Conjugated linoleic acid inhibits cell proliferation and ErbB3 signaling in HT-29 human colon cell line

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Conjugated linoleic acid inhibits cell proliferation and ErbB3 signaling in the HT-29 human colon cell line. Am J Physiol Gastrointest Liver Physiol 284: G996–G1005, 2003. First published February 5, 2003; 10.1152/ajpgi.00347.2002.—Conjugated linoleic acid (CLA) has chemoprotective properties in experimental cancer models, and in vitro studies have shown that CLA inhibits HT-29 colon cancer cell growth. ErbB2 and ErbB3 have been implicated in the development of colon cancer, and both proteins are expressed at high levels in the HT-29 cell line. Activation of ErbB2/ErbB3 heterodimers is regulated by the ErbB3 ligand heregulin. To examine CLA regulation of HT-29 cell proliferation and apoptosis and the influence of CLA on the ErbB3 signaling pathway, HT-29 cells were cultured in the presence of CLA and/or heregulin. CLA inhibited DNA synthesis and induced apoptosis of HT-29 cells. Although the addition of heregulin-α led to an increase in cell number, it was not able to counteract the negative growth regulatory effect of CLA. Immunoprecipitation/Western blot studies revealed that CLA inhibited heregulin-α-stimulated phosphorylation of ErbB2 and ErbB3, recruitment of the p85 subunit of phosphoinositol 3-kinase (PI3-kinase) to the ErbB3 receptor, ErbB3-associated PI3-kinase activities, and phosphorylation of Akt. CLA decreased ErbB2 and ErbB3 mRNA and protein levels in a dose-dependent manner. In conclusion, we demonstrate that CLA inhibits cell proliferation and stimulates apoptosis in HT-29 cells and that this may be mediated by its ability to downregulate ErbB3 signaling and the PI3-kinase/Akt pathway.

Inhibition of mammary tumors in rats is effective, regardless of type or amount of dietary fat (19). We (37) previously reported that dietary CLA inhibited colon cancer incidence in rats treated with 1,2-dimethylhydrazine. In addition to animal studies, in vitro studies have shown that CLA inhibits the growth of the human colon cancer cells, SW480 (34) and HT-29 (35, 42). Strong evidence for the anticancer abilities of CLA indicates a need to study the mechanisms of chemoprotection by CLA.

The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR) or ErbB1, -2, -3, and -4. Activation of these receptors regulates a number of processes including cell proliferation, survival, and differentiation. Ligands that bind to and activate ErbB receptors belong to two classes, those that bind the EGFR, such as EGF and transforming growth factor-α (2), and those that bind to ErbB3 and -4, the heregulins (HRGs) (45). Although it is devoid of any kinase activity (14), ErbB3 is an important mediator of HRG actions. HRG binding induces ErbB3 to associate with other members of the ErbB family to form receptor heterodimers (2).

Overexpression of ErbB genes, particularly ErbB2, has been observed in several types of human cancer (15, 41, 49). In colon cancer, the expression of mRNA for ErbB2 and -3 as well as the corresponding proteins was increased compared with normal mucosa (6, 30, 40). However, no difference in EGFR protein levels was evident between normal colon and cancer (30). In addition, HRG is coexpressed with ErbB2 proteins in human colon cancer specimens and autocrine activation of ErbB2 occurs through heterodimerization with ErbB3 in GEO colon cancer cells (48). These results suggest that regulation of the HRG/ErbB2/ErbB3 pathway may be important modulators of aberrant growth in colon cancer (21, 33).

One of the many initial events that occur after growth factors bind to their cognate growth factor receptor tyrosine kinases is the recruitment and acti-
viation of phosphoinositide 3-kinase (PI3-kinase) (47). PI3-kinase phosphorylates inositol phospholipids at position 3 of the inositol ring and PI3-kinase lipid products interact with certain proteins and modulate their localization and/or activity (46). PKB or Akt is known as an important downstream target for PI3-kinase, which can be activated by a variety of growth factors and cytokines via phosphorylation on serine and threonine residues (7, 17, 25) and may participate in growth factor-stimulated cell cycle (10, 31) and inhibition of apoptosis (27). Disturbance of normal PKB/Akt signaling has been reported in several human cancers (20, 32). In addition to the PI3-kinase/Akt pathway, MAPK, also known as extracellular signal-regulated kinases (ERKs), are protein serine/threonine kinases that play a critical role in the regulation of cell growth and differentiation (18, 29). Both pathways are known to be regulated by HRG.

