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RESEARCH PAPER

Oleic acid promotes MMP-9 secretion and invasion in breast cancer cells

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Abstract Epidemiological and animal studies suggest an association between dietary fatty acids and an increase risk of developing breast cancer. Obesity, which is characterized by hyperlipidemia and an elevation of circulating free fatty acids (FFAs), is also associated with enhanced cancer risk. In breast cancer cells, the FFA oleic acid (OA) induces migration, proliferation, prolong survival, invasion, an increase in cellular Ca^{2+} concentration, MEK1/2, ERK1/2, FAK and Src activation. However, the role of OA on MMP-9 secretion and invasion has not been studied in detail. We demonstrate here that stimulation of MDA-MB-231 breast cancer cells with 200 μM OA induces an increase on MMP-9 secretion through a PKC, Src, and EGFR-dependent pathway, as revealed by gelatin zymography assays. Furthermore, microtubule network mediates MMP-9 secretion induced by OA. In contrast, OA does not induce an increase on MMP-9 secretion in MCF10A cells, whereas it does not induce MMP-9 secretion in MCF12A mammary non-tumorigenic epithelial cells. In addition, OA induces invasion through an EGFR, Gi/Go proteins, MMPs, PKC and Src-dependent pathway, but it is not able to promote invasion in non-invasive MCF-7 breast cancer cells. In summary, our findings demonstrate that OA promotes an increase on MMP-9 secretion and invasion through a PKC, Src, and EGFR-dependent pathway in breast cancer cells.

Keywords Breast cancer · Oleic acid · MMP-9 · Invasion

Introduction

Epidemiological and animal studies suggest an association between dietary fatty acids and an increased risk of developing breast cancer [1–4]. Obesity, which is characterized by hyperlipidemia and an elevation of circulating free fatty acids (FFAs), has been also associated with enhanced cancer risk [5, 6]. FFAs are energy source for the body, act as signaling molecules, and they bind to nuclear peroxisomal proliferated-activated receptors (PPARs), mediating expression of genes involved in glucose and lipid metabolism. However, FFAs also mediate biological effects independent of PPARs, such as proliferation and migration [7–10].

GPR40, GPR41, GPR43 and GPR120 are G protein-coupled receptors (GPCRs) activated by FFAs. GPR40 and GPR120 are receptors for medium and long chain FFAs, whereas GPR41 and GPR43 are receptors for short chain FFAs [11–16]. GPR40 and GPR120 are expressed in MDA-MB-231 and MCF-7 breast cancer cells and in the mammary non-tumorigenic epithelial cells MCF10A [11, 17, 18]. In breast cancer cells, oleic acid (OA) induces migration, proliferation, prolong survival, invasion, an increase in cellular Ca^{2+} concentration, MEK1/2, ERK1/2, FAK and Src activation [10, 18–23]. Moreover, arachidonic acid

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(AA) promotes FAK activation and cell migration in breast cancer cells and epithelial-to-mesenchymal transition in mammary epithelial cells MCF10A [17, 24].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that collectively are capable of degrading all extracellular matrix (ECM) components, and have been implicated in several aspects of tumor progression, including angiogenesis, tumor cell growth and invasion through basement membranes (BMs) and interstitial matrices [25–27]. MMPs gene family consists of at least 20 members and is subgrouped into different types based on sequence characteristic and substrate specificity [28, 29]. MMPs are synthesized and secreted as zymogens that require activation to become proteolytically active. Thus, activation is a critical step in the regulation of MMP-dependent proteolytic activity. In particular, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are associated with tumor progression and metastasis due to their ability to degrade type IV collagen, the main component of BM, and their elevated expression in malignant tumors. In breast cancer, both gelatinases are highly expressed, and is suggested that play an important role in breast cancer invasion, metastasis and tumor angiogenesis [30–33].

In the present study, we demonstrate that OA promotes MMP-9 secretion through a PKC, Src and EGFR-dependent pathway, whereas it induces invasion via an EGFR, Gi/Go proteins, MMPs, PKC and Src-dependent pathway in breast cancer cells. In contrast, OA does not induce an increase on MMP-9 secretion in MCF10A cells, and it does not induce MMP-9 secretion in mammary non-tumorigenic epithelial cells MCF12A.

Materials and methods

Reagents

OA sodium salt, epidermal growth factor (EGF), phorbol 12,13-dibutirate (PDB), GF109203X (GF-I), SU6656 and *Pertussis toxin* (PTX) were obtained from Sigma (St. Louis, MO, USA). Fatty acid-free bovine serum albumin (FAF-BSA) was obtained from Research Organics (Cleveland, OH, USA). AG1478, PP2 and GM6001 were obtained from Calbiochem (San Diego, CA, USA). MMP-9 monoclonal antibody (Ab) H-129 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Actin monoclonal Ab was kindly provided for Dr. Manuel Hernandez (Cinvestav-IPN). Basement membrane matrix (BD Matrigel) was obtained from BD Biosciences (Bedford, MA, USA). Free fatty acid quantification kit was obtained from BioVision (Mountain View, CA, USA). ECL reagent was from Amersham Pharmacia Biotech. All other reagents used were of the highest grade available.

Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3.7 g/L sodium bicarbonate, 5% fetal bovine serum (FBS) and antibiotics, in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

The non-tumorigenic epithelial cell lines MCF12A and MCF10A were cultured in DMEM/F12 medium (1:1 and 3:1, respectively) supplemented with 5% FBS, 0.4 μg/ml hydrocortisone, 4.18 μg/ml insulin, 10 ng/ml recombinant EGF and antibiotics, in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

For experimental purposes, breast cancer cells were serum-starved for 12 h before treatment with inhibitors and/or OA, whereas MCF12A and MCF10A cells were starved for 4 h in DMEM without FBS, EGF, insulin and hydrocortisone before treatment with inhibitors and/or OA.

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 PDB, inhibitors and/or OA bound to FAF-BSA (BSA-OA) for the times or concentrations indicated. BSA-OA was prepared by stirring OA sodium salt at 37°C with 5% FAF-BSA as described before [20]. After being adjusted to pH 7.4, the solution was filtered through a 0.22 μm filter and the fatty acid concentration was measured using a fatty acid assay kit (Bio Vision). When BSA-OA was added to serum-free culture medium, the final concentration of BSA was adjusted to 0.005%.

Zymography

Cells were stimulated and conditioned medium was collected and concentrated using chemicom tubes (Millipore). Moreover, cells were lysed and cell lysates were analyzed by western blotting with anti-actin Ab as loading control. Proteolytic activity was assayed on conditioned medium using gelatin-substrate gels as described previously [34]. Briefly, same volume of non-heated samples were mixed with sample buffer (2.5% SDS, 1% sucrose and 4 μg/ml phenol red), without reducing agent and applied to 8% acrylamide gels copolymerized with gelatin at 1 mg/ml. After electrophoresis at 72 V for 2 h, the gels were rinsed twice in 2.5% Triton X-100 to remove SDS and then incubated in 50 mM Tris–HCl pH 7.4 and 5 mM $CaCl_2$ assay buffer at 37°C for 24 h. Gels were fixed and stained with 0.25% Coomassie Brilliant Blue G-250 in 10% acetic acid and 30% methanol. Proteolytic activity was detected as clear bands against the background stain of undigested substrate in the gel.

Western blotting

Equal amounts of protein were separated by SDS-PAGE using 8% 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, and incubated overnight at 4°C with the primary Ab as indicated. The membranes were washed three times with PBS/0.1% Tween 20, and then incubated with secondary Abs (horseradish peroxidase-conjugated, donkey Abs to rabbit) (1:5,000) for 2 h at 22°C. After washing three times with PBS/0.1% Tween 20, the immunoreactive bands were visualized using ECL detection reagents. Autoradiograms were scanned and the labeled bands were quantified using the Sigma-Gel software (Jandel Scientific).

Invasion assay

Matrigel invasion assays were performed by the modified Boyden chamber method in 24-well plates containing 12 cell culture inserts with 8 μm pore size (Costar, Corning, Inc). Briefly, 30 μl BD Matrigel was added into culture inserts and kept overnight at 37°C to form a semisolid matrix. Untreated or treated cells with inhibitors were plated at 1×10^5 cells per insert in serum-free medium on the top chamber. The lower chamber of transwells contained 600 μl DMEM without or with 200 μM OA. Cells were incubated for 48 h at 37°C in a 5% CO_2 atmosphere. Following incubation, cells and matrigel on the upper surface of membrane were removed with cotton swabs, and cells on the lower surface of membrane were washed and fixed with methanol for 5 min. The number of invaded cells was estimated by staining with 0.1% crystal violet in PBS. The dye was eluted with 500 μl 10% acetic acid, and the absorbance at 600 nm was measured. Background value was obtained from wells without cells.

Statistical analysis

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

Results

Oleic acid promotes MMP-9 secretion in breast cancer cells

Since, OA promotes proliferation of breast cancer cells and that MMP-9 is proposal to play an important role in breast cancer invasion, metastasis and tumor angiogenesis [20, 30, 31], we decided to examine whether OA induces MMP-9 secretion in breast cancer cells. First, to determine whether OA induces MMP-9 secretion, MDA-MB-231 cells were treated with 200 μM OA for various times and subsequently, conditioned medium was harvested. The medium was subjected to gelatin zymography and Western-blotting using an Ab against MMP-9. Moreover, cells were lysed and cell lysates were analyzed by western blotting with anti-actin Ab as loading control. As shown in Fig. 1a (upper and middle panel), treatment of cells with OA induced a marked increase on MMP-9 secretion that reached a maximum at 20 h of treatment. In contrast, MDA-MB-231 cells have a constitutive secretion of MMP-2 and treatment with OA did not induce an increase on its secretion (data not shown). It has been described that PKC strongly stimulates MMP-9 expression and secretion [35–37]. Then, a positive control of MMP-9 secretion was prepared by treatment of MDA-MB-231 cells with 100 ng/ml PDB, a strong activator of PKC, for 24 h (Fig. 1a). Moreover, OA also induced MMP-9 secretion in a concentration-dependent manner (Fig. 1b, upper and middle panel). Western blotting with anti-actin Ab of cell lysates confirmed that similar number of cells was present in the absence or presence of OA (Fig. 1a, b, lower panel).

