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Arachidonic Acid Induces an Increase of β-1,4-Galactosyltransferase I Expression in MDA-MB-231 Breast Cancer Cells

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ABSTRACT

Arachidonic acid (AA) is a common dietary n-6 *cis* polyunsaturated fatty acid that under physiological conditions is present in an esterified form in cell membrane phospholipids, and it might be present in the extracellular microenvironment. AA and its metabolites are implicated in FAK activation and cell migration in MDA-MB-231 breast cancer cells, and an epithelial-to-mesenchymal-like transition process in mammary non-tumorigenic epithelial cells MCF10A. During malignant transformation is present an altered expression of glycosiltransferases, which promote changes on the glycosilation of cell-surface proteins. The β-1,4-galactosyltransferase I (GalT I) is an enzyme that participates in a variety of biological functions including cell growth, migration, and spreading. However, the participation of AA in the regulation of GalT I expression and the role of this enzyme in the cell adhesion process in breast cancer cells remains to be investigated. In the present study, we demonstrate that AA induces an increase of GalT I expression through a PLA2α, Src, ERK1/2, and LOXs activities-dependent pathway in MDA-MB-231 breast cancer cells. Moreover, MDA-MB-231 cells adhere to laminin via GalT I expression and pretreatment of cells with AA induces an increase of cell adhesion to laminin. In conclusion, our findings demonstrate, for the first time, that AA promotes an increase of GalT I expression through an AA metabolism, Src and ERK1/2 activities-dependent pathway, and that GalT I plays a pivotal role in cell adhesion to laminin in MDA-MB-231 breast cancer cells. J. Cell. Biochem. 113: 3330–3341, 2012.

KEY WORDS: BREAST CANCER; GALT I; ARACHIDONIC ACID; ADHESION

In the mammary gland, free fatty acids (FFAs) are taken up and used as energy source and milk lipid synthesis by epithelial cells. However, epidemiological studies and animal models strongly suggest that dietary fatty acids are associated with an increased risk of developing breast cancer [Fay et al., 1997; Lee and Lin, 2000]. FFAs bind to nuclear peroxisome proliferator-activated receptors (PPARs), and mediate expression of genes involved in glucose and lipid metabolism, however they also induce biological processes that are independent of PPARs [Ferre, 2004; Yonezawa et al., 2004]. Particularly, FFAs induce proliferation, invasion, migration and the activation of signal transduction pathways in breast cancer cells [Yonezawa et al., 2004; Navarro-Tito et al., 2008; Soto-Guzman et al., 2008; Soto-Guzman et al., 2010].

Arachidonic acid (AA) is a common dietary n-6 *cis* polyunsaturated fatty acid that under physiological conditions is present in an esterified form in cell membrane phospholipids. It is released by the assistance of at least one of the three different enzymes, namely cytosolic phospholipase A2 (cPLA2), phospholipase C (PLC), and phospholipase D (PLD) [Glaser et al., 1993; Rebecchi and Pentyala, 2000; Harizi et al., 2008]. Free AA is enzymatically metabolized by three major pathways: cyclooxygenases (COXs), lipoxygenases (LOXs), and cytochrome P450 (CYP) epoxygenases. LOXs pathway produces several hydroperoxyeicosatetraenoic acids (HPETEs) and hydroxyeicosatetraenoic acids (HETEs). COXs pathway is mediated by two enzymes namely COX-1 and COX-2, which produce prostaglandins (PGs) and thromboxanes (TXs), whereas CYP epoxigenases pathway produces HETEs and epoxides [Piomelli, 1993; Brash, 2001]. AA and its metabolites are implicated in a variety of biological processes including chemotaxis, atherosclerosis, cancer, rheumatoid arthritis, and the activation of signal

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transduction pathways [Goetzl et al., 1995; Brash, 2001; Touqui and Alaoui-El-Azher, 2001].

The GalT I is an enzyme localized in two distinct subcellular compartments, the *trans*-golgi apparatus and the cell surface. In the *trans*-golgi, GalT I catalyzes the transfer of galactose (Gal) in a β-1,4-linkage from UDP-Gal to terminal *N*-acetylglucosamine (GlcNAc) residues on elongating oligosaccharide chains. However, a portion of the total cellular GalT I is localized on the plasma membrane, and participates like a receptor for extracellular matrix (ECM) proteins, as well as in cellular interactions by binding to appropriate glycoside substrates on the adjacent cell surface [Lopez et al., 1989; Shur, 1993]. GalT I mediates a variety of biological functions, including neurite extension, cell growth, sperm–egg interactions, spreading and cell migration on basal lamina [Gong et al., 1995; Rodeheffer and Shur, 2002].

In the present study we demonstrate that AA induces an increase of GalT I expression through a PLA2α, Src, ERK1/2, and LOX activities-dependent pathway and an increase of GalT I cell surface level in MDA-MB-231 breast cancer cells. Moreover, MDA-MB-231 cells adhere to laminin via GalT I expression and pretreatment of cells with AA induces an increase of cell adhesion to laminin.

MATERIALS AND METHODS

MATERIALS

AA sodium salt, laminin, cycloheximide (CHX), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). PP2, PP3, U73122, nordihydroguaiaretic acid (NDGA), 5-bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl) thiophene (DuP-697), *N*-{(2*S*,4*R*)-4-(Biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl]acrylamide,HCl (cPLA2α inhibitor), and 3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione, HCl (ERK inhibitor) were obtained from Calbiochem-Novabiochem (San Diego, CA). GalT I antibody (Ab) G-15, major histocompatibility complex class I (MHC-I) Ab BRA23/9, GalT I siRNA, and scramble siRNA kits were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ERK1/2 polyclonal Ab and phosphospecific monoclonal Ab E10 to threonine (Thr)-202 and tyrosine (Tyr)-204 of ERK1/2 (anti-P-ERK1/2) were obtained from Cell Signaling Technology (Beverly, MA). A micro Bradford protein assay reagent was from Bio-Rad (Hercules, CA).

CELL CULTURE

MDA-MB-231 and MCF-7 breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/L sodium bicarbonate, 5% fetal bovine serum (FBS), in a humidified atmosphere containing 5% CO_2, and 95% air at 37°C. For experimental purposes, cells were serum starved for 24 h, before treatment with inhibitors and/or AA.

CELL STIMULATION

Confluent cultures were washed twice with DMEM without FBS, equilibrated in the same medium at 37°C for at least 30 min, and then treated with inhibitors and/or AA for the times or concentrations indicated. The stimulation was terminated by aspirating the medium and cell surface proteins were obtained or cells were solubilized in RIPA buffer (50 mM HEPES pH 7.4, 150 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 1.5 mM $MgCl_2$, 0.1% SDS, and protease inhibitors). Lysates were clarified by centrifugation at 12,000 rpm for 10 min at 4°C. Supernatants were transferred to fresh tubes and the protein level of each sample was determined by the micro Bradford protein assay.