Several studies suggest that CLA may have therapeutic benefits in individuals predisposed to developing colon cancer. The present study was designed to identify mechanisms underlying CLA regulation of growth and survival of colon adenocarcinoma cells. We confirmed that CLA inhibits DNA synthesis and stimulates apoptosis in HT-29 cells and determined that CLA regulation of proliferation and survival may be regulated by modulation of ErbB3 receptor signaling.

**MATERIALS AND METHODS**

Reagents. The following reagents were purchased from the indicated suppliers: DMEM/Ham’s F-12 nutrient mixture (DMEM/F-12), essentially fatty acid-free BSA, ascorbic acid, α-tocopherol phosphate, a mixture of CLA isomers (36), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylenetetrazolium bromide (MTT, Sigma, St. Louis, MO); FBS, trypsin-EDTA, penicillin-streptomycin, and selenium (Life Technologies, Gaithersburg, MD); [methyl-3H]thymidine (5 Ci/mmol), horse-radish peroxidase (HRP)-conjugated anti-rabbit and antimouse Ig (Amersham); anti-PI3-kinase p85 antibody (Upstate Biotechnology); anti-Akt (29752), anti-phospho-Akt (p-Akt, 473), and γ-[32P]ATP (New England Nuclear-Life Sciences); anti-phosphotyrosine-RC20 antibody linked to HRP (PY20-HRP; Transduction Laboratories); recombinant human HRG-α EGF domain (R&D Systems, Minneapolis, MN); antibodies against phospho-p44/42 MAPK (p-MAPK, Thr202/Tyr203) and p44/42 MAPK (Cell Signaling Technology); antibodies against EGFR (1005), Neu (C-18), ErbB-3 (C-17), HRG (C-20), and ErbB-4 (C-18) (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell culture. The HT-29 cell line was purchased from the American Type Culture Collection (Manassas, VA) and was maintained in DMEM/F-12 containing 10% FBS with 100 U/ml penicillin and 100 µg/ml streptomycin. HT-29 cells between passages 135 and 145 were used in these studies. To examine the effect of CLA and HRG-α, cells were plated in 24-well plates at 50,000 cells/well with DMEM/F-12 containing 10% FBS. Before CLA treatment, the cell monolayers were rinsed and serum-starved for 24 h with DMEM/F-12 supplemented with 5 µg/ml transferrin, 1 mg/ml BSA, and 5 ng/ml sodium selenite (serum-free medium). After serum starvation, fresh serum-free medium containing the indicated concentrations of CLA and/or recombinant human HRG-α was replaced. Fatty acids were complexed to essentially fatty acid-free BSA, with the molar ratio of fatty acid to BSA being 4:1 (24). Media were changed every 2 days. The basal serum-free medium containing 0.15 µM linoleic acid was chosen to eliminate the possibility of an essential fatty acid deficiency. All cultures contained ascorbic acid (50 mg/ml) and α-tocopherol phosphate (20 mg/ml) to protect fatty acids from peroxidation. Viable cell numbers were estimated by the MTT assay as described previously (24).

To determine [3H]thymidine incorporation, HT-29 cells were plated in 96-well plates at the density of 6,000 cells/well, serum-starved, and treated with CLA for 3 days as described above. At this time, 0.5 µCi [3H]thymidine was added to each well, and the incubation was continued for an additional 14 h at 37°C. [3H]Thymidine incorporation into the DNA of HT-29 cells was determined as described previously (24).

