M + 100 nM); Image i, i': AA+PGD2 (100 μ M + 10 ng/ml). MDA-MB-231 cells that migrated into the wound-region were counted and results are shown in Table-1.

Fig. (5). Methyl- β -cyclodextrin blocks the migration of MDA-MB-231 cells.

Approximately 1×10^5 cells were grown in a 12-well plate in growth medium supplemented with 5% FBS and treated with AA (100 μ M), AA (100 μ M) + NDGA (5 μ M), NDGA, or MBCD (1 mM) for 24 h. Panel A: Effect of MBCD (1 mM) on the migration of MDA-MB-231 (Images a and b) and MCF-7 (Images c and d) cells. Panel B: Graphical representation of migration results shown in panel A. Data represent the average (mean $\pm$ SE) of the two separate experiments, and each experiment was carried out in duplicate.

MBCD-treated samples were compared with controls and P values were calculated based on Student's t test using the Graph Pad (** p < 0.001).

Fig. (6). Arachidonic acid induces the expression of caveolin-1 in MDA-MB-231 cells.

Panel A: Immunoblot analysis using caveolin-1 antibody. Tubulin antibody (Sigma) was used as a loading control. Panel B: Densitometric analysis of the immunoblot. Panel C: Immunostaining of caveolae using anti-caveolin-1 antibody. Image a: control; image b: AA (100 μM) treatment; Image c: treatment with NDGA (5 μM); and Image d: treatment with AA + NDGA (100 μM+5 μM). The arrow denotes the membrane, and the asterisk indicates the nucleus. Bar: 10 μM. Panel D: analysis of fluorescence intensities using Zeiss ZEN confocal software (2009). Experiments were repeated three times, and the results expressed in mean ± SE. p values shown in panel B and D were calculated based on Student's t test and compared with the control. *p < 0.05.

Fig. (7). Arachidonic acid promotes the colocalization of BLT1 receptors with lipid rafts in MDA-MB-231 cells

Panel A: Cells were treated with AA (100 μ M) and MBCD (1mM) for 24 h followed by staining of MDA-MB-231 and MCF-7 cells with anti-BLT1 antibody (red, images a–f) and alexa fluor 488-conjugated CTXB for LRs (green, Images a'–f'). The colocalization of CTXB and BLT1 antibody with DAPI are shown in images a''–f''. CM, cell membrane; N, nucleus (stained with DAPI, blue). Bar: 10 μ M. Panels B and C: The fluorescence intensities of CTXB and BLT1 for control and treated cells (MDA-MB-231 and MCF-7) were measured using Zeiss ZEN confocal software (2009). AA and MBCD treated samples were compared with their respective control cells (MDA-MB-231 or MCF-7 immunostained with CTXB or BLT1 antibody, respectively). Results were expressed in mean $\pm$ SE and p values were analyzed by One-way ANOVA test followed by the Holm-Šídák method. *p < 0.05, **p < 0.01; *** p < 0.001; NS = Not significant. Panel D: Scattergrams showing colocalization of the CTXB and BLT1 in MDA-MB-231 and MCF-7 cell lines.

Fig. (8). Arachidonic acid stimulates the production of phospho NFκB (p65), IL-6 and IL-8 in MDA-MB-231 cells.

MDA-MB-231 and MCF-7 cells were cultured and treated with AA, AA+NDGA, or NDGA before measuring the phospho-NFκB, IL-6, and IL-8 levels using commercially available assay kits, as described in Materials and Methods. Data (mean ± SE) represent the average of four independent experiments carried out in triplicate. Treated and control samples of respective cells (MDA-MB-231 or MCF-7) were compared and p values were calculated by One-way ANOVA test followed by the Holm-Šidák method. *p < 0.05; NS = Not significant.

Table 1

Migration analysis of MDA-MB-231 cells after the treatment with 5-LOX inhibitors

Treatment	MDA-MB-231, 0 h	MDA-MB-231, 24 h
	Mean $\pm$ SD	Mean $\pm$ SD
Control	2.3 $\pm$ 1.3	79 $\pm$ 7.6
AA	3.4 $\pm$ 2.2	119.7 $\pm$ 18.9
NDGA	2 $\pm$ 1.4	9 $\pm$ 1.7
AA + NDGA	4 $\pm$ 1.5	84.7 $\pm$ 18.9
Zil	2.2 $\pm$ 1.1	40 $\pm$ 5.2
AA + Zil	2.3 $\pm$ 1	69 $\pm$ 10
AA + LTB4	1.6 $\pm$ 1.2	138.3 $\pm$ 4.7
LTB4 + NDGA	1 $\pm$ 1.3	48.3 $\pm$ 1.7
AA + NDGA+ LTB4	2.3 $\pm$ 2.2	67.3 $\pm$ 10.8
LTB4 + Zil	0.3 $\pm$ 1.1	62.7 $\pm$ 5.8
AA + Zil + LTB4	2.6 $\pm$ 1.5	68 $\pm$ 12.4
AA + PGD2	4.3 $\pm$ 1	62 $\pm$ 6.2
PGD2 + NDGA	2.3 $\pm$ 1.5	44.3 $\pm$ 5.2
AA + NDGA+ PGD2	1.5 $\pm$ 2.6	58.7 $\pm$ 18.1
PGD2 + Zil	1.6 $\pm$ 2.3	61.6 $\pm$ 15.7
AA + Zil + PGD2	3.6 $\pm$ 1.2	43 $\pm$ 14.8

Results shown here are the average values ($\pm$ SD) obtained from migration assays after various treatments. The experiments were repeated twice in triplicate on two different days. SD, Standard deviation; LTB4, leukotriene B4; NDGA, nordihydroguaiaretic acid; PGD2, prostaglandin D2.