SEPARATION OF CELL SURFACE PROTEINS

Separation of cell surface proteins was performed as described previously with some modifications [Bordier, 1981; Friedrichson and Kurzchalia, 1998]. Briefly, 2×10^6 cells were washed twice with ice-cold PBS and lysed. Lysates were incubated for 20 min at 4°C and 10 min at 37°C in 1 ml of Triton X-114 lysis buffer (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-114, and protease inhibitors). Lysates were collected, chilled on ice and cleared by centrifugation at 15,000*g* for 15 min at 4°C. Cleared lysates were subjected to temperature-induced phase separation for 5 min at 37°C. Aqueous and detergent phases were separated by centrifugation for 3 min at 13,000*g* at room temperature. To the detergent phase, 0.9 ml Triton X-114 wash buffer (150 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.06% Triton X-114, and protease inhibitors) was added and vortexed before centrifugation for 15 min at 15,000*g* at 4°C, and other two rounds of phase separation were performed. Proteins were precipitated from detergent phase with cold acetone for 3 h at −20°C and centrifugation for 20 min at 15,000*g* and 4°C. Pellets of cell surface proteins were reconstituted in RIPA buffer, and the protein level of each sample was determined by the micro Bradford protein assay.

WESTERN BLOTTING

Equal amounts of protein (80 μg) were separated by SDS–PAGE using 10% separating gels followed by transfer to nitrocellulose membranes. After transfer, membranes were blocked using 5% non-fat dried milk in phosphate buffered saline (PBS) pH 7.2/0.1% Tween 20 (wash buffer), and incubated overnight at 4°C with primary Ab. The membranes were washed three times with wash buffer and incubated with secondary Ab (horseradish peroxidase-conjugated; 1:5,000) for 2 h at 22°C. After washing three times with wash buffer, the immunoreactive bands were visualized using ECL detection reagent. Autoradiograms were scanned and the labeled bands were quantified using the Image J software (NIH, USA).

FLOW CYTOMETRY

Confluent cultures were washed twice with PBS, scraped off the dishes, collected by centrifugation and fixed for 10 min with 2% formaldehyde in PBS. Cells were incubated overnight at 4°C in PBS-1% BSA containing 0.4 μg/ml Ab directed against GalT I or MHC-I. Cells were washed twice with PBS and incubated with PBS-1% BSA containing the secondary FITC-coupled-donkey anti-goat or goat anti-mouse and incubated for 30 min at room temperature in the dark. After washing the cells three times with cold PBS-1% BSA, cells were re-suspended in 200 μl PBS-1% BSA. One control of secondary Ab without primary Ab was included. Cells were analyzed

with a cytometer FACScan (Dako Cytomation®). Data analysis was performed with the Summit 5.1 software.

RNA INTERFERENCE

GalT I expression was silenced in MDA-MB-231 cells by using the Silencer siRNA kit from Santa Cruz Biotechnology, according the manufacturer's guidelines. One control of scramble siRNAs was included according to the manufacturer's guidelines.

CELL ADHESION ASSAY

Twenty four-well plates coated with 2 $\mu g/cm^2$ laminin were washed and blocked with 2% BSA solution for 1 h at room temperature. MDA-MB-231 cells without and with stimulation with 15 μM AA for 3 and 10 h were dissociated, washed and resuspended in assay buffer (serum free DMEM supplemented with 20 mM HEPES and 0.1% BSA, pH 7.4), and incubated at 37°C in a humidified 5% CO_2 incubator for 20 min to allow cells to recover from the detachment process. In parallel, the wells were aspirated, washed and incubated with 600 μl assay buffer. Next, 1×10^5 cells/ml were plated in triplicates and incubated at 37°C for several times as indicated. After incubation, medium was aspirated and the wells were washed twice with PBS to remove non-adherent cells. Cells were fixed with 4% paraformaldehyde in PBS for 20 min and stained for 10 min with a solution of 0.1% (w/v) crystal violet and quantified by measuring the absorbance at 590 nm in a plate reader. Absorbance readings from blank wells were used to subtract the background binding of crystal violet to plastic.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD. Data were statistically analyzed using one-way ANOVA and the pairwise comparison was performed using Newman–Keuls multiple comparison test. Control comparison was performed using Dunnett's test. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

AA INDUCES AN INCREASE OF GALT I EXPRESSION IN BREAST CANCER CELLS

First, we examined whether AA induces an increase of GalT I expression in breast cancer cells. MDA-MB-231 cells were treated with 15 μM AA for various times and lysed. Cell lysates were analyzed by Western blotting with anti-GalT I Ab. As shown in Figure 1A (upper panel), treatment of cells with AA induced an increase of GalT I expression that reached a maximum between 2 and 4 h of treatment. In addition, AA also induced an increase of GalT I expression in a concentration-dependent manner (Fig. 1B, upper panel). Western blotting with anti-actin Ab of the same membranes confirmed that similar amounts of protein were recovered in the absence or presence of AA (Fig. 1A,B, lower panel).

Next, we determined whether AA induces an increase of GalT I expression in another breast cancer cell line (MCF-7). Cultures of MCF-7 cells were stimulated with 15 μM AA for various times or for

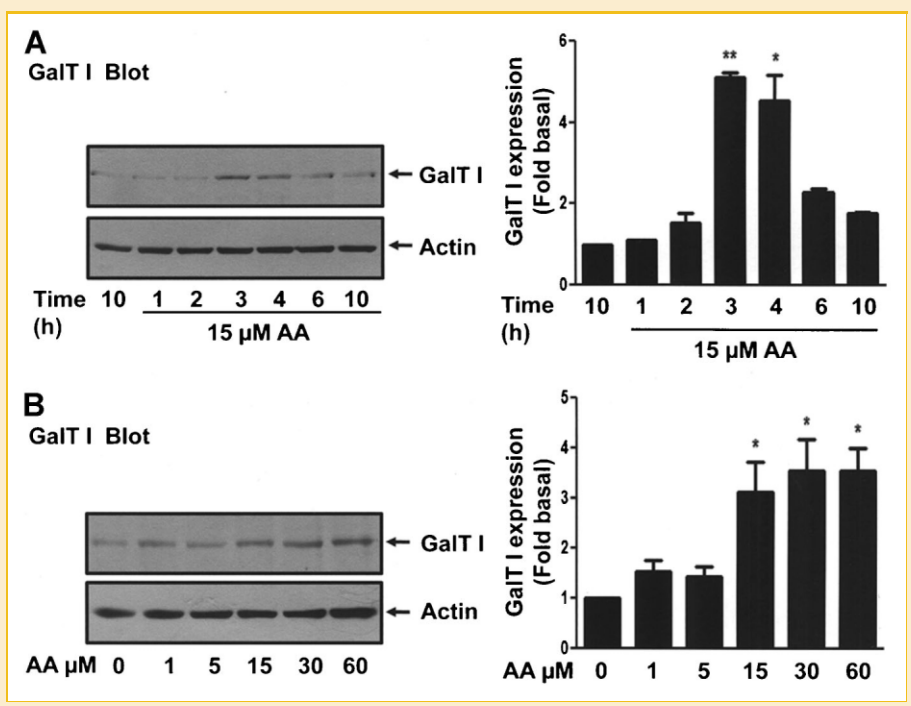

Fig. 1. Arachidonic acid induces an increase of GalT I expression in MDA-MB-231 breast cancer cells. Panel A: MDA-MB-231 cells were treated with 15 μM arachidonic acid (AA) for various times and lysates were obtained. Panel B: MDA-MB-231 cells were treated for 3 h with various concentrations of AA and lysates were obtained. GalT I expression was analyzed by Western blotting using anti-GalT I Ab. The membranes were analyzed further by Western blotting using anti-actin Ab as loading control. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the fold expression above control values. Asterisks denote comparisons made to unstimulated cells. $^*P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA.

