Evidence from epidemiological studies and animal models suggests a link between high levels of dietary fat intake and risk of breast cancer. In addition, obesity, in which circulating lipids are elevated, is associated with increased risk of various cancers. Relative to this point, we previously showed that oleate stimulates the proliferation of breast cancer cells and that phosphatidylinositol 3-kinase plays a role in this process. Nonetheless, questions remain regarding the precise mechanism(s) by which oleate promotes breast cancer cell growth. Pharmacological inhibitors of the G protein-coupled receptor (GPCR), a fatty acid receptor, amplified oleate-induced proliferation, whereas silencing the GPR40 gene using RNA interference decreased it. Overexpressing in these cells the G protein-coupled receptor GPR40, a fatty acid receptor, amplified oleate-induced proliferation, whereas silencing the GPR40 gene using RNA interference decreased it. Overexpressing GPR40 in T47D and MCF-7 breast cancer cells that are poorly responsive to oleate allowed a robust proliferative action of oleate. The data indicate that the phospholipase C, MEK 1/2, Src, and phosphatidylinositol 3-kinase/protein kinase B signaling pathways are implicated in the proliferative signal induced by oleate and that these effects are mediated at least in part via the G protein-coupled receptor GPR40. The results suggest that GPR40 is implicated in the control of breast cancer cell growth by fatty acids and that phosphatidylinositol 3-kinase regulates the MAPK pathway.

Oleate Promotes the Proliferation of Breast Cancer Cells via the G Protein-coupled Receptor GPR40*

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Evidence from epidemiological studies and animal models suggests a link between high levels of dietary fat intake and risk of breast cancer. In addition, obesity, in which circulating lipids are elevated, is associated with increased risk of various cancers. Relative to this point, we previously showed that oleate stimulates the proliferation of breast cancer cells and that phosphatidylinositol 3-kinase plays a role in this process. Nonetheless, questions remain regarding the precise mechanism(s) by which oleate promotes breast cancer cell growth. Pharmacological inhibitors of the G protein-coupled receptor (GPCR), a fatty acid receptor, amplified oleate-induced proliferation, whereas silencing the GPR40 gene using RNA interference decreased it. Overexpressing GPR40 in T47D and MCF-7 breast cancer cells that are poorly responsive to oleate allowed a robust proliferative action of oleate. The data indicate that the phospholipase C, MEK 1/2, Src, and phosphatidylinositol 3-kinase/protein kinase B signaling pathways are implicated in the proliferative signal induced by oleate and that these effects are mediated at least in part via the G protein-coupled receptor GPR40. The results suggest that GPR40 is implicated in the control of breast cancer cell growth by fatty acids and that phosphatidylinositol 3-kinase regulates the MAPK pathway.

Unsaturated FFAs including oleate but not saturated FFAs have been shown to trigger tyrosine phosphorylation and epithelial growth factor receptor (EGFR) activation in an endothelial cell line (14). GPCRs for fatty acid derivatives such as prostaglandins (15), leukotrienes (16), lysophosphatidic acid (LPA) (17), sphingosine 1-phosphate (18), and eicosatetraenoic acid are well characterized, but their signaling pathways are less well understood. However, recent studies have provided evidence that GPR40, a G protein-coupled receptor (GPCR), is involved in the control of breast cancer cell proliferation. In this study, we investigated the role of GPR40 in the proliferation of breast cancer cells in response to oleate and other fatty acids.

Materials and Methods

Cell Culture

MDA-MB-231 and MCF-7 breast cancer cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. T47D cells were cultured in RPMI supplemented with 10% FBS and penicillin/streptomycin. Cells were grown in a humidified atmosphere containing 5% CO2 at 37°C.

GPR40 Expression

The expression of GPR40 was confirmed by RT-PCR and Western blotting. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen). The expression of GPR40 was determined by quantitative RT-PCR using the LightCycler 480 System (Roche Diagnostics). The primers and probes used were as follows: forward primer, 5’-GAGACTGCGTCTTCAGTTGT-3’; reverse primer, 5’-AGAGAGGAGTGCAGAGGG-3’; and probe, 5’-FAM-TTCACAGTTGCAGTCAACTACAAGATTG-TAMRA-3’.