In order to substantiate further our results, we examined whether OA promotes MMP-9 secretion in MCF-7 breast cancer cells and in the non-tumorigenic epithelial cells MCF10A and MCF12A. Cultures of MCF-7, MCF10A and MCF12A cells were stimulated with 200 μM OA for various times and subsequently, conditioned medium was harvested. The medium was subjected to gelatin zymography. In agreement with our previous results, our findings showed that stimulation of MCF-7 cells with OA induced MMP-9 secretion in a time-dependent manner (Fig. 1c). In contrast, in the non-tumorigenic epithelial cell line MCF10A, untreated cells showed a little bit of MMP-9 secretion and treatment with OA did not induce an increase on MMP-9 secretion (Fig. 1d), whereas in MCF12A cells, treatment with OA also did not induce MMP-9 secretion (Fig. 1e).

Oleic acid induces MMP-9 secretion through a PKC, Src, EGFR and Gi/Go protein-dependent pathway

Oleic acid promotes Src activation in MDA-MB-231 cells, and it induces EGFR activation in endothelial cells [18, 38]. In breast cancer cells, type IV collagen promotes MMP-9 secretion via an Src-dependent pathway, fibroblast growth factor and heregulin-beta-1 mediate MMP-9 secretion through a PKC-dependent pathway, and EGF induces MMP-9 expression in SKBR3 breast cancer cells [34, 39, 40, 41]. To examine the involvement of Src, PKC

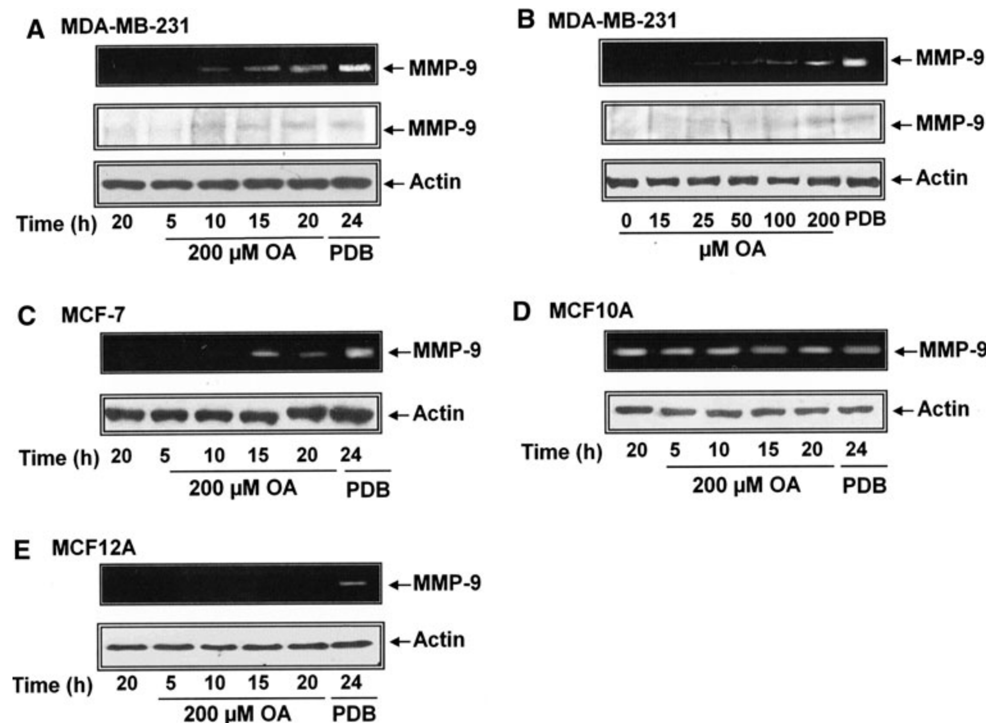

Fig. 1 Oleic acid induces MMP-9 secretion in breast cancer cells. **a** MDA-MB-231 cells were treated without or with 200 μM oleic acid (OA) for various times as indicated. **b** MDA-MB-231 cells were treated for 20 h without or with various concentrations of OA as indicated. Conditioned medium was obtained, and MMP-9 secretion was analyzed on conditioned medium using gelatin-substrate gels and western blotting using anti-MMP-9 Ab. **c** MCF-7 cells were treated without or with 200 μM OA for various times as indicated. **d**, **e** MCF10A and MCF12A cells were treated without or with 200 μM OA for various times as indicated. Conditioned medium was obtained, and MMP-9 secretion was analyzed on conditioned medium using gelatin-substrate gels. Cell lysates were obtained, and equal volumes were analyzed by western blotting using anti-actin Ab as loading control. A positive control of MMP-9 secretion was included, which was prepared by treatment of MDA-MB-231 cells with 100 ng/ml PDB for 24 h. The results shown are representative of at least three independent experiments

