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Stearate Inhibition of Breast Cancer Cell Proliferation

A Mechanism Involving Epidermal Growth Factor Receptor and G-Proteins

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Long chain saturated fatty acids are known to inhibit breast cancer cell proliferation; however, the mechanism of this inhibition is not known. Treatment of Hs578T breast cancer cells with long chain saturated fatty acids (0.15 mmol/L for 6 hours) before epidermal growth factor (EGF) treatment inhibited EGF-induced cell proliferation in a chain-length-dependent manner. Stearate (C:18) completely inhibited the EGF-induced cell proliferation, whereas palmitate (C:16) inhibited by 67 ± 8% and myristate (C:14) had no effect. In contrast, stearate had little effect on insulin-like growth factor-1-stimulated cell proliferation. The inhibitory effect of stearate on cell proliferation was dose and time dependent and independent of EGF receptor (EGFR) tyrosine phosphorylation. Pretreatment of cells with pertussis toxin (0.1 μg/ml for 24 hours) inhibited the EGF-induced cell growth by 50 ± 8%, also independent of EGFR tyrosine phosphorylation. A pertussis-toxin-sensitive, 41-kd G-protein was specifically co-immunoprecipitated with the EGFR. Pretreatment of cells with 0.15 mmol/L stearate from 0 to 6 hours inhibits, in parallel, both the EGF-induced cell proliferation and pertussis-toxin-catalyzed ADP ribosylation of the G-protein associated with the EGFR. These studies suggest that long chain saturated fatty acids inhibit EGF-induced breast cancer cell growth via a mechanism involving an EGFR-G-protein signaling pathway. (Am J Pathol 1996, 148:987–995)

Numerous studies have demonstrated that certain types of dietary fat affect the pathogenesis of breast cancer. Generally, high levels of dietary long chain saturated fatty acids (LCSFAs) suppress the development of mammary tumors compared with long chain unsaturated fatty acids in the carcinogen-induced rodent mammary tumor model.[1] Supplementing saturated fatty acids with a small amount of long chain unsaturated fatty acids reverses the inhibition of mammary tumor cell proliferation of such fats,[2,3] but this reversal is dependent on the amount of unsaturated fat added.[4] It has been suggested that this inhibition is simply due to inadequate linoleic acid content in the high LCSFA diet. However, when rats were fed high levels of LCSFAs, they were typically fed palm oil (11% linoleic acid) or lard (9% linoleic acid), which have approximately 10 times the normal recommended essential fatty acid requirement for normal growth.[5] An inhibitory effect of LCSFAs on cell proliferation and promotion has also been demonstrated in spontaneously derived and transplantable mammary tumors in mice and rats[1] as well as breast cancer cell culture studies.[6–9] The mechanism for this inhibition is unknown.

The epidermal growth factor receptor (EGFR) is overexpressed in some breast cancers. Its presence is associated with a more aggressive clinical course, suggesting that it has an important growth regulatory function in breast cancer.[10–13] Furthermore, an increased proliferation rate of breast tumors correlates with a poor relapse-free survival time and overall patient survival.[14] EGF initiates a cascade of intra-

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cellular events through specific interactions with the EGFR, which ultimately leads to DNA synthesis and cell proliferation. EGF activates its receptor by inducing tyrosine phosphorylation of the receptor and subsequent phosphorylation of endogenous substrates.

Recent evidence suggests that pertussis-toxin-sensitive G-proteins mediate the EGFR signaling pathways in both normal and neoplastic cells.[15–18] EGF-induced cell division in BALB/c3T3 cells has been shown to involve Giα translocation from plasma membrane to the nucleus.[17] An association of G-proteins with EGFR has been shown in hepatocytes[15] and breast cancer cells.[16,18] Furthermore, Giα has been shown to bind phospholipase Cγ in an EGF-dependent manner.[15]

We have identified an EGFR-G-protein signaling mechanism likely to be involved in breast cancer cell proliferation that is inhibited by LCSFAs.

Materials and Methods

Human recombinant EGF was purchased from R & D Systems (Minneapolis, MN). [methyl-^{3}H]Thymidine was from ICN Biomedicals (Irvine, CA). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Antibody to EGFR (monoclonal) used for immunoprecipitation was from Oncogene Science (Uniondale, NY). Fetal bovine serum, antiphosphotyrosine antibody, and EGFR antibody (monoclonal) used for immunoblotting as well as insulin-like growth factor (IGF)-1 were from UBI (Lake Placid, NY). Goat anti-mouse IgG-gold conjugate was from Bio-Rad (Richmond, CA). Tissue culture media were purchased from GIBCO (Grand Island, NY). Bovine serum albumin (BSA), fatty acids, and dibutyryl cyclic AMP were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained from standard sources.