Western Blotting

Protein expression was analyzed by Western blotting. Cells were harvested in lysis buffer containing protease and phosphatase inhibitors. Total protein was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were loaded onto a 10% SDS-PAGE gel and blotted onto a PVDF membrane. The membranes were blocked with 5% nonfat dry milk in TBST for 1 h and then incubated with the following antibodies: anti-GPR40 (1:1000), anti-β-actin (1:1000), and anti-phospho-ERK1/2 (1:1000) overnight at 4°C. After washing, the membranes were incubated with the secondary antibody (1:5000) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences).

Results

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Discussion

GPR40 has been shown to play a role in the proliferation of breast cancer cells. In this study, we investigated the role of GPR40 in the proliferation of breast cancer cells in response to oleate and other fatty acids. Our results showed that GPR40 expression was increased in breast cancer cells in response to oleate. Overexpression of GPR40 in MDA-MB-231 cells increased cell proliferation, whereas silencing GPR40 using RNA interference decreased cell proliferation. These findings suggest that GPR40 is involved in the control of breast cancer cell proliferation.

Conclusion

In conclusion, our results provide new insights into the role of GPR40 in the proliferation of breast cancer cells. GPR40 expression is increased in breast cancer cells in response to oleate, and this effect is mediated at least in part via the G protein-coupled receptor GPR40. These findings have implications for the development of new therapeutic strategies for the treatment of breast cancer.
acid (19) are well characterized. Agonist stimulation of these GPRs induces a variety of cellular responses, including cell proliferation (18). These effects implicate the activation of a wide variety of signaling pathways, including the modulation of adenylyl cyclases, phospholipases, ion channels, and mito-
gen-activated protein kinases (20). Recently, three independent groups found that the orphan receptor GPR40 is activated by medium and long chain FFAs (21–23). Using a ligand-fishing strategy based on measurement of intracellular calcium concent-
rations ([Ca\textsuperscript{2+}],), they showed that FFAs in the absence of bovine serum albumin (BSA) increased [Ca\textsuperscript{2+}], in GPR40-over-
expressing cells. GPR40 is highly expressed in pancreatic \beta-cells (21, 22), but it is also present in other tissues (23). Interestingly, GPR40 is expressed in the human breast cancer cell line MCF-7 in which unsaturated, but not saturated, FFAs
bound to BSA increase [Ca\textsuperscript{2+}], (24).

In the present study, we investigated the mechanisms by which oleate increases the proliferation of the breast cancer cell line MDA-MB-231. The results suggest that multiple pathways are involved in the proliferative action of oleate in these cells and that the oleate effect implicates a GPCR. In addition, evidence is provided that the oleate-induced proliferation of breast cancer cells is mediated at least in part through GPR40.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sodium salts of fatty acids were purchased from Nu-
Check Prep (Elysian, MN). Fatty acid-free BSA (fraction V) was
obtained from Sigma. Fura-2/AM was from Molecular Probes Inc.
(Eugene, OR). [\textsuperscript{3}H]thymidine (specific activity, 71 Ci/mmol) was
obtained from PerkinElmer Life Sciences. Lipofectamine 2000 and
FuGENE 6 were purchased from Invitrogen and from Roche Applied
Sciences, respectively. U73122, PD98059, PP1, LPA, H-89, wortman-
nin, protein kinase C (PKC) \beta \textsubscript{I} peptide inhibitor were from Biomol
(Plymouth Meeting, PA). Pertussis toxin and AG1478 were from
Cellbiochem (La Jolla, CA).

**Cell Culture**—The human breast cancer cell lines MDA-MB-231,
T47D, and MCF-7 were obtained from the American Type Culture
Collection. Cells were cultured at 37 °C with 5% CO\textsubscript{2} in phenol red-
free minimal essential medium containing non-essential amino acids, 2 mM
glutamine, 10 mM Hepes (pH 7.4), and 5% heat-inactivated fetal bovine
serum (Atlanta Biologicals). BSA-bound fatty acids were prepared by
stirring fatty acid sodium salts (≥99% purity) at 37 °C with 5% fatty
acid-free BSA as described before (25). After being adjusted to pH 7.4,
the solution was filtered through a 0.22-\mu m filter, and the fatty acid
concentration was measured using a NEFA C kit (GmbH; Wako
Chemicals). When BSA-bound fatty acids were added to serum-free culture
medium, the final concentration of BSA was adjusted to 0.5%.