3 h with various concentrations of AA. Cells were lysed and analyzed by Western blotting with anti-GalT I Ab. In agreement with our previous results, treatment of MCF-7 cells with AA induced an increase of GalT I expression in a time- and concentration-dependent manner (Fig. 2A,B). In contrast, AA did not induce an increase of GalT I expression in mammary non-tumorigenic epithelial cells MCF10A (Supplementary Material, Fig. 1S).

In order to substantiate further that AA induces an increase of GalT I expression, we analyzed the effect of CHX, which is a inhibitor of translation in eukaryotes [Schneider-Poetsch et al., 2010], on the increase of GalT I expression. MDA-MB-231 cells were treated for 1 h with 70 μM CHX and then stimulated with 15 μM AA for 3 h. Cells were lysed and analyzed by Western blot with anti-GalT I Ab. Our results showed that treatment with CHX completely inhibit the increase of GalT I expression induced by AA (Fig. 2C).

AA INDUCES AN INCREASE OF GALT I-CELL SURFACE LEVEL

In order to determine whether AA induces an increase of GalT I-cell surface level. MDA-MB-231 cells were treated with 15 μM AA for various times and GalT I-cell surface level was evaluated by Western blotting of cell surface proteins and by flow cytometric analysis of unpermeabilized cells using the anti-GalT I Ab. As loading and cell surface protein controls anti-MHC-I Ab was included. As illustrated in Figure 3A (upper panel), treatment of cells with AA induced an increase of GalT I in cell surface protein extracts that reached a maximum at 10 h of treatment. Western blotting with anti-MHC-I Ab of the same membranes confirmed that similar amounts of cell surface proteins were recovered in the absence or presence of AA (Fig. 3A, lower panel). Furthermore, AA induced an increase of GalT I-cell surface level that reached a maximum at 10 h of treatment (Fig. 3B, black bars). Control of cell surface proteins expression

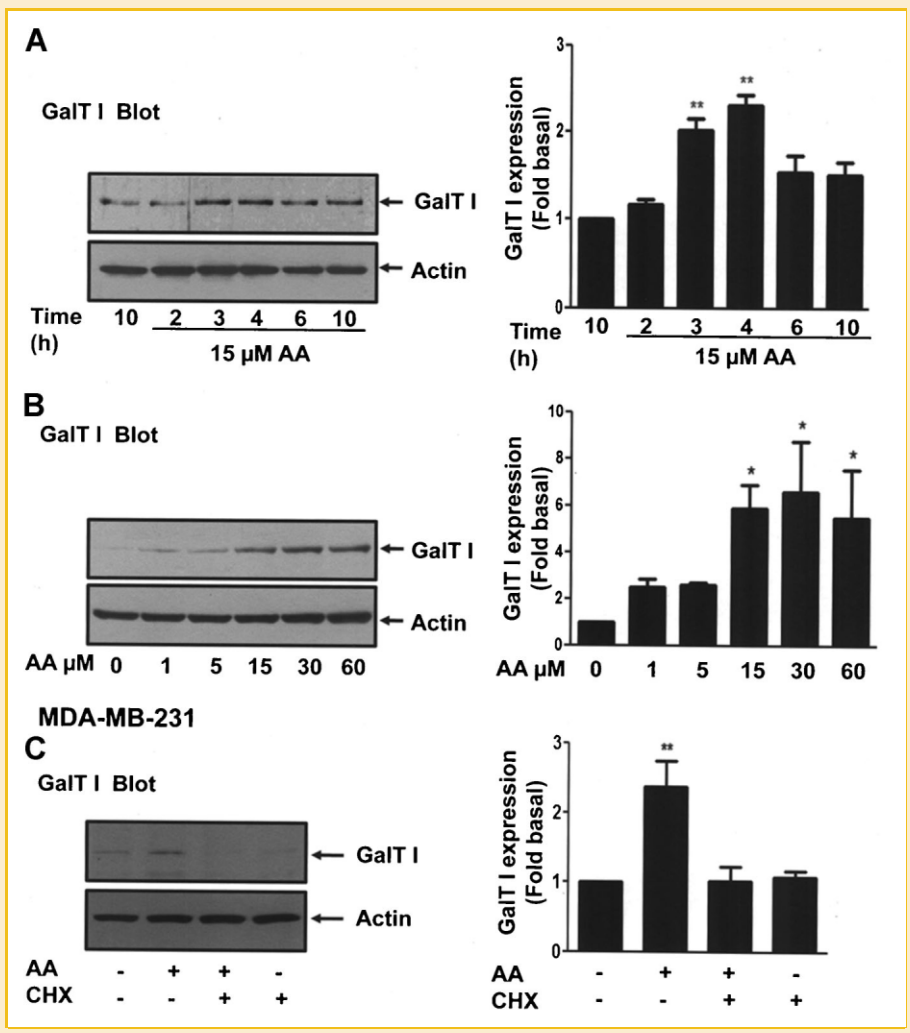

Fig. 2. Arachidonic acid induces an increase of GalT I expression in MCF-7 cells. Panel A: MCF-7 cells were treated with 15 μM AA for various times and lysates were obtained. Panel B: MCF-7 cells were treated for 3 h with various concentrations of AA and lysates were obtained. Panel C: MDA-MB-231 cells were treated for 1 h in the absence (−) or presence (+) of 70 μM cycloheximide (CHX), and then stimulated without (−) or with (+) 15 μM AA for another 3 h and lysates were obtained. GalT I expression was analyzed by Western blotting using anti-GalT I Ab. The membranes were analyzed further by Western blotting using anti-actin Ab as loading control. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the fold expression above control values. Asterisks denote comparisons made to unstimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA.