**Immunoprecipitation and immunoblotting analyses.** Cells were grown in 100-mm culture dishes, washed briefly with ice-cold PBS containing 1 mM sodium orthovanadate (Na3VO4) and 1 mM PMSF, and solubilized for 40 min at 4°C with lysis buffer containing (in mM) 20 HEPES, pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 100 NaF, 10 sodium pyrophosphate, and 1 Na3VO4, plus 1% Triton X-100. The following protease inhibitors were used (in µg/ml): 20 aprotinin, 10 antipain, 10 leupeptin, and 50 benzamidine HCl, plus 0.2 µM PMSF. The insoluble material was removed by centrifugation at 13,000 g for 10 min and protein content was determined by using the BCA protein assay kit (Pierce, Rockford, IL). Supernatant (0.75 mg/ml) was preclarified by incubating on a rotating platform for 1 h at 4°C with 1 µg of normal rabbit IgG and 50 µl of resuspended volume of protein A-Sepharose beads (Amersham Pharmacia Biotech) and centrifuged at 2,500 rpm for 5 min at 4°C. The supernatants were incubated with 1 µg anti-ErbB3 or anti-ErbB2 antibody for 2 h at 4°C. Protein A-Sepharose beads were added to lystate-antibody mix followed by incubation for 2 h at 4°C. The beads were washed four times with lysis buffer. The immunoprecipitate or total cell lysates were resolved on a SDS-PAGE (4–20%) and transferred onto polyvinylidene fluoride membrane (Millipore). The blots were blocked for 1 h in 1% BSA in 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 (TBST) or 5% milk TBST and incubated for 1 h with either PY20-HRP (1:5,000), anti-EGFR (1:250), anti-ErbB2 (1:500), anti-ErbB3 (1:1,000), anti-ErbB4 (1:500), anti-PI3-kinase (1:1,000), anti-Akt (1:1,000), anti-p-Akt (1:1,000), anti-MAPK (1:1,000), anti-p-MAPK (1:1,000), anti-HRGC (1:500), or anti-β-actin (1:1,000) antibody. The blot was then incubated with anti-mouse or anti-rabbit HRP-conjugated antibody. Signals were detected by using the enhanced chemiluminescence method using SuperSignal West Dura Extended Duration Substrate (Pierce, IL).

**RT-PCR.** Total RNA was isolated by using the guanidinium isothiocyanate-phenol-chloroform method and RT-PCR was performed as previously described (28). Each PCR cycle consisted of denaturing at 94°C for 1 min, annealing at temperatures listed in Table 1 for 1 min, and extension at 72°C for 1 min. Sequences for PCR primer sets and numbers of cycles used for PCR amplification are listed in Table 1. The levels of mRNA were corrected as a ratio to the corresponding β-actin level.

**DNA laddering.** HT-29 cells were cultured and treated as described above and were extracted for 2 h in extraction buffer (50 mM Tris, pH 7.5, 20 mM EDTA, and 1% Nonidet P-40). SDS was then added to 1%, and the mixture was incubated for 2 h with 500 µg/ml RNase at 37°C followed by incubation for 2 h with 500 µg/ml proteinase K at 42°C. The mixture was then extracted with phenol-chloroform-isoamylalcohol (25:24:1) and the DNA was precipitated with 0.3 M sodium acetate and 2.5 vol of absolute ethanol. Equal amounts of DNA samples (30 µg) were electrophoresed on a
We examined the effect of CLA on viability of HT-29 cells. Cells in monolayer culture were treated with CLA (0–20 \( \mu \)M) for 2 or 4 days in serum-free medium, and the viable cell number was estimated. As illustrated in Fig. 1A, CLA decreased the viable HT-29 cell numbers in a dose-dependent manner with a 55 ± 2% decrease in cell number 4 days after the addition of 20 \( \mu \)M CLA. To examine whether CLA inhibits DNA synthesis of HT-29 cells, cells in monolayer culture were treated with CLA for 3 days, and \(^{3}H\)thymidine incorporation was estimated. As illustrated in Fig. 1B, CLA decreased the incorporation of \(^{3}H\)thymidine into DNA of HT-29 cells in a dose-dependent manner with an 86 ± 3% decrease in \(^{3}H\)thymidine incorporation after the addition of 20 \( \mu \)M CLA.