and EGFR activity on MMP-9 secretion induced by OA, we studied the effect of SU6656, GF-I and AG1478, which are selective inhibitors that have been used previously to inhibit the activity of Src, PKC and EGFR, respectively, in MDA-MB-231 cells [18, 20, 42–46]. MDA-MB-231 cells were treated for 30 min with 10 μM SU6656, 300 nM AG1478 or 3.4 μM GF-I and then stimulated with 200 μM OA for another 20 h. Conditioned medium was harvested and subjected to gelatin-zymography. Our results showed that treatment of MDA-MB-231 cells with Src, PKC and EGFR inhibitors completely prevented MMP-9 secretion (Fig. 2a, b).

Since, OA is known to act via GPCRs and that GPR40 is coupled with both the Gi/Go and Gq families in MCF-7 cells [10, 13, 20], we studied the effect of PTX, an inhibitor of Gi/Go proteins [47], on MMP-9 secretion. MDA-MB-231 cells were treated for 12 h in the absence or presence of 100 ng/ml PTX and then stimulated with 200 μM OA for another 20 h. Conditioned medium was harvested and subjected to gelatin-zymography. As shown in Fig. 2c, treatment with PTX did not inhibit MMP-9 secretion induced by OA.

In order to determine whether OA induces MMP-9 secretion through a protein synthesis-dependent pathway, we studied the effect of cycloheximide, an antibiotic that its main biological activity is translation inhibition in eukaryotes resulting in inhibition of proteins synthesis [48, 49], on MMP-9 secretion. Confluent cultures of MDA-MB-231 cells were treated for 1 h with 30 μg/ml cycloheximide (CHX) and then stimulated with 200 μM OA for another 20 h. Conditioned medium was harvested and subjected to gelatin-zymography. Our results showed that treatment with cycloheximide inhibit MMP-9 secretion induced by OA (Fig. 2d).

Role of cytoskeleton integrity on MMP-9 secretion

In order to determine the contribution of cytoskeleton on MMP-9 secretion, we determined the effect of cytochalasin D and colchicine on MMP-9 secretion induced by OA. Cytochalasin D promotes depolymerization of actin fibers, whereas colchicine is a selective inhibitor of tubulin polymerization [50, 51]. MDA-MB-231 cells were treated for 2 h in the absence or presence of 2.4 μM cytochalasin

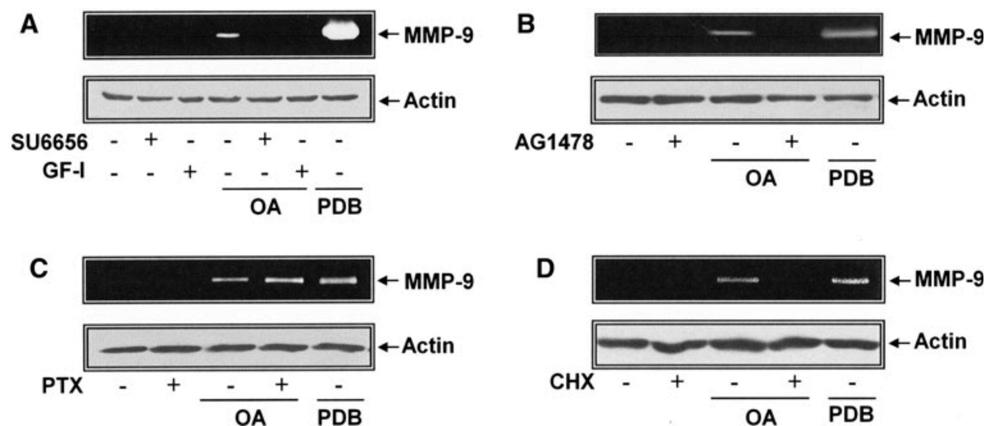

Fig. 2 Oleic acid induces MMP-9 secretion through an EGFR, Src, PKC and Gi/Go proteins-dependent pathway. **a**, **b** MDA-MB-231 cells were treated for 30 min in the absence (−) or presence (+) of 10 μM SU6656, 3.4 μM GF-I or 300 nM AG1478 as indicated and then stimulated without or with 200 μM OA for 20 h. **c** MDA-MB-231 cells were treated for 12 h in the absence (−) or presence (+) of 100 ng/ml PTX as indicated and then stimulated without or with 200 μM OA for 20 h. **d** MDA-MB-231 cells were treated for 1 h in the absence (−) or presence (+) of 30 μg/ml cycloheximide (CHX) as indicated and then stimulated without or with 200 μM OA for 20 h. Conditioned medium was obtained, and MMP-9 secretion was analyzed on conditioned medium using gelatin-substrate gels. Cell lysates were obtained and equal volumes were analyzed by western blotting using anti-actin Ab as loading control. A positive control of MMP-9 secretion was included, which was prepared by treatment of MDA-MB-231 cells with 100 ng/ml PDB for 24 h. The results shown are representative of at least three independent experiments