Cell Culture

The Hs578T cell line is derived from a carcinoma of the breast and was obtained from the American Type Culture Collection (Rockville, MD). These cells express relatively large amounts of EGFR.[19] Cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose supplemented with 10% fetal bovine serum and 10 μg/ml bovine insulin. Cells were passaged at 80 to 90% confluency with trypsin-EDTA and used before passage 15.

Preparation of Fatty Acid-BSA

Fatty acids were added to the culture medium in association with BSA. Fatty-acid-free BSA was prepared by the acid-charcoal treatment method of Chen,[20] and selected fatty acids were added back to the fatty-acid-free BSA using the inert carrier, celite, as described by Spector and Hoak.[21] Aqueous fatty acid concentrations were determined using an enzymatic colorimetric assay (WAKO Pure Chemicals, Osaka, Japan). Viability of cells treated with stearate was determined by the trypan blue dye exclusion assay.[22]

Effect of EGF on Cell Proliferation

Cells were plated in the normal growth medium in 12-well tissue culture dishes and grown to 60 to 70% confluency. The medium was then replaced with the starvation medium containing phenol-red-free DMEM and 0.5% heat-inactivated fetal bovine serum for 32 hours. The cells were then treated with or without EGF or IGF-1 as indicated for 16 hours and labeled with [^{3}H]thymidine (0.4 μCi/ml) for 4 hours. The medium was aspirated and cells were fixed in 100% methanol for 10 minutes and washed three times with phosphate-buffered saline (PBS). The cells were then treated with 10% trichloroacetic acid for 10 minutes, washed three times with PBS and solubilized in 1 N NaOH for 1 hour. The solution was neutralized with 1 N HCl, mixed with 10 ml of Ecoscint, and counted in a liquid scintillation counter. Proteins were measured using the Bradford protein assay (Bio-Rad).

For determination of the cell number, cells were treated in the same manner as for the [^{3}H]thymidine uptake study, trypsinized three days after treatment with EGF, suspended in Hanks' balanced salt solution, and counted using a Coulter counter.

Immunoprecipitation

Cells grown to 60 to 70% confluence in normal medium in 100-mm plates were serum starved for 48 hours with medium containing phenol-red-free DMEM and 0.5% heat-inactivated serum. The cells were then treated with EGF (1.0 nmol/L) for the indicated times at 37°C, immediately washed three times with ice-cold PBS, and lysed at 4°C for 1 hour with solubilization buffer containing 50 mmol/L HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mmol/L NaCl, 1 mmol/L sodium orthovanadate, and protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin). The remaining steps were carried out at 4°C. The lysates were centrifuged at 14,000 rpm for 30 minutes. Pellets were discarded, and the supernatants were precleared by incubating with 5 μg of control IgG (nonimmune)

preabsorbed to 20 μl of 50% v/v protein-A-Sepharose suspension in solubilization buffer for 30 minutes on a rotator and centrifuged at 14,000 rpm for 2 minutes. The precleared supernatants were then incubated with 5 μg of either control IgG or immunoprecipitating antibody (EGFR) coupled to protein-A-Sepharose beads for 1 hour. The immune complex was washed five times with solubilization buffer.

Immunoblot Analysis

Immunoblots were performed on immunoprecipitates or cell lysates as indicated. The immunoprecipitates or cell lysates were treated with sodium dodecyl sulfate (SDS) sample buffer and separated by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli.[23] The proteins were transferred to polyvinylidene difluoride by blotting for 16 hours at 30 V and then blocked for 2 hours by incubation in Tris-buffered saline containing 2% BSA and 0.1% gelatin. The blots were then incubated overnight at 4°C with the specific monoclonal antibody and washed extensively with Tris-buffered saline containing 0.05% Tween 20. The blots were developed with the secondary antibody reagent, goat anti-mouse IgG-gold conjugate (GAM-Gold) according to the manufacturer's (Bio-Rad) protocol.

Pertussis-Toxin-Catalyzed ADP Ribosylation

The immunoprecipitates were ADP ribosylated in an 80-μl assay volume containing 1 μg of pertussis toxin (preactivated with 20 mmol/L dithiothreitol at 37°C), 50 μmol/L nicotinamide-adenine dinucleotide and 20 μCi of [^{32}P] nicotinamide-adenine dinucleotide for 1 hour at 30°C. The reaction was stopped by the addition of SDS sample buffer[23] followed by boiling for 5 minutes to release the immunoprecipitated proteins. The proteins were separated by SDS-PAGE, electrotransferred onto a polyvinylidene difluoride membrane (Immobilon), and visualized by autoradiography.