Table 1. Primer sequences used for PCR amplification

<table>
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<tr>
<th>mRNA</th>
<th>Primer sequences</th>
<th>Annealing Temperature</th>
<th>Number of Cycles</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
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<td>EGFR</td>
<td>forward: 5'-aaccgacctgaagggagctgc-3'</td>
<td>55°C</td>
<td>27</td>
<td>392</td>
</tr>
<tr>
<td></td>
<td>backward: 5'-agcttggtcctctgcggat-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ErbB2</td>
<td>forward: 5'-agtttcacagtggagggcgatgcc-3'</td>
<td>64°C</td>
<td>27</td>
<td>390</td>
</tr>
<tr>
<td></td>
<td>backward: 5'-ttctccctacggtcctacggccc-3'</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ErbB3</td>
<td>forward: 5'-ggctggctggcctttt-3'</td>
<td>57°C</td>
<td>27</td>
<td>365</td>
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<td>backward: 5'-gctgctggagggctggtaa-3'</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>( \beta )-actin</td>
<td>forward: 5'-gttgagacccctcaacccc-3'</td>
<td>60°C</td>
<td>32</td>
<td>318</td>
</tr>
<tr>
<td></td>
<td>backward: 5'-gtgcccctcctcctgctga-3'</td>
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2% agarose gel in Tris-borate EDTA buffer and visualized by ethidium bromide staining.

Fluorescence-activated cell sorting analysis. To estimate apoptotic cell number, cells were plated in 24-well plates and incubated in the absence or presence of various concentrations of CLA. After 3 days, cells were trypsinized and incubated with phycoerythrin-conjugated Annexin V and 7-amino-actinomycin D (Pharmingen, Franklin Lakes, NJ) for 15 min at room temperature in the dark. Apoptotic cells were analyzed by flow cytometry within 1 h utilizing FACScan (Becton Dickinson, Franklin Lake, NJ). The data were analyzed by using ModFit version 1.2 software.

PI3-kinase assay. An assay for PI3-kinase activity was performed as previously described (13). Cell lysate (750 \( \mu \)g) was immunoprecipitated with polyclonal antibody against ErbB3 followed by incubation with protein A-Sepharose beads. The immunoprecipitates were washed twice with 1% Nonident P-40-PBS, twice with 100 mM Tris-HCl (pH 7.2) containing 500 mM LiCl and 1 mM Na3VO3, and twice with 50 mM Tris-HCl (pH 7.2) containing 150 mM NaCl. After the last wash, the beads were resuspended in 20 \( \mu \)l kinase buffer (in mM: 20 HEPES, pH 7.2, 50 NaCl, 1 EGTA) containing 4 \( \mu \)g of phosphatidylinositol (Sigma), 10 \( \mu \)M ATP, 5 \( \mu \)M MnCl2, and 10 \( \mu \)Ci of [\( ^{32} \)P]ATP and incubated for 20 min at 30°C. The resulting [\( ^{32} \)P]phosphatidylinositol 3-phosphate (PIP) lipids were separated from other reaction products by thin-layer chromatography and were visualized by autoradiography. The PIP signals were quantitated by densitometry by using the Bio-profile Bio-1D application (Vilber-Lourmat, France).

Statistical analyses. Data were expressed as means ± SE and analyzed by ANOVA. Differences between treatment groups were analyzed by Duncan’s multiple range test.

RESULTS

CLA inhibits proliferation and induces apoptosis of HT-29 cells. We examined the effect of CLA on viability of HT-29 cells. Cells in monolayer culture were treated with CLA (0–20 \( \mu \)M) for 2 or 4 days in serum-free medium, and the viable cell number was estimated. As illustrated in Fig. 1A, CLA decreased the viable HT-29 cell numbers in a dose-dependent manner with a 55 ± 2% decrease in cell number 4 days after the addition of 20 \( \mu \)M CLA. To examine whether CLA inhibits DNA synthesis of HT-29 cells, cells in monolayer culture were treated with CLA for 3 days, and \(^{3}H\)thymidine incorporation was estimated. As illustrated in Fig. 1B, CLA decreased the incorporation of \(^{3}H\)thymidine into DNA of HT-29 cells in a dose-dependent manner with an 86 ± 3% decrease in \(^{3}H\)thymidine incorporation after the addition of 20 \( \mu \)M CLA.