D or 10 μM colchicine and then stimulated with 200 μM OA for another 20 h. Conditioned medium was harvested and subjected to gelatin-zymography. Our results showed that treatment with colchicine completely prevented MMP-9 secretion (Fig. 3a), and cytochalasin D did not inhibit MMP-9 secretion induced by OA (Fig. 3b).

Oleic acid promotes invasiveness through an EGFR transactivation and Src-dependent pathway

Since, Src modulates migration and invasion and that OA induces Src activation in breast cancer cells [18, 52], we examined whether OA promotes invasion and the role of Src kinase activity in MDA-MB-231 cells. MDA-MB-231 cells were untreated or treated for 30 min with PP2 and SU6656, which are selective inhibitors of Src family kinases and have been used previously to inhibit Src kinase family members activity in MDA-MB-231 cells [53–55], and then cells were loaded onto the upper chamber and stimulated with 200 μM OA for 48 h. Cells that penetrated the membranes were fixed and stained. As shown in Fig. 4a, b, MDA-MB-231 cells showed a clear invasion, whereas cells treated with PP2 and SU6656 were not able to produce invasion. In contrast, OA did not induce invasion on non-invasive MCF-7 breast cancer cells (Fig. 4d).

Since, EGFR and αvβ3 integrin promote carcinoma cell invasion and metastasis and that OA promotes proliferation through an EGFR-dependent pathway in breast cancer cells [18, 56], we studied the role of EGFR on invasiveness induced by OA in MDA-MB-231 cells. MDA-MB-231 cells were treated with 300 nM AG1478, and then cells were loaded onto the upper chamber and stimulated with 200 μM OA for 48 h. As shown in Fig. 4c, inhibition of EGFR activity prevented cell invasion induced by OA.

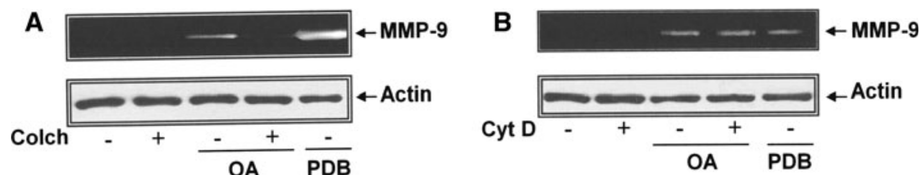

Fig. 3 Role of cytoskeleton on MMP-9 secretion. **a** MDA-MB-231 cells were treated for 2 h in the absence (−) or presence (+) of 10 μM colchicine (Colch), as indicated and then stimulated without or with 200 μM OA for 20 h. **b** MDA-MB-231 cells were treated for 2 h in the absence (−) or presence (+) of 2.4 μM cytochalasin D (Cyt D), as indicated and then stimulated without or with 200 μM OA for 20 h. Conditioned medium was obtained, and MMP-9 secretion was analyzed on conditioned medium using gelatin-substrate gels. Cell lysates were obtained and equal volumes were analyzed by western blotting using anti-actin Ab as loading control. A positive control of MMP-9 secretion was included, which was prepared by treatment of MDA-MB-231 cells with 100 ng/ml PDB for 24 h. The results shown are representative of at least three independent experiments