**Cell Proliferation**—For cell growth assay, 5000 cells/well were seeded
in 96-well plates and incubated for 24 h in standard medium (10). After
a 24-h starvation period in medium without serum, cells were incubated
without or with BSA-bound fatty acids for 24 h. DNA synthesis was then
assayed with a pulse of [\textsuperscript{3}H]thymidine (1 \muCi/well) during the last 4 h
of the incubation. Cells were harvested with a PHD cell harvester from
Dulbecco’s PBS containing 5 mM glucose, 20 mM Hepes (pH 7.4), 2.5 mM
probenecid, and 3 mM fura-2/AM. After 30 min of fura-2/AM loading at 25 °C, cells were washed and resuspended as above but without fura-2/AM and were dispensed at 2 × 10\textsuperscript{4} cells/well into 96-well plates. [Ca\textsuperscript{2+}], was measured at 37 °C by the ratiometric
method (emission fluorescence at 500 nm and excitation wavelengths at
340 and 380 nm) using a FLUOstar OPTIMA microplate reader (BMG
Labsystems Inc., Durham, NC). Calcium concentrations were calcu-
lated as described by Grynkiewicz et al. (27).

**Cell Transfection**—Cells seeded in 6-well plates at 4 × 10\textsuperscript{5} cells/well
were incubated for 24 h in standard medium. Cells were transiently
transfected with 5 μg of the plasmid pIRESpuro-GPR40 expressing the
human GPR40 (provided by Bjorn Olde, Wallenberg Neuroscience
Center, Lund, Sweden) or a control plasmid expressing Renilla luciferase
(CMV-RLuc) using Lipofectamine 2000 (MDA-MB-231) and FuGENE 6
(T47D and MCF-7) according to the manufacturer’s instructions. Five
hours post-transfection, cells were seeded into 96-well plates at 5000
cells/well and assayed for cell growth as described above.

**Real-time Quantitative Interference—Vectors expressing small interfering RNAs (siRNA) under the control of the human H1 promoter were
constructed by inserting pairs of annealed DNA oligonucleotides into
the solution was filtered through a 0.22-\mu m filter, and the fatty acid
concentration was measured using a NEFA C kit (GmbH; Wako
Chemicals). When BSA-bound fatty acids were added to serum-free culture
medium, the final concentration of BSA was adjusted to 0.5%.

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Labsystems Inc., Durham, NC). Calcium concentrations were calcu-
lated as described by Grynkiewicz et al. (27).

**RESULTS**

**Oleate-induced Cell Proliferation Does Not Involve EGFR Activation**—We previously showed that the monounsaturated fatty acid oleate stimulates the proliferation of three different breast cancer cell lines and that PI3-K is implicated in this effect (10). Fig. 1A confirms this observation because the PI3-K inhibitor wortmannin (28) markedly curtailed oleate-induced proliferation of MDA-MB-231 cells. In addition, we showed that PI3-K is rapidly activated by oleate, suggesting signaling through a receptor (10). Because the EGFR has been reported to be activated by oleate in endothelial cells (14), we first examined whether the activation of the EGFR could be involved in oleate-induced proliferation of MDA-MB-231 cells. AG1478, a pharmacologic inhibitor of EGFR activity (29), did not significantly affect oleate-induced [\textsuperscript{3}H]thymidine incorpo-
ration (Fig. 1B). However, AG1478 efficiently prevented the phosphorylation of EGFR induced by EGF (Fig. 2A). In addi-
tion, an examination of the time course of EGFR phosphoryla-
tion induced by oleate revealed no activation of EGFR over 240 min of treatment (Fig. 2B). However, EGF induced a strong
activation of the EGFR at 2 min that became maximal at 15 min and decreased thereafter. Interestingly, LPA transiently
produced a modest increase in EGFR phosphorylation resulting probably from transactivation of the EGFR following LPA
binding to its receptor (30). These results establish that the EGFR is not implicated in oleate-induced proliferation of MDA-MB-231 cells.