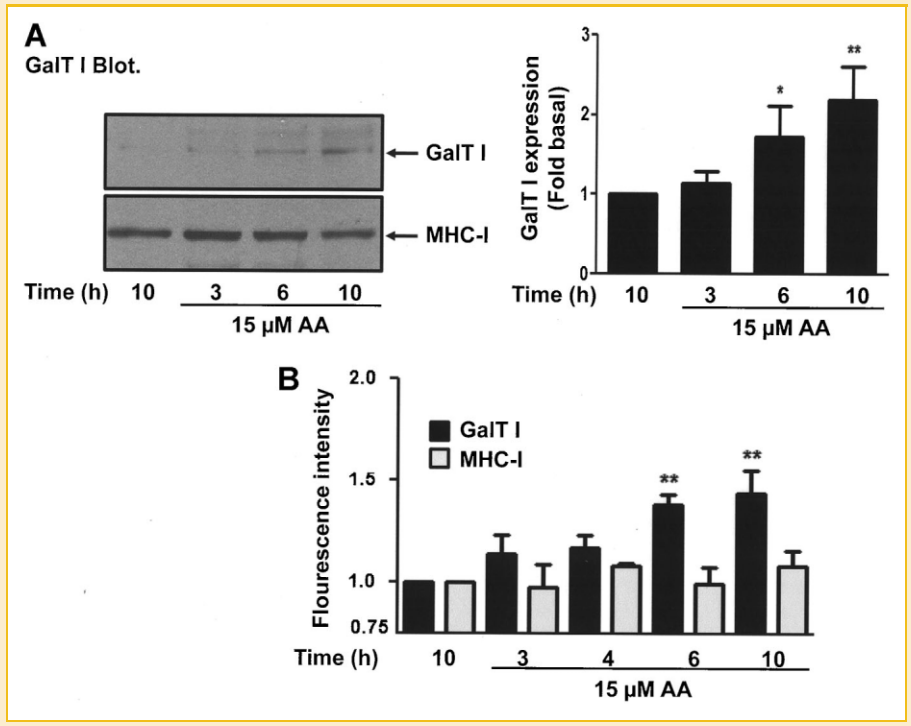

Fig. 3. Arachidonic acid induces an increase of GalT I cell surface level. MDA-MB-231 cells were treated with 15 μM AA for various times Panel A: Cell surface proteins were obtained. GalT I and MHC-I expressions were analyzed by Western blotting using anti-GalT I and anti-MHC-I Abs respectively. The graph represents the mean ± SD of at least three independent experiments and is expressed as the fold expression above unstimulated values. Panel B: GalT I and MHC-I cell surface levels were analyzed by flow cytometric analysis. The graph represents mean fluorescence intensities ± SD for each experimental condition and they were obtained from at least three independent experiments performed in triplicate. Asterisks denote comparisons made to unstimulated cells. $^{*}P < 0.05$, $^{**}P < 0.001$ by one-way ANOVA.

corresponding to MHC-I showed that similar amount of proteins were present on cell surface in the absence or presence of AA (Fig. 3B, white bars).

ROLE OF SRC AND ERK1/2 ON GALT I EXPRESSION

In order to study the role of Src kinase activity on GalT I expression, MDA-MB-231 cells were treated for 30 min with 10 μM PP2, a selective inhibitor of Src family members, or with 10 μM PP3, which is a structurally related but inactive analog of PP2, and then stimulated with 15 μM AA for 3 h. Cell lysates were obtained and analyzed by Western blotting with anti-GalT I Ab. Our results showed that treatment of cells with PP2 inhibit the increase of GalT I expression, whereas treatment with PP3 did not inhibit the increase of GalT I expression in response to AA (Fig. 4A,B).

To determine whether AA induces ERK1/2 activation and the contribution of ERK1/2 in the increase of GalT I expression induced by AA. First, MDA-MB-231 cells were treated with 15 μM AA for various times and lysed. Cell lysates were analyzed by Western blotting with anti-P-ERK1/2 Ab. As shown in Figure 4C (upper panel), treatment of cells with AA induced an increase of ERK1/2 activation, given by the phosphorylation of ERK1/2 at Thr-202 and Tyr-204 [Payne et al., 1991], that reached at maximum between 20 and 45 min of treatment. Next, MDA-MB-231 cells were treated for 1 h with 50 μM ERK inhibitor (ERK-I), and then stimulated with 15 μM AA for 3 h. Cell lysates were obtained and analyzed by Western blotting with anti-GalT I Ab. Our findings demonstrated that treatment with ERK-I prevented the increase of GalT I expression induced by AA (Fig. 4D).

AA INDUCES GALT I EXPRESSION THROUGH AN AA METABOLISM-DEPENDENT PATHWAY

In order to determine the contribution of AA metabolism on the increase of GalT I expression induced by AA. First, we determined whether the increase of GalT I expression requires AA production. Since, AA is mainly generated by the action of cPLA2 and PLC pathways [Chau and Tai, 1981; Dessen, 2000; Rebecchi and Pentyala, 2000]. We studied the effect of the compounds cPLA2αI and U73122, which are selective inhibitors of cPLA2α and PLC respectively [Tatrai et al., 1994; Seno et al., 2000], on the increase of GalT I expression. MDA-MB-231 cells were treated with 1 μM cPLA2αI for 30 min or with 2 μM U73122 for 24 h and then stimulated with 15 μM AA for 3 h. Cell lysates were obtained and analyzed by Western blotting with anti-GalT I Ab. Our findings showed that treatment of cells with cPLAαI completely prevented the increase of GalT I expression, whereas treatment with U73122 did not inhibit the increase of GalT I expression induced by AA (Fig. 5A,B).

Next, we determined the role of AA oxidation on the increase of GalT I expression. We analyzed the effect of the compounds DuP-697 and NDGA, which are selective inhibitors of COXs and LOXs respectively. MDA-MB-231 cells were treated for 24 h with 5 μM DuP-697 or 10 μM NDGA and then stimulated with 15 μM AA for