To examine whether CLA induces apoptosis, HT-29 cells were similarly treated, and genomic DNA was prepared. DNA isolated from cells treated with 10 \( \mu \)M CLA displayed an oligonucleosomal ladder pattern characteristic of apoptotic cell death (Fig. 2A). The intensity of oligonucleosomal ladder was increased to a greater degree in cells treated with 20 \( \mu \)M CLA. To quantify the apoptotic cells in the early stage, phosphatidylserine on the outer leaflet of apoptotic cell membranes was analyzed by using Annexin-V followed by flow cytometry. As shown in Fig. 2B, a small increase in early apoptotic cell numbers was observed after treatment with 10 \( \mu \)M CLA. The numbers of early

Fig. 1. Effect of conjugated linoleic acid (CLA) on HT-29 cell growth. HT-29 cells were plated, cultured, and serum-starved as described in MATERIALS AND METHODS. A: viable cell numbers. After serum starvation, cells were incubated for 2 or 4 days in serum-free medium containing 0, 5, 10, or 20 \( \mu \)M CLA, and cell numbers were estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. B: \(^{3}H\)thymidine incorporation. Cells were treated with CLA for 3 days, \(^{3}H\)thymidine was then added, and the incubation was continued for another 14 h to measure the incorporation into DNA. Each bar represents the mean ± SE (n = 6). Comparisons between groups that yielded significant differences (P < 0.05) are indicated by different letters above each bar (a–d). cpm, Cycles/min.
apotheotic cells were further increased after cells were treated with 20 μM CLA.

**CLA regulates ErbB receptor signaling.** ErbB receptors play important roles in regulating epithelial cell proliferation and survival, so we investigated whether CLA influences ErbB receptor expression in HT-29 cells. Total cell lysates were immunoblotted with antibodies specific for EGFR, ErbB2, -3, or -4, and HT-29 cells were found to express EGFR, ErbB2, and -3 (Fig. 3). ErbB4 was not detectable under the conditions of the present experiment. Treatment of HT-29 cells with increasing concentrations of CLA led to decreased EGFR, ErbB2, and -3 levels. HRG was also detected, but CLA did not affect HRG levels. Immunoblots were probed with an antibody for β-actin as a control for protein loading. To determine whether CLA regulates the expression of EGFR, ErbB2, and -3 at a transcriptional level, cells were similarly treated with CLA, total RNA was isolated, and mRNA levels were determined by RT-PCR analysis. As shown in Fig. 4, CLA decreased EGFR, ErbB2, and ErbB3 transcripts in a concentration-dependent manner.

Because significant levels of ErbB3 were detected in HT-29 cells, we determined whether exogenous HRG would be able to counteract growth inhibition induced by CLA. Serum-starved cells were incubated with or without 100 ng/ml HRG-α in the absence or presence of 10 μM CLA for 2 or 4 days. Viable cell numbers were significantly decreased in the presence of 10 μM CLA, and HRG-α mitigated the effect of CLA at 2 days (Fig. 5). HRG-α alone also increased cell number at both 2 and 4 days. However, at 4 days, the viable cell number was not higher in the CLA + HRG-treated group com-

![Fig. 2](http://example.com/fig2.png)

**Fig. 2.** Effect of CLA on apoptosis of HT-29 cells. HT-29 cells were cultured and treated with CLA for 3 days as described in Fig. 1. A: DNA fragmentation. Genomic DNA samples were prepared and analyzed by agarose gel electrophoresis (30 μg/lane). A photograph of the ethidium bromide-stained gel, which is representative of 3 independent experiments, is shown. B: fluorescence-activated cell sorting. Cells were trypsinized, loaded with 7-amino-actinomycin D and Annexin V, and then analyzed by flow cytometry. The number of living cells and early apoptotic cells is expressed as a percentage of total cell number. Each bar represents the mean ± SE from 6 independent experiments. Comparisons between groups that yielded significant differences (P < 0.05) are indicated by different letters (A–C, a–c) above each bar.

![Fig. 3](http://example.com/fig3.png)

**Fig. 3.** Effect of CLA on the protein expression of epidermal growth factor receptor (EGFR), ErbB2, ErbB3, heregulin (HRG), and the p85 subunit of phosphoinositide 3-kinase (PI3K). HT-29 cells were treated with CLA for 3 days as described in Fig. 1. Cell lysates were subjected to immunoblotting with an antibody against EGFR, ErbB2, ErbB3, p85 subunit of PI3K-kinase, HRG, or β-actin. A: photographs of chemiluminescent detection of the blots, which were representative of 3 independent experiments, are shown. B: quantitative analysis of immunoblots. The relative abundance of each band was estimated by densitometric scanning of the exposed films. Each bar represents the mean ± SE (n = 3).
pared with the CLA-treated group. Thus HRG-α stimulation of ErbB3 signaling is not sufficient to counteract the negative influence of CLA on HT-29 cell growth and viability. We found that the mean IC<sub>50</sub> dose of CLA in HT-29 cells was 17.6 ± 0.08 μM, and the value did not change after the addition of 100 ng/ml HRG (18.3 ± 0.53 μM).