**Oleate-induced MDA-MB-231 Cell Proliferation Is Decreased by Specific Inhibitors of Gi/Go, Phospholipase C, Src, MEK1/2, and PKCζ—**Several fatty acid derivatives such as LPA are known to act via GPCRs and to activate PI3-K via G proteins (13). We first studied the effect of pertussis toxin, an inhibitor of Gi/Go proteins (31), on oleate-induced cell proliferation. As shown in Fig. 1C, pertussis toxin decreased oleate-induced [3H]thymidine incorporation by 70%. The same extent of inhibition was observed with LPA, which has been shown to increase cell proliferation through GPCR activation via Gi/Go proteins (32). This strongly supports that the effect of oleate on breast cancer cell proliferation is mediated at least in part by GPCR(s), because the action of pertussis toxin to inhibit Gi/Go is considered highly specific (31).

Agonist stimulation of GPCR caused the activation of a wide variety of signaling pathways, including modulation of phospholipases and protein kinases (20). To assess a possible role of these different signal transduction pathways in the action of oleate on breast cancer cell proliferation, we tested the effect of different classes of specific inhibitors. Treating cells with the phospholipase C (PLC) inhibitor U73122 (33) resulted in an 80% reduction of oleate- or LPA-induced proliferation (Fig. 1D). PP1 and PD98059, which are pharmacological inhibitors of the Src-like family (34) and of mitogenic-extracellular signal-regulated kinase 1/2 (MEK1/2) (35), respectively, reduced the proliferative effect of oleate by about 40% (Fig. 1, E and F). Moreover, PKCζ, a downstream effector of PI3-K, appears also to be implicated in the proliferative effect of oleate because a specific membrane-permeant peptide inhibitor of PKCζ (36) blocked the oleate-induced proliferation (Fig. 1G). However, this peptide inhibitor also decreased the proliferation in the control situation, suggesting that PKCζ is important for the growth of MDA-MB-231 cells. Blocking protein kinase A, another downstream effector of GPCR, with H-89 (37) did not affect oleate-induced DNA synthesis (Fig. 1H). Taken together, the results are consistent with the view that oleate signals at least in part via GPCR(s) and that many downstream signal transduction pathways, including PLC, Src, MEK1/2, and PKCζ, may participate in its proliferative effect.
Oleate induces a rapid increase in AKT phosphorylation—ERK1/2 and AKT, the direct downstream effectors of MEK1/2 and PI3-K, respectively, are major regulators of cell proliferation (38). We therefore examined the role of ERK1/2 and AKT in oleate-induced cell proliferation by using antibodies recognizing the activated/phosphorylated forms of Thr-202/Tyr-204-ERK1/2 and Ser-473-AKT. Because basal ERK1/2 activity is very high in MDA-MB-231 cells as previously described (39), we were unable to detect significant increases in ERK1/2 phosphorylation in response to either oleate or the usual positive control EGF (data not shown). In sharp contrast, both oleate and EGF stimulated AKT phosphorylation (Fig. 3, A and B). Oleate-induced AKT phosphorylation, which increased more than 2-fold during the first 5 min of treatment, peaked at 30 min and then slowly declined. The same extent of AKT activation was observed with five different preparations of oleate BSA. In agreement with our previous data showing that oleate, but not palmitate, activates PI3-K (10), AKT phosphorylation was not induced by two different preparations of palmitate-BSA that had been shown to be active in inducing apoptosis (data not shown). Considering the PI3-K inhibitor data described in Fig. 1A, the results indicate that the proliferative signal induced by oleate is mediated at least in part via PI3-K/AKT activation.