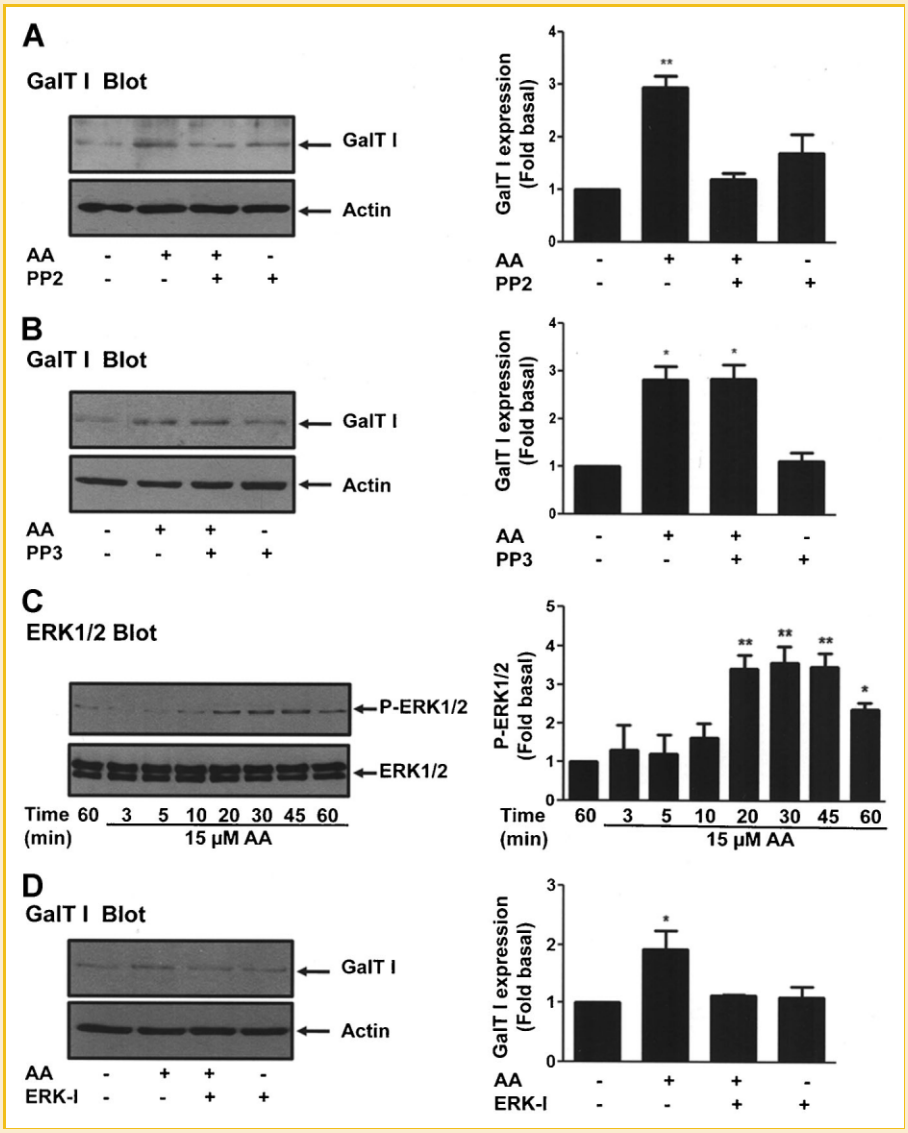

Fig. 4. Src and ERK1/2 mediate the increase of GalT I expression induced by arachidonic acid. Panels A,B: MDA-MB-231 cells were treated for 30 min in the absence (−) or presence (+) of 10 μM PP2 or 10 μM PP3, and then stimulated without (−) or with (+) 15 μM AA for another 3 h and lysates were obtained. GalT I expression was analyzed by Western blotting using anti-GalT I Ab. The membranes were analyzed further by Western blotting using anti-actin Ab as loading control. Panel C: MDA-MB-231 cells were treated with 15 μM AA for various times and lysates were obtained. ERK1/2 phosphorylation at Thr-202 and Tyr-204 was analyzed by Western blotting using anti-P-ERK1/2 Ab. The membranes were analyzed further by Western blotting using anti-ERK1/2 Ab. Panel D: MDA-MB-231 cells were treated for 1 h in the absence (−) or presence (+) of 50 μM ERK inhibitor (ERK-I), and then stimulated without (−) or with (+) 15 μM AA for another 3 h and lysates were obtained. GalT I expression was analyzed by Western blotting using anti-GalT I Ab. The membranes were analyzed further by Western blotting using anti-actin Ab as loading control. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the fold expression or phosphorylation above control values. Asterisks denote comparisons made to unstimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA.

3 h. Cell lysates were obtained and analyzed by Western blotting with anti-GalT I Ab. As shown in Figure 6A,B, treatment with COXs inhibitor DuP-697 did not inhibit the increase of GalT I expression, whereas treatment with LOXs inhibitor NDGA completely prevented the increase of GalT I expression induced by AA.

Since, the increase of GalT I expression induced by AA requires ERK1/2 activity, we determined the role of LOXs and Src on ERK1/2 activation. MDA-MB-231 cells were treated with 10 μM NDGA or 10 μM PP2, and then stimulated with 15 μM AA for 30 min. Cells were lysed and ERK1/2 activity was determined. Our findings showed that inhibition of LOXs and Src activities completely inhibit ERK1/2 activation induced by AA (Fig. 6C,D).

ROLE OF GALT I ON CELL ADHESION INDUCED BY AA

Cell adhesion and migration are critical steps involved in invasiveness and metastasis. We studied whether AA induces an increase of cell adhesion to laminin, and the role of GalT I. First, we determined the cell adhesion kinetic to laminin of MDA-MB-231 cells. As illustrated in Figure 7A,B, MDA-MB-231 cells adhere to

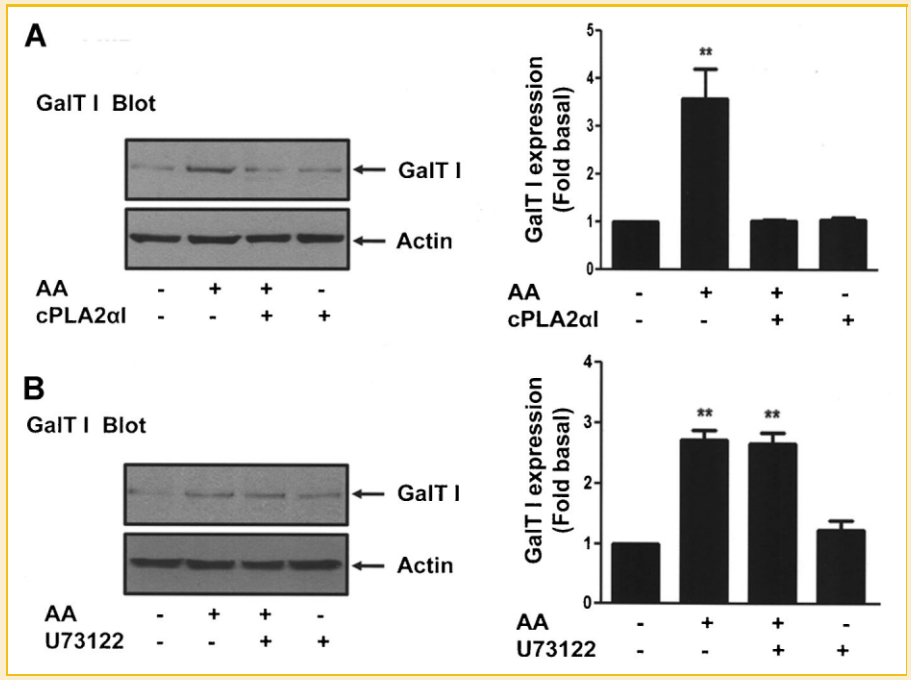

Fig. 5. Arachidonic acid induces an increase of GalT I expression through a cPLA2-dependent pathway. MDA-MB-231 cells were treated without (−) or with (+) 1 μM cPLA2αI for 30 min (Panel A), or 2 μM U73122 for 24 h (Panel B), and then stimulated without (−) or with (+) 15 μM AA for another 3 h and lysates were obtained. GalT I expression was analyzed by Western blotting using anti-GalT I Ab. The membranes were analyzed further by Western blotting using anti-actin Ab as loading control. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the fold expression above control values. Asterisks denote comparisons made to unstimulated cells. $^{**}P < 0.01$ by one-way ANOVA.

laminin in a time-dependent manner, reaching a maximum of adhesion at 120 min (Fig. 7A,B).