Receptor tyrosine kinase subunits become tyrosine phosphorylated after activation by ligand. To determine whether CLA affects ErbB3 tyrosine phosphorylation, cells were treated in the absence or presence of 10 μM CLA for 3 days and lysates were prepared after 0, 1, 5, or 60 min of stimulation with HRG-α. Immunoblot analysis of lysates with anti-phosphotyrosine antibody revealed that HRG-α induced a time-dependent increase in the tyrosine phosphorylation of high molecular mass proteins (~180 kDa) with the expected mobility of ErbB family receptors, with peak levels of tyrosine phosphorylation being detected at 5 min. Tyrosine phosphorylation was significantly reduced in cells treated with 10 μM CLA (Fig. 6A). We have performed a similar experiment after treating cells with 10 μM CLA for 1 day. There were small but consistent decreases in tyrosine phosphorylation of these proteins in cells treated with CLA, although there were no changes in ErbB protein levels (data not shown).

By using immunoprecipitation followed by immunoblotting, we measured levels of ErbB receptor expression and phosphorylation. After the addition of HRG-α alone, ErbB3 levels remained constant, and increased ErbB3 phosphorylation was detected at 1 and 5 min, indicating that the receptor was being activated by the ligand (Fig. 6B). ErbB3 protein levels were decreased by ~25% in cells treated with 10 μM CLA. However, there were only slight increases in ErbB3 tyrosine phosphorylation by HRG in cells treated with CLA indicating that cells treated with CLA had a reduced ability to respond to the stimulation of HRG-α, which is consistent with the finding that CLA blunted the effect of exogenous HRG-α on HT-29 cell growth.

ErbB3 lacks intrinsic kinase activity and its activation is mediated by heterodimerization with and transphosphorylation by other tyrosine kinases, with ErbB2 being its preferred partner (12, 45). This heterodimer formation has been observed in the colonic epithelial cell line T84 (23). To examine whether HRG regulates ErbB2 activity in the HT-29 cell line, cell lysates were immunoprecipitated with anti-ErbB2 antibody and the immunoprecipitated proteins were probed with anti-phosphotyrosine antibody. HRG-induced phosphorylation of ErbB2 in a time-dependent manner in HT-29 cells with maximum stimulation at 1 min. ErbB2 expression levels were ~26% lower in cells treated with 10 μM CLA. HRG-induced phosphorylation of ErbB2 was very small in cells treated with 10 μM CLA (Fig. 6C). Thus CLA appears to negatively regulate both ErbB2 and -3 phosphorylation by causing a decrease in the expression levels and phosphorylation of these two signaling proteins.
HRG-α-mediated activation of PI3-kinase and Akt is inhibited by CLA. Activation of ErbB receptor signaling leads to activation of PI3-kinase. We examined expression of the p85 regulatory subunit of PI3-kinase in HT-29 cells treated with increasing amounts of CLA by using immunoblotting (Fig. 3). Although decreased levels of ErbB receptors were detected, expression of PI3-kinase was not altered by the addition of CLA. We investigated whether CLA influences HRG-induced association of ErbB3 and PI3-kinase. Immunoprecipitations were performed with an anti-ErbB3 antibody followed by immunoblotting with the p85 antibody. HRG-α stimulated association of p85 regulatory subunit of PI3-kinase with ErbB3, and the association was reduced in CLA-treated cells (Fig. 7A). The levels of PI3-kinase associated with ErbB3 were normalized to ErbB3 expression levels to differentiate whether the reduced association after CLA treatment is a result of decreased ErbB3 levels or the result of an inhibition of p85 recruitment. After normalization, the reduced association after CLA addition is still apparent (Fig. 7B), indicating that the decrease in PI3-kinase association to ErbB3 is a result of both decreased ErbB3 levels and an inhibition of p85 recruitment.
PI3-kinase activity in the anti-ErbB3 immunoprecipitates was analyzed by in vitro kinase assays, in which the PIP product was resolved by thin-layer chromatography. The spots of [32P]-PIP on thin-layer chromatography plates are shown in Fig. 8A with their activities plotted in Fig. 8B. In accordance with p85 Akt antibody-specificity, phosphorylated Akt (p-Akt) was mainly detected at 5 min after HRG stimulation. However, the magnitude of changes in DNA synthesis due to CLA treatment (data not shown).