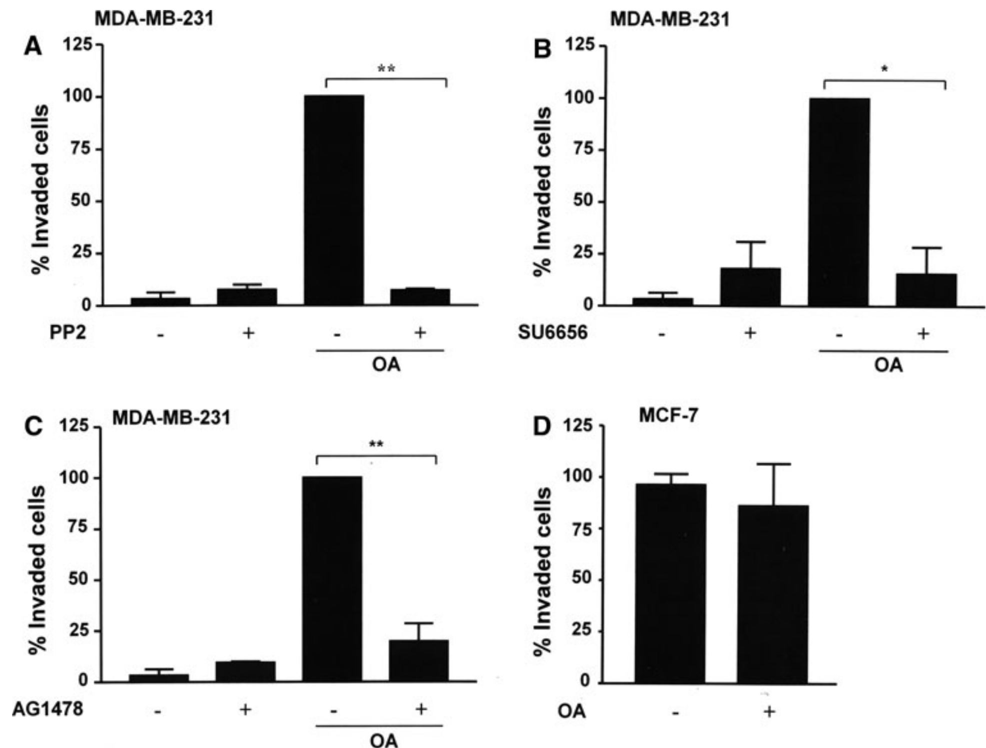

Fig. 4 Oleic acid induces invasiveness through an EGFR and Src-dependent pathway. **a–c** MDA-MB-231 cells held in suspension were treated for 30 min in the absence (−) or presence (+) of 10 μM PP2, 10 μM SU6656 or 300 nM AG1478, as indicated and then cells were plated on the *top* of matrigel and treated with 200 μM OA for 48 h. **d** MCF-7 cells held in suspension were plated on the top of matrigel and treated with 200 μM OA for 48 h. Cell invasion was evaluated after 48 h of incubation. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the percentage of maximum invaded cells. *Asterisks* denote comparisons made to control cultures (unstimulated). * $P < 0.05$ and ** $P < 0.005$ by one-way ANOVA

Role of MMPs, PKC and Gi/Go proteins on invasiveness

MMPs are implicated in tumor progression, including invasion through BM and interstitial matrices [26, 57], FGF-2 and TPA induces MMP-9 secretion through a PKC activation pathway [40]. We studied the role of MMPs and PKC activity on invasiveness induced by OA in MDA-MB-231 cells. MDA-MB-231 cells were treated with 10 μM GM6001 or 3.4 μM GF-I, which are selective inhibitors of MMPs and PKC activity, respectively, and have been used previously to inhibit MMPs and PKC activity in MDA-MB-231 cells [58, 59], and then cells were loaded onto the upper chamber and stimulated with 200 μM OA for 48 h. Cells that penetrated the membranes were fixed and stained. Our results showed that inhibition of MMPs activity prevented invasion (Fig. 5a), whereas treatment with PKC inhibitor partly prevented invasion induced by OA (Fig. 5b).

OA acts via GPR40 pathway, and it is coupled with both Gi/Go and Gq in MCF-7 cells [10, 13, 20], whereas AA induces cell migration through a Gi/Go-dependent pathway in MDA-MB-231 cells [17]. We studied the role of Gi/Go proteins on invasiveness induced by OA in MDA-MB-231 cells. MDA-MB-231 cells were treated for 12 h with 100 ng/ml PTX, an inhibitor of Gi/Go proteins [47], and then cells were loaded onto the upper chamber and stimulated with 200 μM OA for 48 h. As illustrated in Fig. 5c, treatment with PTX inhibited invasion in MDA-MB-231 cells stimulated with OA.

Discussion

Numerous studies in women suggest that certain dietary factors such as higher intake of fat and meat increase the risk of breast cancer, whereas obesity has been associated with enhanced cancer risk [6, 60, 61]. However, the role of FFAs, such as OA, in tumor progression through regulation of cell migration, MMPs secretion, invasion, epithelial to mesenchymal transition and angiogenesis has not been studied in detail.