Oleate increases [Ca\(^{2+}\)](i) in the presence and absence of BSA—GPR40 was first recognized to be activated by medium and long chain FFA (not bound to BSA) by assays measuring [Ca\(^{2+}\)](i) in GPR40-overexpressing cells (21–23). Because GPR40 is expressed in MDA-MB-231 breast cancer cells at about 12,000 copies/μg of total RNA as we evaluated by real-time PCR, we measured changes of [Ca\(^{2+}\)](i) in fura-2/AM-loaded cells following oleate or palmitate treatments. In the absence of BSA, oleate and palmitate caused a rapid increase in [Ca\(^{2+}\)](i) by 2- and 1.5-fold, respectively (Fig. 4A). Interestingly, in the presence of BSA, a more physiological condition, oleate still caused a rapid increase in [Ca\(^{2+}\)](i) by 2-fold (Fig. 4B). In contrast, palmitate did not affect [Ca\(^{2+}\)](i) in the presence of BSA.

GPR40 is involved in oleate-induced cell proliferation—To determine whether GPR40 is implicated in the proliferative action of oleate in MDA-MB-231 cells, we first attempted to reduce the expression level of GPR40 in MDA-MB-231 cells using RNA interference. Because the efficiency of transfection is about 60% in this cell line (data not shown), cells were co-transfected with a plasmid encoding GFP together with a plasmid encoding an siRNA against GPR40 or with a control scrambled siRNA, followed by selection of cells expressing GFP with a fluorescent-activated cell sorter. At the end of the selection, GPR40 mRNA expression was reduced by 80% in siRNA-transfected cells as determined by real-time PCR (Table I). Using a standard protocol to evaluate cell proliferation, fluorescent-activated cell sorter-selected cells were serum-starved for 24 h and then incubated for an additional 24 h in serum-free medium supplemented with 0.5% BSA (control) or various concentrations of BSA-bound oleate. The increase in \(^{3}H\)thymidine incorporation following oleate stimulation was signific-
GPR40 expression was assessed using real-time reverse transcription-PCR. Results are expressed as -fold change over mock and have been normalized with respect to glyceraldehyde-3-phosphate dehydrogenase expression. Means ± S.E. of two to three independent experiments. N/A, not applicable. *, p < 0.01 versus Renilla luciferase; **, p < 0.01 versus siRNA control.

| Transfection          | MDA-MB-231 | T47D       | MCF-7       |
|-----------------------|------------|------------|------------|
| Mock                  | 1.00       | 1.00       | 1.00       |
| Renilla lucerase      | 0.85 ± 0.12| 0.75 ± 0.20| 0.99 ± 0.26|
| GPR40 siRNA           | 14,300 ± 1,760* | 236,100 ± 52,990* | 153,400 ± 52,100* |
| siRNA control         | 1.11 ± 0.07 | N/A        | N/A        |
| siRNA GPR40           | 0.22 ± 0.06** | N/A        | N/A        |

**TABLE 1**

GPR40 expression in transfected breast cancer cells

Fig. 5. GPR40 is implicated in oleate-induced cell proliferation. MDA-MB-231 cells were transiently co-transfected for 24 h with a plasmid encoding GFP and with a plasmid encoding an siRNA against GPR40 (GPR40 siRNA) or a scrambled siRNA (Cont siRNA) for 24 h follow- ing by selection on a fluorescent cell sorter of cells expressing GFP (A) or transfected for 24 h with a plasmid encoding either GPR40 or Renilla luciferase (RLuc) (B). T47D cells and MCF-7 cells were transiently transfected for 24 h with a plasmid encoding GPR40 or Renilla luciferase (RLuc) (C and D). After 24 h of serum-starvation in minimal essential medium, cells were incubated for an additional 24 h in serum-free medium supplemented with 0.5% BSA (control) or various concentrations of BSA-bound oleate. During the last 4 h of incubation, cells were labeled with [3H]thymidine. Means ± S.E. of two to three independent experiments performed in triplicate or quadruplicate. *, p < 0.01 versus control (A) or Renilla luciferase (B, C, and D).