Next, we examined whether AA induces an increase of cell adhesion to laminin and the role of GalT I on cell adhesion. MDA-MB-231 cells and cells in which GalT I protein was silenced by siRNA (Fig. 7C), were pretreated with 15 μM AA for 3 and 10 h. Cells were detached and then cell adhesion assays to laminin were performed at 45 min. Our results showed that cells treated with AA for 10 h present the maximum of adhesion to laminin, whereas inhibition of GalT I expression prevented the cell adhesion to laminin in treated and untreated cells for 3 and 10 h with AA. Specificity of inhibition was demonstrated by transfection with scramble siRNAs, which did not inhibit cell adhesion to laminin in treated and untreated cells with AA (Fig. 7D).

DISCUSSION

Metastasis is a complex process involving a series of sequential events including release of cells from primary tumor, invasion through ECM including basement membrane and intravasation. It requires the interaction between tumor cells and the ECM, which constitutes an important microenvironment factor that regulates a lot of cellular processes [Liotta and Kohn, 2001]. Moreover, malignant transformation involves an altered expression of glycosyltransferases at the transcriptional and translational levels and changes of cell-surface protein glycosilation, where sugar residues and oligosaccharide chains of glycoproteins play diverse and crucial roles in adhesion, growth and metastasis [Asada et al., 1997]. However, the contribution of AA in the expression of glycotransferases and the role of these enzymes in cell adhesion of breast cancer cells has not been studied in detail.

The adhesion and migration through ECM are associated with enhanced expression of receptors that recognize ECM components including integrins (αVβ3, α3β1, α5β1), immunoglobulin superfamily members (ICAM-1 and MUC18), as well as cell adhesion molecules that recognize carbohydrate ligands, such as GalT I [Edward, 1995]. Particularly, GalT I functions as a surface receptor during cell migration, because it associates with the cytoskeleton and localizes to the leading edge of the cell where facilitates spreading and migration [Eckstein and Shur, 1989]. Furthermore, GalT I has been implicated in cancer, because it is up-regulated in metastatic tumors and is involved in tumor cell migration and invasion. GalT I also is expressed at high levels in a number of metastatic murine and human cell lines [Passaniti and Hart, 1990; Johnson and Shur, 1999; Zhu et al., 2005]. Therefore, we studied whether AA induces an increase of GalT I expression in breast cancer cells. Our findings demonstrate that MDA-MB-231 and MCF-7 cells express GalT I, and that one portion of this protein is expressed on cell surface. Moreover, AA promotes an increase of GalT I expression level, with an increase on cell surface in MDA-MB-231 cells. In contrast, AA does not induce an increase of GalT I expression level in mammary non-tumorgenic epithelial cells MCF10A. We propose that AA plays an important role in breast cancer progression via the

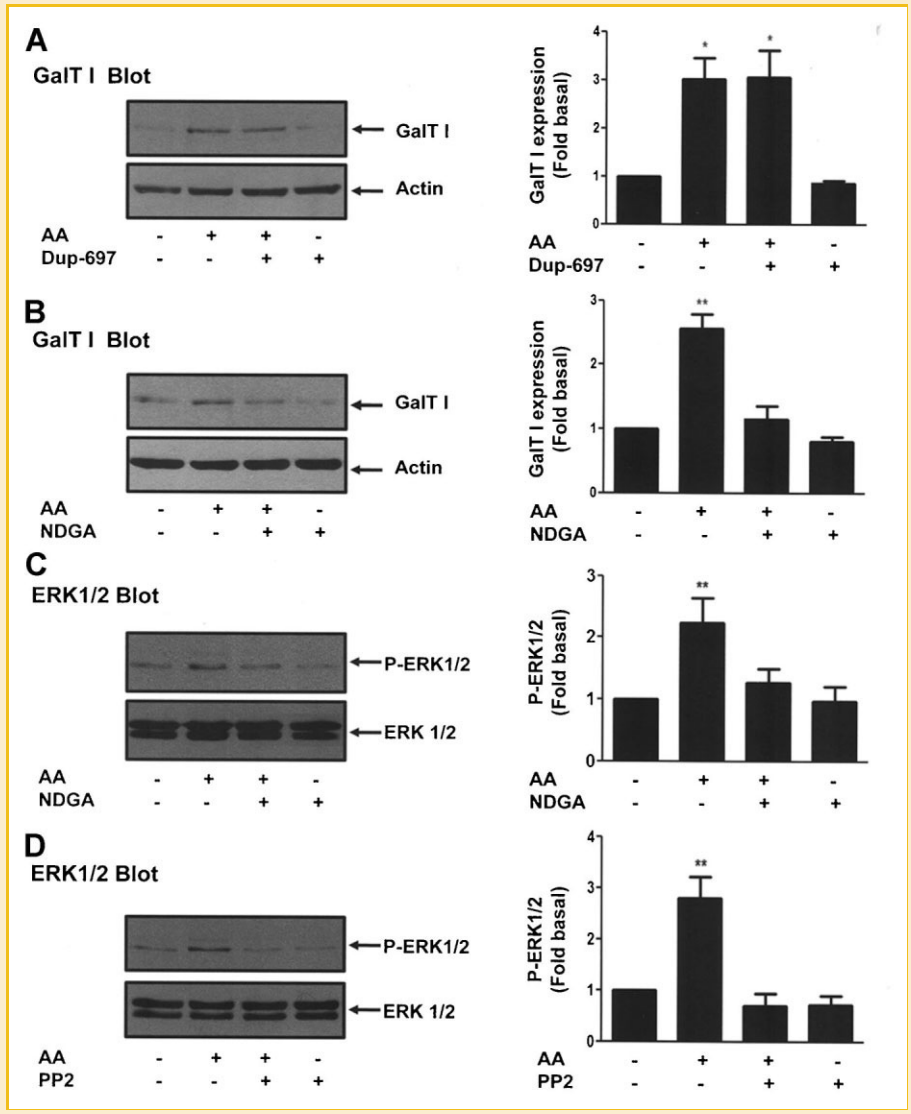

Fig. 6. LOXs mediates the increase of GalT I expression and ERK1/2 activation induced by arachidonic acid. Panels A–C: MDA-MB-231 cells were treated for 24 h in the absence (−) or presence (+) of 5 μM Dup-697 or 10 μM NDGA and then stimulated without (−) or with (+) 15 μM AA for 3 h or 30 min and cell lysates were obtained. Panel D. MDA-MB-231 cells were treated for 30 min in the absence (−) or presence (+) of 10 μM PP2 and then stimulated without (−) or with (+) 15 μM AA for another 30 min and lysates were obtained. GalT I expression and ERK1/2 activation were analyzed by Western blotting using anti-GalT I and anti-P-ERK1/2 Abs, respectively. The membranes were analyzed further by Western blotting using anti-actin Ab or anti-ERK1/2 Ab as loading controls. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the fold expression or phosphorylation above control values. Asterisks denote comparisons made to unstimulated cells. $^{*}P < 0.05$, $^{**}P < 0.01$ by one-way ANOVA.