Type IV collagen is the main component of BM and is the first protein that must be degraded in order to produce invasion and metastasis. BM is degraded mostly by MMP-2 and MMP-9, and it has been proposed that these MMPs play a critical a role in the conversion of in situ cancer to invasive lesions [27, 32]. Furthermore, OA induces proliferation through a MMPs-dependent pathway and invasion in breast cancer cells [18, 62]. Consequently, we considered the possibility that OA regulates MMP-9 secretion and therefore invasion through a MMPs-dependent pathway in breast cancer cells. In the present study, we determined that OA promotes MMP-9 secretion through a PKC, Src and EGFR-dependent pathway in MDA-MB-231 breast cancer cells. Our findings also show that Gi/Go proteins do not participate on MMP-9 secretion induced by OA, because treatment with PTX does not prevent MMP-9 secretion. Since OA promotes proliferation in the breast cancer cell lines MCF-7 and MDA-MB-231 and it is mediated at least in part through GPR40 and that GPR40 is coupled with Gi/Go and Gq

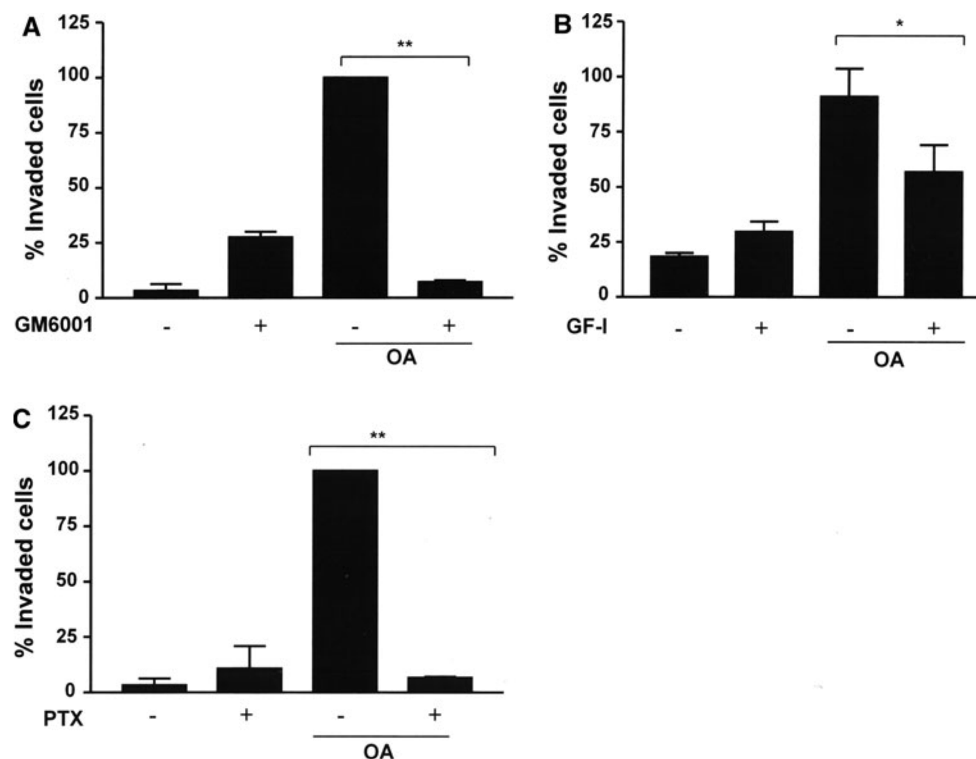

Fig. 5 Oleic acid induces invasiveness through MMPs, PKC and Gi/Go proteins-dependent pathway. **a**, **b** MDA-MB-231 cells held in suspension were treated for 30 min in the absence (−) or presence (+) of 10 μM GM6001 or 3.4 μM GF-I as indicated and then cells were plated on the *top* of matrigel and treated with 200 μM OA for 48 h. **c** MDA-MB-231 cells held in suspension were treated for 12 h in the absence (−) or presence (+) of 100 ng/ml PTX and then cells were plated on the *top* of matrigel, and treated with 200 μM OA for 48 h. Cell invasion was evaluated after 48 h of incubation. The graphs represent the mean ± SD of at least three independent experiments and are expressed as the percentage of maximum invaded cells. *Asterisks* denote comparisons made to control cultures (unstimulated). * $P < 0.05$ and ** $P < 0.005$ by one-way ANOVA

[10, 12, 20], our findings suggest that OA induces MMP-9 secretion via GPR40 activation coupled with Gq or that OA induces GPR120 activation, because GPR120 is the receptor for medium and long chain FFAs, such as OA [11]. In addition, our results show that protein synthesis is required for MMP-9 secretion induced by OA. It suggests that OA induces up-regulation of MMP-9 expression and/or that OA induces synthesis of proteins involved on MMP-9 secretion.

Human non-tumorigenic mammary epithelial cells MCF10A have a constitutive secretion of MMP-9 and treatment with OA does not promote an increase on its secretion. Previously, it has been reported that untreated MCF10A cells constitutively secrete MMP-9 [63, 64]. It is in agreement with the constitutive secretion that we detected. In addition, OA does not induce MMP-9 secretion on non-tumorigenic mammary epithelial cells MCF12A. Since GPR40 and GPR120 are expressed in MCF-7, MDA-MB-231 and MCF10A cells [10, 17, 18], we propose that these receptors are activated by treatment with OA in MCF-7 and MDA-MB-231 breast cancer cells, but they are not activated in the human breast epithelial cells MCF10A and MCF12A cells. Furthermore, our findings also suggest that OA may contribute to invasiveness and therefore metastasis process in breast cancer, because it has been suggested that MMP-2 and MMP-9 play a pivotal role in metastasis and invasion in breast cancer [30, 32]. In contrast to these results, previous reports show that OA induces inhibition of MMP-2 activity in human bronchial epithelial cells, and a reduction on MMP-2 and MMP-9 activity in mice bearing implants of metastatic colon carcinoma cells [65, 66]. We propose that OA mediates different signal transduction pathways and cellular responses on the diverse types of human cancers.