Pretreatment of Cells with Pertussis Toxin

Pertussis toxin treatment of cells was performed for 24 hours at 37°C using a toxin concentration of 0.1 μg/ml. After treatment of cells with the toxin, medium was replaced with fresh starvation medium and the cells were treated with or without EGF for the indicated times.

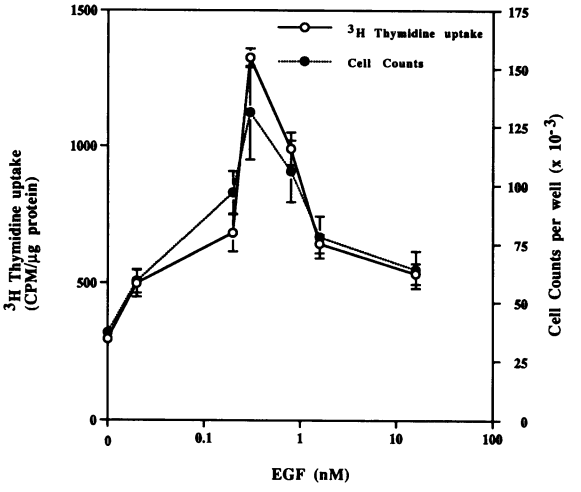

Figure 1. *EGF induces cell proliferation in Hs578T breast cancer cells in a concentration-dependent manner. Cells were grown and [^{3}H]thymidine incorporation and cell proliferation were determined as described in Materials and Methods. The results shown are the mean ± SEM obtained from three independent experiments, each done in triplicate.*

Results

LCSFAs Inhibit EGF-Induced Cell Proliferation in a Chain-Length- and Dose-Dependent Manner

The effects of EGF on cell proliferation and DNA synthesis in Hs578T cells were determined. Treatment of cells with increasing concentrations of EGF (0 to 16 nmol/L) resulted in increased cell proliferation as assessed by cell number and [^{3}H]thymidine uptake. Figure 1 shows the dose response for EGF as a function of [^{3}H]thymidine incorporation and cell number. The effects of EGF on DNA synthesis and cell division of these cells were biphasic, reaching a maximal 3- to 4.5-fold increase at 0.3 nmol/L concentration and decreasing at higher concentrations. As the results of the cell proliferation agree with that of [^{3}H]thymidine incorporation, the latter was used as an indicator of cell proliferation in subsequent studies.

To determine the effects of fatty acid pretreatment on EGF-stimulated [^{3}H]thymidine incorporation, cells were pretreated with or without individual fatty acids (0.15 mmol/L for 6 hours) and treated with or without EGF, and [^{3}H]thymidine incorporation was assayed. As shown in Figure 2A, EGF-stimulated [^{3}H]thymidine incorporation was inhibited by fatty acids in a chain-length-dependent manner. Stearate (C:18) completely inhibited the EGF-induced thymidine incorporation whereas palmitate (C:16) inhibited by 67%, and myristate had no effect.