increase of GalT I expression. Supporting our proposal, we previously demonstrated that AA induces FAK activation and cell migration in breast cancer cells, whereas it promotes an epithelial-mesenchymal-like transition in mammary non-tumorigenic epithelial cells MCF10A [Navarro-Tito et al., 2008; Martinez-Orozco et al., 2010]. Moreover, GalT I is highly expressed in hepatoma and HBx, a regulatory protein that contributes to the development of hepatocellular carcinoma, induces upregulation of GalTI, which mediates tumor formation in vivo; whereas in ovarian cancer cells, GalT I expression mediates adhesion and invasion [Yamashita et al., 2003; Wei et al., 2008].

In breast cancer cells, oleic acid (OA) induces ERK1/2 activation and cell proliferation via MEK1/2 and ERK1/2 activity [Hardy et al., 2005; Soto-Guzman et al., 2008]. In line with this notion, we demonstrate that AA induces ERK1/2 activation in a time-dependent manner in MDA-MB-231 cells. Our findings are in agreement with previous reports showing that AA induces MAP kinase family members activation in MDA-MB-231 and vascular smooth muscle cells [Rao et al., 1994; Paine et al., 2000]. In addition, our findings show that AA induces ERK1/2 activation and one increase of GalT I expression via Src kinase activity in MDA-MB-231 cells. We propose that AA promotes Src activation and then Src promotes

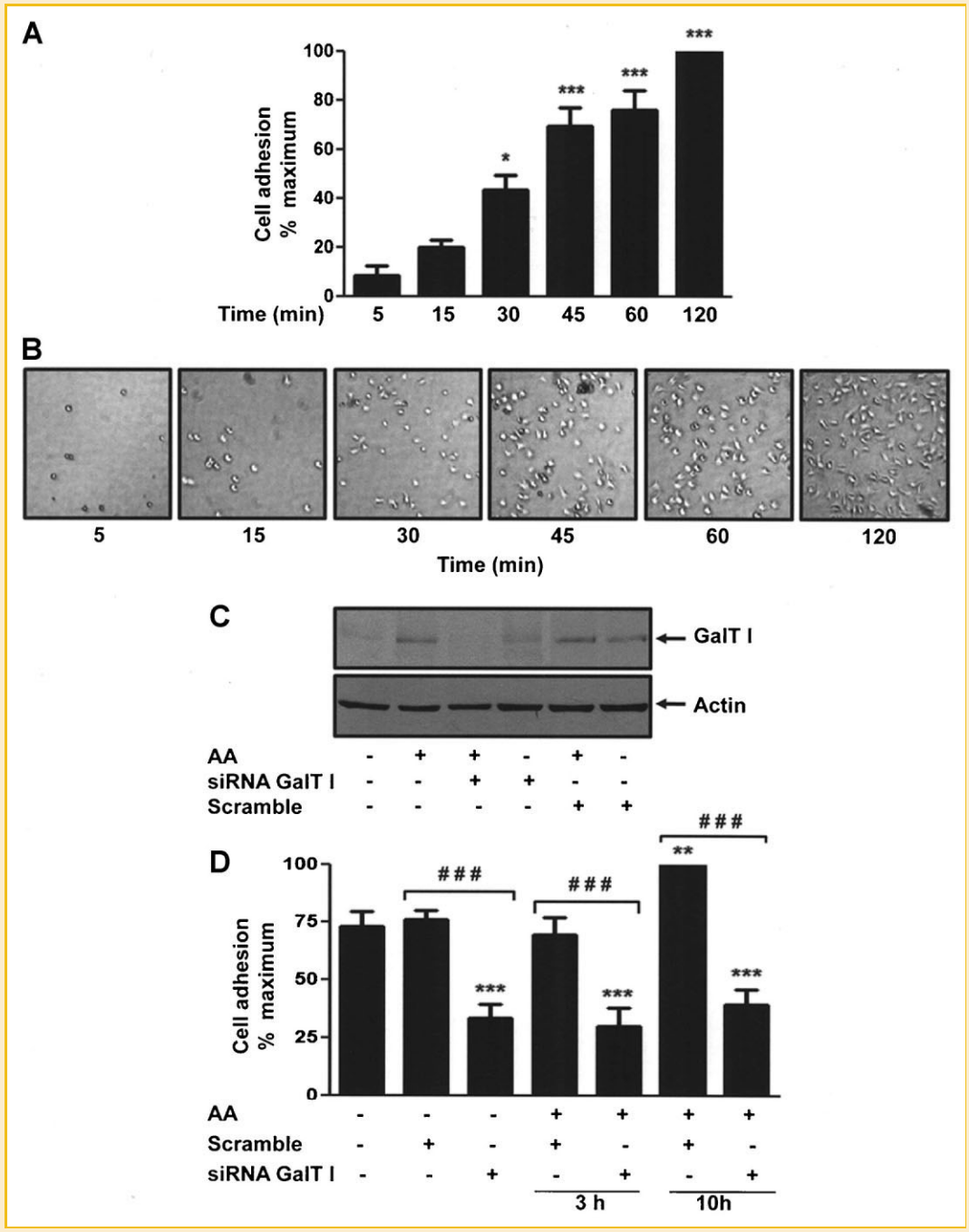

Fig. 7. GalT I mediates cell adhesion to laminin induced by arachidonic acid. Panel A,B: Cell adhesion assays to laminin were performed for several times using MDA-MB-231 cells. Pictures were taken at the several times as indicated. The graph represents the mean ± SD of at least three independent experiments and is expressed as percentage of cell adhesion maximum. Asterisks denote comparisons made to 5 min of cell adhesion. Panel C: MDA-MB-231 cells were transfected with GalT I-specific or scramble siRNAs. GalT I expression was analyzed by Western blotting of cell lysates using anti-GalT I Ab. Panel D: MDA-MB-231 cells and cells transfected with GalT I-specific or scramble siRNAs were pretreated without (−) or with (+) 15 μM AA for 3 and 10 h. Cells were detached and cell adhesion assays to laminin were performed at 45 min. The graphs represent the mean ± SD of at least three independent experiments and are expressed as percentage of maximum of cell adhesion. Asterisks denote comparisons made to control cells (unstimulated and without transfection cells). Number signs denote comparisons between the groups of cells that are indicated by the brackets $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#\#\#}P < 0.001$ by one-way ANOVA.