**DISCUSSION**

This study was prompted by the intriguing link between the incidence of breast cancer and obesity noted in both animal and human studies (1–3). The mechanism for this potential link, however, is enigmatic. To address this issue, we focused a series of studies on the abundant fatty acid, oleate, because it has been shown to stimulate the proliferation of breast cancer cells (10). Inhibition of individual signaling cascades, at the level of G/Gs proteins, Src, MEK1/2, PI3-K, or PLC resulted in a substantial reduction of oleate-induced DNA synthesis, suggesting that integration or cooperation of signals from multiple pathways is necessary to drive cells to enter S-phase in response to oleate. We also found that a GPCR is implicated in the proliferative signal transduction induced by this monounsaturated FFA. Thus, pertussis toxin, a very specific inhibitor of some G proteins (31), attenuated both LPA and oleate-induced proliferation, suggesting the implication of G/Gs proteins in this process. LPA has previously been shown to induce the proliferation of fibroblasts via GPCRs coupled to Gp (32). In addition, the fact that we provide evidence (see below) that the GPCR GPR40 is implicated in the proliferative action of oleate is in itself strong evidence for a role of a GPCR in oleate action.

It is noteworthy that, except for a possible role in insulin secretion, no other physiological action has been attributed to the GPCR GPR40 (21). Several lines of evidence demonstrate that GPR40 is implicated in oleate-induced proliferation of breast cancer cells. First, [Ca2+]i is increased in response to oleate treatment in GPR40-expressing MDA-MB-231 cells. Second, overexpression of GPR40 amplified the oleate-induced cell proliferation of MDA-MB-231 cells is mediated at least in part through GPR40.

**GPR40 and Breast Cancer Cell Proliferation**

GPR40 is implicated in oleate-induced cell proliferation.
proliferation in three breast cancer cell lines, suggesting that oleate acts via this FFA receptor in these cells. Third, treatment of MDA-MB-231 cells with an siRNA-targeting GPR40 reduced the proliferative effect of oleate. Activation of GPCR signaling leading to cell growth has previously been documented for different GPCR agonists, including lysophospholipids and thrombin (18). Because only a partial inhibition of the proliferative effect of oleate with siRNA-targeting GPR40 was observed here, we could on one hand speculate that additional receptors for oleate or other signaling pathways participate in oleate-induced cell proliferation. For instance, LPA interacts with several GPCRs to mediate its biological actions (40), and it may also act via a receptor-independent pathway to cause cell growth (41). Interestingly, another FFA-activated GPCR, GPR120, was recently identified (42), and its contribution to oleate-induced proliferation of breast cancer cells remains to be studied. On the other hand, the importance of GPR40 in oleate-induced proliferation is likely to be underestimated because the siRNA did not completely block its expression. Moreover, as we do not have information on GPR40 protein level and stability in these cells, it is difficult to draw quantitative conclusions.

The marked decrease in oleate-induced proliferation with the PLC inhibitor suggests a significant role of this phospholipase in this process. PLC is a major contributor of GPCR signaling (43). One might speculate that oleate binding to the receptor activates PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol trisphosphate, which, respectively, activates PKC and mobilizes calcium from the endoplasmic reticulum (44). Both PKC and calcium are effectors that have been reported to modulate processes required for breast cancer cell proliferation (45). Moreover, several studies have shown that unsaturated FFAs, including oleate, stimulate PLCγ activity (46). In addition, our results show that oleate and palmitate caused a rapid and transient increase in \([Ca^{2+}]_i\), in MDA-MB-231 cells in the absence of BSA. However, in the presence of BSA, palmitate did not increase \([Ca^{2+}]_i\) in contrast to oleate, which still elicited a robust \([Ca^{2+}]_i\) response. Similar results have been observed in MCF-7 breast cancer cells (24). The difference observed between these two fatty acids might be explained in part by a better availability of unbound oleate for GPR40. In comparison to palmitate, oleate has a slightly lower affinity for BSA (47) and lower partition coefficient into cellular membranes (48). Thus, oleate may promote breast cancer cell proliferation by binding more efficiently to GPR40 than palmitate. In addition, as shown in our previous studies (10, 11), differences in the metabolism of these two fatty acids are involved in their opposite effects on cell fate: the saturated fatty acid palmitate induces apoptosis of breast cancer cells via a mechanism that possibly implicates enhanced cardiolipin turnover and a reduction of this mitochondrial phospholipid, whereas the unsaturated fatty acid oleate, by sustaining cardiolipin synthesis, permits cell proliferation.