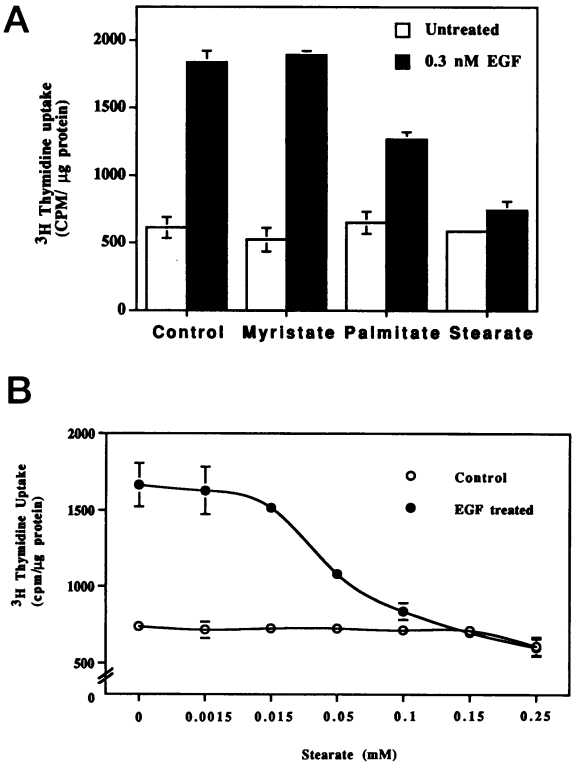

Figure 2. *LCSFAs inhibit EGF-induced cell proliferation in a chain-length- and concentration-dependent manner. [^{3}H]Thymidine incorporation was determined as described in Materials and Methods. The mean ± SEM of three independent experiments, each done in triplicate, are shown.* **A:** *Fatty acids (0.15 mmol/L) were added for 6 hours at 37°C as described in the Materials and Methods. Control cells were incubated with fatty-acid-free BSA at the same concentration as the fatty-acid-treated cells.* **B:** *Cells were incubated with medium containing stearate (0 to 0.25 mmol/L) for 6 hours at 37°C.*

To determine whether the stearate inhibition of cell proliferation is dose dependent, cells were pretreated with increasing concentrations of stearate (0 to 0.25 mmol/L) for 6 hours and [^{3}H]thymidine incorporation was studied. As seen in Figure 2B, concentrations of less than 0.05 mmol/L had no effect and concentrations of more than 0.05 mmol/L progressively inhibited EGF-induced thymidine incorporation with complete inhibition at a concentration of 0.15 mmol/L.

To determine whether the LCSFAs have any toxic effects, cells treated with or without 0.15 mmol/L stearate for 6 hours were trypsinized and assayed for viability using the trypan blue dye exclusion assay.[22] There was no significant difference between the viability of control cells (100%) and stearate-treated cells (98 ± 2%).

In experiments identical to the EGF-induced cell proliferation experiments, except that IGF-1 was used instead of EGF, there was a 3.01 ± 0.72-fold increase in the control cells plus IGF-1 group,

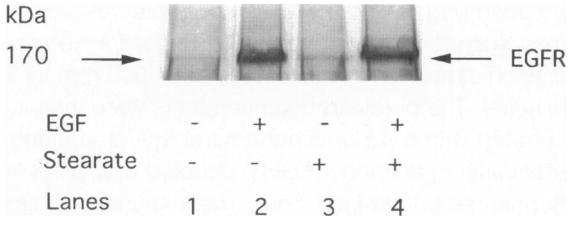

Figure 3. *Stearate pretreatment does not alter EGFR tyrosine phosphorylation. Cells were grown to 60 to 70% confluence, serum starved for 42 hours, and treated with 0.15 mmol/L stearate* (lanes 3 *and* 4) *or with fatty-acid-free BSA* (lanes 1 *and* 2) *for 6 hours. The medium was then replaced with fresh starvation medium, and the cells were treated with* (lanes 2 *and* 4) *or without* (lanes 1 *and* 3) *1.0 nmol/L EGF for 1 minute. Cells were lysed, subjected to SDS-PAGE, and immunoblotted. The blots were probed for tyrosine-phosphorylated proteins using a monoclonal antiphosphotyrosine antibody (UBI) and developed with goat anti-mouse IgG-gold conjugate (Bio-Rad). A representative immunoblot of three experiments is shown.*

whereas the stearate (pretreatment for 6 hours with 0.15 mmol/L stearate at 37°C) plus IGF-1 group showed a 2.23 ± 0.53-fold increase (n = 3). The difference is not significant ($P > 0.13$), whereas under identical conditions stearate completely inhibited EGF-induced cell proliferation ($P < 0.01$; see Figure 2A).

Stearate Pretreatment Does Not Alter EGFR Tyrosine Phosphorylation

As EGFR tyrosine kinase mediates many of the effects of EGF, one possible pathway by which stearate could inhibit the EGF-induced cell proliferation is by altering the EGFR tyrosine phosphorylation. To determine whether the tyrosine phosphorylation of the EGFR was inhibited by stearate, cells were pretreated with fatty-acid-free or stearate-containing (0.15 mmol/L) media for 6 hours, treated with or without 1.0 nmol/L EGF, and lysed. The whole cell lysates were subjected to SDS-PAGE and immunoblotted, and the blots were probed with antiphosphotyrosine antibody. As seen in Figure 3, basal or EGF-stimulated EGFR tyrosine phosphorylation was not inhibited by stearate pretreatment. Densitometric scans of the three similar experiments indicated the following fold increase with EGF treatment: 5.9 ± 0.7 *versus* 5.7 ± 0.7, with or without stearate, respectively.