MAPKs activation including ERK1/2, which promotes the activation of transcription factors involved in GalT I expression. In support of our proposal, AA induces Src activation [Navarro-Tito et al., 2008], and we demonstrate that Src mediates ERK1/2 activation, whereas ERK1/2 activity mediates the increase of GalT I expression induced by AA in MDA-MB-231 cells. Furthermore, an overexpression of ERK1 promotes an increase of GalT I expression in PGLH7 human lung cancer cells [Zhu et al., 2005], whereas ERK

activity mediates GalT I expression in rat Schwann cells [Yang et al., 2009].

AA promotes adhesion to type IV collagen through a 15(S)-LOX-2-dependent pathway, whereas it induces FAK activation and cell migration through a LOXs-dependent pathway in MDA-MB-231 cells [Nony et al., 2005; Navarro-Tito et al., 2008]. Since, our findings demonstrate that AA induces an increase of GalT I expression by a pathway dependent on cPLA2α and LOXs activities and that cPLA2α is the central regulator of stimulus-coupled cellular AA release [Ghosh et al., 2006]. We propose that AA induces the release of AA from membrane phospholipids via cPLA2α activity and then free AA is metabolized by LOXs and then LOXs metabolites induces the activation of signal transduction pathways that mediate the increase of GalT I expression in MDA-MB-231 cells. Supporting our proposal, we demonstrate that AA induces ERK1/2 activation via LOXs activity and that ERK1/2 mediates the increase of GalT I expression in MDA-MB-231 cells.

LOXs constitute a family of nonheme iron dioxygenases, including 5-, 8-, 12- and 15-LOX whose main products are 5(S)-, 8(S)-, 12(S)- and 15(S)-HETE [Brash, 1999]. Particularly, 12(S)-HETE promotes the formation of focal adhesions, leading to enhanced adhesion of murine B16 amelanotic melanoma cells to fibronectin via a G-protein-coupled receptor (GPCR) coupled to Gi/Go [Liu et al., 1995], whereas in prostate cancer cells, 12(S)-HETE stimulates ERK1/2 phosphorylation via a GPCR coupled to Gi/Go [McCabe et al., 2006]. Since, the increase of GalT I expression induced by AA is dependent of LOXs activities, we propose that 12-LOX and its metabolite 12(S)-HETE mediate the increase of GalT I expression via a GPCR coupled to Gi/Go. However, it remains to be investigated.

FFAR1 (GPR40) and GPR120 are GPCRs activated by medium and long chain FFAs, such as AA, and are expressed in MDA-MB-231 and MCF-7 breast cancer cells and in the mammary non-tumorigenic epithelial cells MCF10A [Yonezawa et al., 2004; Navarro-Tito et al., 2008; Soto-Guzman et al., 2008]. In breast cancer cells, OA induces an increase in cellular Ca^{2+} concentration and cell proliferation via FFAR1 coupled with Gi/Go [Yonezawa et al., 2004; Hardy et al., 2005]. We propose that AA induce the increase of GalT I expression via activation of GPR40 and/or GPR120 in MDA-MB-231 cells. However, it remains to be investigated.

GalT I on the plasma membrane functions as a laminin receptor by binding to E8 domain of *N*-linked oligosaccharides, and mediates cell migration on basal lamina matrices [Begovac et al., 1991; Appeddu and Shur, 1994]. In addition, GalT I expressed on cell surface mediates cell migration, whereas the level of GalT I on cell surface also contributes to the invasive phenotype in vitro and to the metastatic phenotype in vivo [Appeddu and Shur, 1994; Johnson and Shur, 1999]. In line with this notion, we demonstrate that cell adhesion to laminin is mediated by GalT I and that treatment of cells with AA for 10 h induces an increase of cell adhesion to laminin via GalT I expression in MDA-MB-231 cells. We propose that increase of GalT I expression mediates cell adhesion through activation of proteins present in focal adhesions including FAK in breast cancer cells. Supporting our proposal, it has been reported that clustering of cell surface GalT I induces a transient tyrosine phosphorylation of FAK followed for a reduction of actin stress fibers and focal adhesion contacts [Wassler and Shur, 2000].

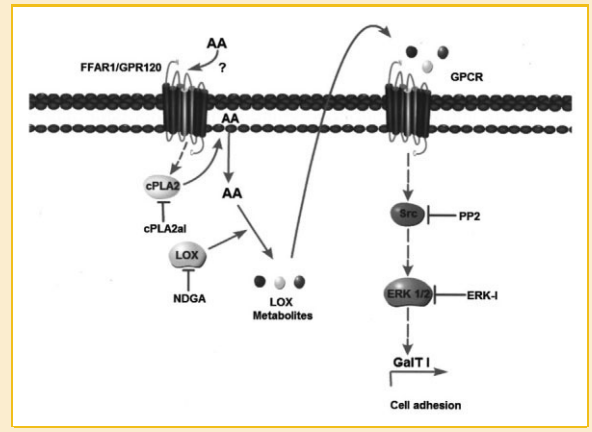

Fig. 8. Model of increase of GalT I expression induced by arachidonic acid in MDA-MB-231 breast cancer cells. Taken together our findings, we propose that stimulation of cells with AA induces the activation of FFAR1/GPR120, which promotes cPLA2α activation and the release of AA from membrane phospholipids. Free AA is metabolized by LOXs and LOXs metabolites are produced and secreted to the medium. LOXs metabolites bind and activate GPCRs, which induces Src and ERK1/2 activation. ERK1/2 promotes the activation of transcription factors that participate in GalT I expression. GalT I on cell surface plays a pivotal role in cell adhesion to laminin.

Taken together, these results suggest that AA and the increase of GalT I expression play a pivotal role in cell migration and invasion in breast cancer cells, and therefore that they also may promotes invasion and metastasis in breast cancer. Moreover, our findings suggest that breast cancer metastasis may be prevented by using drugs that keep a low-level of AA in the tumor microenvironment and that inhibit the increase of GalT I expression. In line with this notion, we propose the inhibition of AA oxidation, particularly LOXs activity, because it will inhibit the increase of GalT I expression. Supporting this proposal we previously demonstrated that OA induces cell migration through a LOXs-dependent pathway and that native type IV collagen induces MMP-2 and MMP-9 secretion via LOXs activity in MDA-MB-231 breast cancer cells [Navarro-Tito et al., 2010; Castro-Sanchez et al., 2011]. However, additional studies are necessary.

In conclusion, our findings demonstrate that increase of GalT I expression induced by AA requires AA oxidation, ERK1/2 and Src kinase activity, as well as GalT I plays a pivotal role in adhesion to laminin of MDA-MB-231 breast cancer cells. These findings are depicted in Figure 8.

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