MT-MMP-1 plays a pivotal role in tumor cell invasion and it associate with p27RF-Rho, which enhances Rho activation and therefore regulates actin structures [67]. In gastric tumor cells, phospholipase C delta 1 expression inhibit their clonogenicity and migration through regulation of cytoskeleton organization [68]. We therefore hypothesize that an intact cytoskeleton is required for MMP-9 secretion. Supporting our hypothesis, this study demonstrates that integrity of microtubule network is required for MMP-9 secretion induced by OA. In contrast, actin cytoskeleton is not necessary for MMP-9 secretion. These results suggest

that cytoskeleton play an important role in cell invasion by regulation of MMPs secretion, such as MMP-9. In support to our proposal, it has been reported that regulation of cytoskeleton is a prerequisite for processes like endocytosis, cell motility and cancer cell invasion [69].

Previous reports demonstrate that OA induces proliferation, migration and invasion in breast cancer cells [20, 23]. We demonstrated here that OA induces invasion through an EGFR, PKC, Src, Gi/Go proteins and MMPs-dependent pathway in MDA-MB-231 cells. In contrast, OA is not able to induce invasion on the non-invasive MCF-7 breast cancer cells. These results strongly suggest that invasion induced by OA is restricted to invasive breast cancer cells and they support our proposal that OA may contribute to invasiveness and therefore metastasis process in breast cancer.

Src kinase family members are implicated in adhesion, migration, invasion and survival in cancer cells [34, 70, 71]. In human neutrophils, tumor necrosis factor mediates MMP-9 release through a PKC and Src-dependent pathway [72]. Our findings show that OA promotes MMP-9 secretion an invasion via an Src-dependent pathway in MDA-MB-231 breast cancer cells. In agreement with our findings, previous reports show that type IV collagen promotes an increase on MMP-9 secretion through an Src-dependent pathway in breast cancer cells [34].

Increased levels of PKC have been associated with malignant transformation in breast cancer cell lines, while a positive correlation between elevated PKC levels and invasive potential of breast cancer cells lines is suggested [73, 74]. Moreover, OA induces glucagon-like-peptide-1 secretion via PCK zeta-dependent pathway on intestinal endocrine L cells [75]. In line with this notion, our results demonstrated that OA promotes MMP-9 secretion and invasion through a PKC-dependent pathway in MDA-MB-231 cells. In agreement with our results, 12-*O*-tretradecanoylphorbol-13-acetate, an activator of PKC, induces expression and secretion of MMP-9 in MCF-7 cells [40, 76], whereas PKC epsilon mediates cell invasion in MDA-MB-231 and MCF-7 breast cancer cells [77].

EGFR and Her-2 overexpression correlates with a reduction on survival and induction of invasion and metastasis in malignant breast cancer. In particular, EGF induces MMP-9 secretion in ovarian and lung cancer [78, 79]. We demonstrated that OA promotes MMP-9 secretion and invasion through an EGFR-dependent pathway. Supporting our results, we previously demonstrate that OA promotes proliferation through an EGFR-dependent pathway in breast cancer cells [18]. Since, Src family kinases are mediators of GPCR-induced EGFR transactivation [80], and that OA promotes Src activation in MCF-7 cells [18], we propose that OA mediates MMP-9 secretion an invasion through an EGFR transactivation-dependent pathway. Our findings also show that OA mediates invasion through a pathway coupled with Gi/Go proteins, because inhibition of Gi/Go proteins by treatment with PTX inhibit invasion. It is in agreement with previous reports showing that OA promotes proliferation and an increase in cellular Ca^{2+} concentration through GPR40 activation via Gi/Go proteins in breast cancer cells [10, 20]. We propose that OA induces invasion via GPR40 coupled with Gi/Go and/or OA induces GPR120 activation. In addition, during invasion process, cells induce the formation of invadipodia protrutions, which are actin microdomains rich in associated-actin proteins and MMPs [40, 81]. In line with this notion, we demonstrated that OA induces invasion through a MMPs-dependent pathway.

In conclusion, our findings demonstrated that OA induces MMP-9 secretion through a PKC, Src and EGFR-dependent pathway, whereas it induces invasion via an EGFR, Gi/Go proteins, MMPs, PKC and Src-dependent pathway in MDA-MB-231 breast cancer cells. These findings strongly suggest that OA may have an important role in the invasion process and metastasis in breast cancer.

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