Pertussis Toxin Inhibits the EGF-Induced [^{3}H]Thymidine Incorporation

To determine whether pertussis-toxin-sensitive G-proteins are involved in EGFR signal transduction, cells were pretreated with pertussis toxin (0.1 µg/ml)

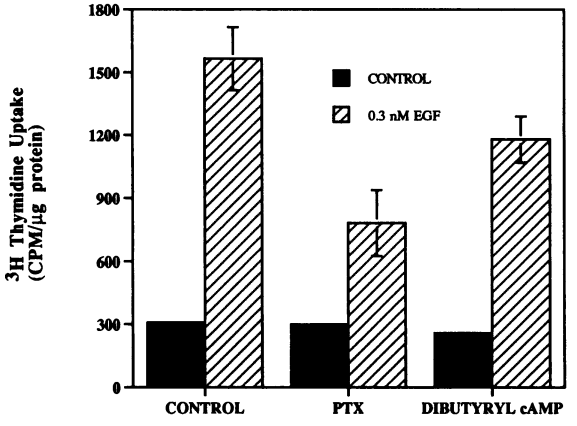

Figure 4. *Pertussis toxin pretreatment inhibits and dibutyryl cAMP has no significant effect on EGF-induced [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation and pertussis toxin pretreatment were performed as described in Materials and Methods. The dibutyryl cAMP group was treated the same as the pertussis toxin-treated group except that pertussis toxin was replaced with 1 mmol/L dibutyryl cAMP. The figure shows the mean of three independent experiments ± SEM.*

for 24 hours and [^{3}H]thymidine incorporation was determined. Pertussis toxin inhibited the EGF-stimulated [^{3}H]thymidine incorporation by 50 ± 8% ($P < 0.015$; Figure 4). To investigate whether the inhibition of [^{3}H]thymidine incorporation by pertussis toxin was mediated through elevated cAMP levels, the cells were pretreated with a permeable cAMP analogue, dibutyryl cAMP, and treated with or without EGF, and mitogenic assays were performed. Prior exposure of cells to 1 mmol/L dibutyryl cAMP had no significant effect ($P > 0.08$) on the EGF-stimulated [^{3}H]thymidine incorporation (Figure 4), suggesting that EGFR-mediated cell proliferation in 578T cells was independent of changes in cAMP. This is further supported by the fact that neither cholera toxin nor forskolin, which are known to cause stimulation of adenylate cyclase and thereby increase cAMP, affected EGF-stimulated DNA synthesis (data not shown). Therefore, it is unlikely that the pertussis toxin effects seen on the Hs-578T cells can be accounted for by stimulation of adenylate cyclase. These results indicate that EGFR regulates the proliferation of these cells, at least partially, by a mechanism involving a pertussis-toxin-sensitive G-protein, independent of the adenylate cyclase pathway.

EGF Stimulates Pertussis-Toxin-Catalyzed ADP Ribosylation of a 41-kd G-Protein That Co-Immunoprecipitates with the EGFR

To investigate the possible association of G-proteins with the EGFR, we immunoprecipitated EGFR from cells treated with or without EGF and ADP-ribosylated the pellet in the presence of pertussis toxin. As shown in Figure 5C, we found that a 41-kd G-protein co-immunoprecipitates with the EGFR, and EGF (1 nmol/L) increased the amount of ADP-ribosylated 41-kd G-protein associated with the EGFR. The co-precipitation of this G-protein with the EGFR was rapid and transient, reaching a maximum at 1 minute of stimulation. In contrast, EGFR tyrosine phosphorylation is maintained at a high level for 10 minutes (Figure 5A). Importantly, the amount of EGFR