The rapid and transient increase in \([Ca^{2+}]_i\), induced by oleate suggests calcium mobilization from intracellular stores that implicates Gαq protein activation in response to many receptor agonists (18). Thus, these results favor the view that oleate stimulates, in part, MDA-MB-231 cell proliferation via GPR40 coupled to a Gαq protein and via the PLC/Ca2+ pathway. GPR40 is mainly coupled to Gαq in MIN6 cells and Chinese hamster ovary cells overexpressing GPR40 (21, 22). However, GPR40 is also coupled to Gαi and Gq in MCF-7 cells (24). Hence, in addition to Gαq, oleate-induced proliferation of MDA-MB-231 cells via GPR40 is likely to implicate Gq/Gi, because pertussis toxin reduced but did not abolish the proliferative effect of oleate in these cells. An orphan GPCR coupled to Gq/Gi proteins has been shown to be activated by the unsaturated FFA arachidonate and to cause a decrease in cAMP levels (19). The subunits of several Gq/Gi proteins are able to inhibit adenylyl cyclases resulting in a decrease in cAMP levels (49). However, cAMP levels were not affected by oleate in MDA-MB-231 cells when compared with LPA, which elicited a reduction in the cellular cAMP content (data not shown). This is consistent with the observation that H-89, a protein kinase A inhibitor, did not impair oleate-induced DNA synthesis. It is well established that many of the diverse biological effects resulting from the activation of Gq/Gi proteins are not mediated via the cAMP transduction system (50). For example, members of the Gq/Gi protein family directly affect Src (50) and Ca2+ channels (51).

The increase in DNA synthesis induced by oleate appears to be mediated in part by the Src and MEK1/2 pathway as supported by the experiments using pharmacological inhibitors. The activity of Src and ERK1/2, the direct downstream effector of MEK1/2, is critical for cell survival and proliferation mediated by diverse growth factors (52, 53). Consistent with this view, a previous study reported that oleate induces ERK1/2 activation via GPR40 in the MIN6 pancreatic β-cell line (21). AKT is rapidly activated by oleate in MDA-MB-231 cells, but not by palmitate. AKT controls cell cycle progression and is well known to participate in cell proliferation and survival (38). Agonist-induced stimulation of GPCRs may lead to the transactivation of the EGFR via Src and subsequently the activation of a wide variety of signaling pathways, including AKT and ERK1/2 (26, 30). However, oleate did not induce EGFR activation in the cellular model we used, and blocking EGFR activity with AG1478 did not affect oleate-induced proliferation. Oleate can activate the EGFR in endothelial cells (14) but had no effect on EGFR tyrosine phosphorylation in Ha578T breast cancer cells (54).

In summary, the molecular, biochemical, and pharmacological results in this study suggest the following cascade of events in response to oleate in MDA-MB-231 cells. The unsaturated FFA binds to GPR40 and possibly other FFA receptor(s) coupled to Gq/Gi, and Gq, resulting in the activation of Src proteins, PI3-K, AKT, and Ca2+ signaling, thus promoting cell growth. It cannot be discounted that other non-GPCR receptors are also implicated in oleate-induced activation of the PLC, Src, PI3-K/AKT, MEK1/2, and PKCζ pathways that may participate in the proliferative effect. Taken together, these data provide a novel mechanism for the action of oleate in breast cancer cells in relation to cell growth by showing that this monounsaturated FFA acts as an extracellular signaling molecule to regulate breast cancer cell proliferation via the FFA receptor GPR40. Thus, GPR40 is not only a receptor that may participate in the control of insulin secretion by FFA, but it might also play an important role in the control of cell growth/apoptosis by some FFA. Hence, the possibility should be considered that GPR40 provides a link between fat and/or obesity and cancer. In this respect, the emerging evidence suggest an important association between insulin resistance, obesity, type 2 diabetes, and several cancers, in particular colon, prostate, and breast cancer (5). Finally, it could be hypothesized that overexpression of GPR40 in a subset of tumors could render them more susceptible to progression in patients eating a diet rich in unsaturated fatty acids.

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