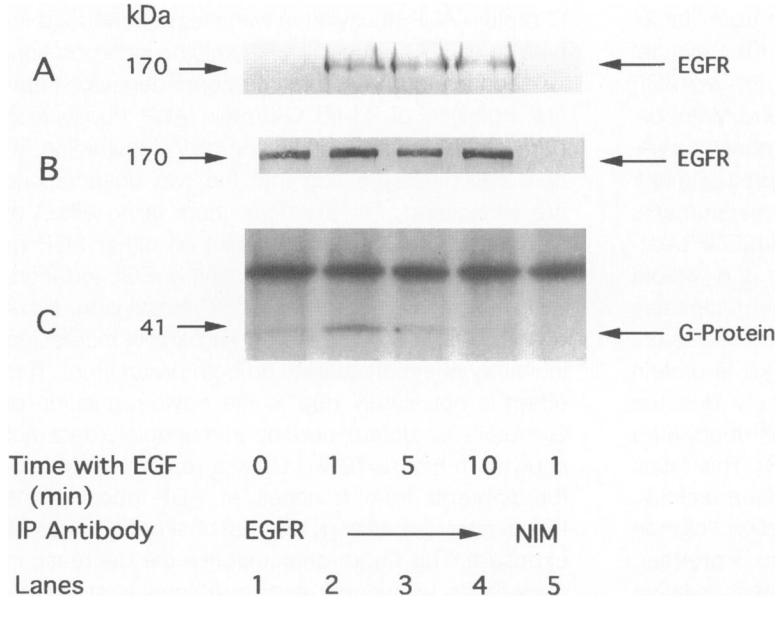

Figure 5. *EGF stimulates pertussis toxin-catalyzed ADP ribosylation of a 41-kd G-protein that co-immunoprecipitates with the EGFR. Cells were grown to 60 to 70% confluence and serum starved for 48 hours. EGFRs were immunoprecipitated with either EGFR antibody* (lanes 1 *to* 4) *or nonimmune mouse IgG 2a* (lane 5) *from lysates of cells treated with* (lanes 2 *to* 5) *or without* (lane 1) *1 nmol/L EGF for the indicated times. To determine EGFR tyrosine phosphorylation, the immunoprecipitated proteins were separated on SDS-PAGE and immunoblotted with phosphotyrosine antibody* (A). *To determine G-protein ADP ribosylation, EGFR immunoprecipitates were ADP ribosylated in the presence of pertussis toxin, separated on SDS-PAGE, blotted, and autoradiographed.* (C). *After obtaining an autoradiogram, the blot was probed with EGFR antibody* (B). *The results are representatives of three experiments. The dark bands in all* lanes *of* C, *above 41 kd, are heavy chains of the antibody used for immunoprecipitation.*

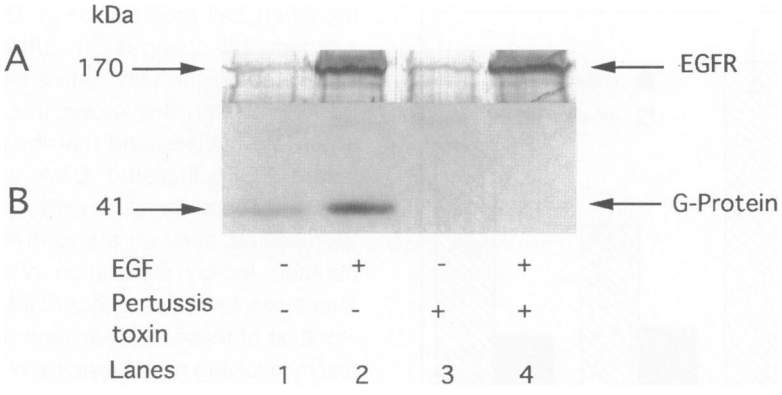

Figure 6. *Pertussis toxin pretreatment inhibits ADP ribosylation of the 41-kd G-protein without affecting EGF-induced EGFR tyrosine phosphorylation. Cells were grown to 60 to 70% confluence, serum starved for 24 hours, treated with or without (control) pertussis toxin (0.1 μg/ml) for 24 hours, treated with or without 1.0 nmol/L EGF, and lysed. To determine EGFR tyrosine phosphorylation, cell lysates were run on SDS-PAGE and immunoblotted with phosphotyrosine antibody. To determine the ADP ribosylation of EGFR-associated G-proteins, EGFRs were immunoprecipitated with the EGFR antibody from lysates of control* (lanes 1 *and* 2), *or pertussis toxin-pretreated* (lanes 3 *and* 4) *cells were stimulated with* (lanes 2 *and* 4) *or without* (lanes 1 *and* 3) *EGF for 1 minute. The immunoprecipitates were ADP ribosylated in the presence of pertussis toxin, separated on SDS-PAGE, blotted, and autoradiographed as described in Materials and Methods.* A: *Effects of pertussis toxin pretreatment on EGFR tyrosine phosphorylation.* B: *Effects of pertussis toxin pretreatment on ADP ribosylation. The results shown are representative of three experiments.*

immunoprecipitated was identical at all time points (Figure 5B).

Pretreatment of Cells with Pertussis Toxin Blocks EGF-Stimulated ADP Ribosylation of the 41-kd G-Protein without Affecting the EGF-Stimulated EGFR Tyrosine Phosphorylation

The effect of treatment of cells with pertussis toxin on the EGF-stimulated ADP ribosylation of the 41-kd G-protein co-immunoprecipitated with the EGFR and the tyrosine phosphorylation of the EGFR tyrosine residues was determined. Cell cultures were incubated with or without pertussis toxin (0.1 μg/ml for 24 hours) and treated with or without EGF for 1 minute. EGFRs were immunoprecipitated and *in vitro* ADP ribosylated as described in Materials and Methods. As shown in Figure 6A, EGFR tyrosine phosphorylation was not affected by pertussis toxin pretreatment. Densitometric scans of the three similar experiments indicated the following fold increase with EGF treatment: 6.1 ± 1.4 *versus* 5.9 ± 0.8, with and without pertussis toxin, respectively. In immunoprecipitates from cells that were pretreated with pertussis toxin, no *in vitro* ADP ribosylation of the 41-kd G-protein could be detected, which is most likely due the EGFR-associated G-protein being ADP ribosylated *in vivo* by the pertussis toxin (Figure 6B). This result suggests that the 41-kd G-protein is a Gi-protein(s). EGF-stimulated tyrosine phosphorylation of the EGFR was not affected by pertussis toxin pretreatment, indicating that EGFR tyrosine phosphorylation is independent of the ADP ribosylation of EGFR-associated G-protein.

Stearate Pretreatment Inhibits the EGF-Induced [^{3}H]Thymidine Incorporation and EGFR-Associated 41-kd G-Protein ADP Ribosylation in a Parallel Time-Dependent Manner

To determine whether stearate inhibition of EGF-induced cell proliferation was occurring via an EGFR-G-protein signaling pathway, we compared the effect of stearate on EGF-induced, EGFR-associated G-protein ADP ribosylation with stearate-induced inhibition of EGF-induced [^{3}H]thymidine incorporation. As seen in Figure 7A, the time dependency of stearate inhibition of 41-kd G-protein ADP ribosylation parallels the stearate inhibition of [^{3}H]thymidine incorporation, suggesting that the two observations are associated. For example, there is no effect of stearate at the 2-hour time point on either EGF-induced [^{3}H]thymidine incorporation or EGF-induced, EGFR-associated G-protein ADP ribosylation. However, at 4 and 6 hours there is a parallel increasing inhibitory effect of stearate on both parameters. This effect is not simply due to the down-regulation of G-protein as determined by immunoblot (data not shown). In Figure 7B we show a representative autoradiograph from a series of ADP ribosylations taken after increasing lengths of time of stearate exposure. This figure demonstrates the decrease in ribosylation with longer exposure times to stearate.

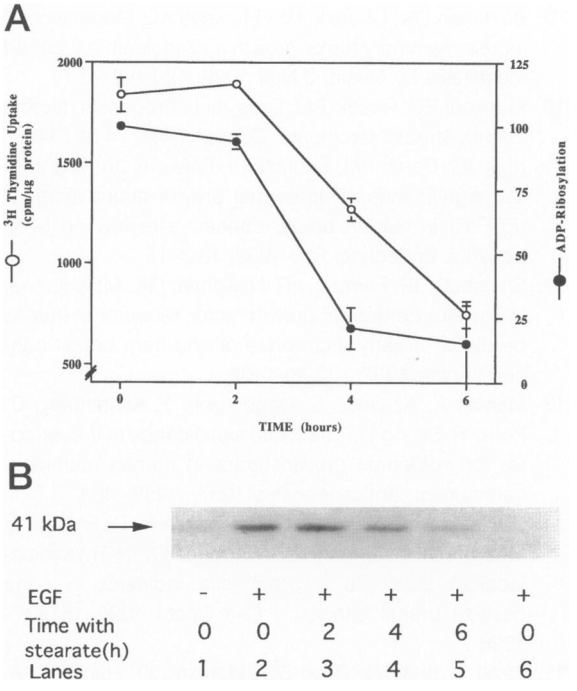

Figure 7. *Stearate pretreatment inhibits EGF-induced [^{3}H]thymidine incorporation and EGFR-associated 41-kd G-protein ADP ribosylation in a parallel, time-dependent manner.* **A:** *Cells were treated with 0.15 mmol/L stearate for the indicated times, and [^{3}H]thymidine incorporation and ADP ribosylation of the EGFR immunoprecipitate in response to 0.3 nmol/L or 1 nmol/L EGF, respectively, were performed as described in Materials and Methods. The mean of three independent experiments, for both [^{3}H]thymidine uptake and ADP ribosylation as indicated, each done in triplicate. Error bars indicate SEM.* **B:** *ADP ribosylation experiment, showing representative autoradiograph of the three ADP ribosylation experiments.* **Lanes 2** *to* **5** *demonstrate the decreasing amount of G-protein associated with the EGFR with increasing time of stearate exposure.* **Lane 1** *is the no stearate control, and* **lane 6** *is the nonimmune IgG2a control immunoprecipitation.*

Discussion

The results presented here demonstrate that the inhibitory effect of stearate on EGF-stimulated cell proliferation is independent of EGFR tyrosine phosphorylation and that this inhibition may involve disruption of an EGFR-G-protein interaction.

Our studies show that EGF stimulates cell proliferation in the Hs578T breast cancer cell line. Cell growth was significantly stimulated at a concentration as low as 0.01 nmol/L EGF, reached a maximum (3- to 4.5-fold) at 0.3 nmol/L, and declined at higher concentrations (>16 nmol/L). The reduced growth stimulation at high EGF concentrations may be due to the internalization of the EGFR complex without the concomitant production of new receptors, or the down-regulation of unoccupied receptors. The down-regulation of EGFR has been demonstrated in other cell types.[24–26]

Myristate, palmitate, and stearate were selected for this study because they are the most prominent LCSFAs present in meat and dairy products commonly found in the Western diet. Under the conditions studied, the basal level of cell proliferation was not altered by any of the LCSFAs investigated, but the EGF-induced proliferation was inhibited in a chain-length-dependent manner (ie, the longer the chain length, the more pronounced the effect). Tinsley et al[27] has shown that, using a spontaneous breast tumor model in the C3H mouse, a statistically significant decrease in time of tumor development was associated with the ingestion of stearic acid. Other saturated fatty acids in their study showed differing but not statistically significant effects. These data support our *in vitro* findings that stearate inhibits breast cancer cell proliferation. Furthermore, these data together with our data indicate that all LCSFAs do not have the same effects on breast cancer cell proliferation both *in vivo* and *in vitro*.

The inhibitory effect of LCSFAs on cell proliferation is dose dependent. Stearate concentrations from 0.0015 to 0.015 mmol/L had no significant effect on the basal or EGF-induced cell proliferation, whereas concentrations of 0.05 mmol/L to 0.15 mmol/L progressively inhibit cell proliferation. Human serum concentrations of non-esterified fatty acids (NEFAs) vary considerably, depending on age, state of fasting, and disease state. In five clinical studies, the mean serum NEFA levels ranged from 0.41 to 0.68 mmol/L for nonobese, nondiabetic, fasting normal subjects.[28–32] As fatty acids are synthesized by breast cells, local NEFA concentrations may be much higher. Stearate constitutes ~10% of total NEFAs[32] and therefore, if the total NEFA concentration was 0.5 mmol/L, then the stearate concentration would be 0.05 mmol/L, which is well within the concentration range shown to be effective at inhibiting breast cancer cell growth (Figure 2B).

The results shown in Figure 5 demonstrate that EGF induces a rapid and transient ADP ribosylation of a 41-kd G-protein associated with the EGFR. This observation supports other studies that have demonstrated an enhanced EGF-induced ADP ribosylation of an EGFR-associated G-protein in rat hepatocytes,[15] in MDA-468 human breast cancer cells,[16] and in BT 20 and MDA-MB 231 human breast cancer cells.[18]

Stearate pretreatment resulting in inhibition of cell proliferation paralleled stearate inhibition of *in vitro* ADP ribosylation of the G-protein associated with the EGFR (Figure 7). Pertussis toxin pretreatment (0.1 μg/ml for 24 hours) inhibited both cell proliferation and ADP ribosylation of the 41-kd G-protein associated with the EGFR. Pertussis toxin did not completely inhibit EGF-induced cell proliferation (Figure

4). This is likely due to other EGF-mediated signaling pathways for cell proliferation.[33] Also, complete inhibition of EGF-stimulated cell proliferation with stearate (Figure 2B) indicates that stearate alters other EGF pathways in addition to the G-protein pathway. Nevertheless, it is very likely that the association of this G-protein with the EGFR is important in EGF signaling of cell proliferation. Several studies have demonstrated the importance of G-proteins in EGF-stimulated cell proliferation (reviewed in Ref. 33). Furthermore, elegant studies by Crouch[17] demonstrated that EGF induced redistribution of Gα from the plasma membrane to perinuclear sites in BALB/c3T3. Our data potentially support this finding and further demonstrate that EGF-induced cell proliferation is inhibited by stearate independently of EGFR tyrosine phosphorylation. An alternative explanation is that G-protein is associated with the EGFR in resting cells in a conformation that is less favorable for ADP ribosylation, and EGF stimulation causes a transient conformational change that is more favorable for ADP ribosylation. Additional studies are required to determine the mechanisms responsible for the observed changes in ADP ribosylation.

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