To examine the effect of CLA on the MAPK pathway, Western blotting was performed with total lysates prepared from cells treated with or without 10 μM CLA and/or 100 ng/ml HRG as described above by using antibody-specific MAPK (ERK1 and -2) and p-MAPK. HRG activation of ErbB3 receptor signaling resulted in a slight increase in MAPK signaling in HT-29 cells. Phosphorylated MAPK levels were increased at 5 min after HRG stimulation. However, the magnitude of the increase was much smaller compared with that of p-Akt, and HRG-induced MAP kinase phosphorylation was not changed by 10 μM CLA. Total MAPK levels did not change after CLA treatment (data not shown).

**DISCUSSION**

The anticarcinogenic effect of CLA has been the focus of many recent investigations. We (37) have previously shown that dietary CLA inhibits the development of colon tumors in rats treated with 1,2-dimethylhydrazine. In vitro studies also have shown that CLA inhibits the growth of SW480 (34) and HT-29 cells (35, 42), the human colon cancer lines. The present study indicates that CLA inhibits HT-29 cell growth by both decreasing cell proliferation and inducing apoptosis. However, the magnitude of changes in DNA synthesis due to CLA was much higher than that of apoptotic cell numbers indicating that the growth inhibitory effect of CLA was mainly due to decreased proliferation. Inhibition of proliferation and induction of apoptosis by CLA have already been shown in other cell types. For example, Ip et al. (19) have shown that CLA inhibited proliferation...
and induced apoptosis of normal mammary epithelial cells, and Palombo et al. (35) reported that trans-10,cis-12 CLA isomer induced caspase-dependent apoptosis in MIP-101 human colorectal carcinoma cells.

In the present study, we provide the first evidence that CLA reduces ErbB2 and ErbB3 protein expression and ErbB3-mediated signaling. When HT-29 cells were incubated with HRG in serum-free medium, the cells responded to HRG by increasing cell number. However, HRG failed to increase the viable cell number when HT-29 cells were treated with 10 μM CLA for 96 h, suggesting that CLA attenuates the HRG-receptor signaling pathway. Indeed, decreased levels of the HRG receptor ErbB3 were noted at 72 h after treatment with CLA. We found that HRG is expressed in HT-29 cells and that the expression of HRG in HT-29 cells was not altered by CLA. Previous studies have shown that HRG is expressed in gastrointestinal tissues (22, 33) and human colon cancer specimens and is an autocrine growth stimulator of GEO human colon cancer cells (48). In addition, production of HRG by mesenchymal cells in the intestine (33) may have an impact on epithelial cells in vivo. HRG may be an autocrine growth regulator of HT-29 cells. However, it is more likely that CLA inhibits cell's ability to respond to HRG without changing the production of this growth factor in HT-29 cells.

CLA decreased ErbB mRNA levels suggesting that CLA regulates the expression of these receptors at the transcriptional level. The present study did not determine the mechanisms responsible for the CLA regulation of ErbB mRNA levels. CLA has been reported to be an activator of the peroxisome proliferator-activated receptor (PPAR)γ (50). CLA or its metabolites may influence transcription of genes that regulate growth by acting as a ligand for the PPAR. Ligands for PPARγ induce apoptosis and exert antiproliferative effects on several carcinoma cell lines (8, 26).

Activation of tyrosine kinase receptors requires tyrosine phosphorylation of receptor subunits. HRG induces tyrosine phosphorylation of ErbB2 and -3 only minimally in cells treated with CLA, suggesting that CLA inhibits activation of these receptors in addition to decreasing these receptor protein levels. Observations that tyrosine phosphorylation of these proteins decreased without changes in protein levels after a short-term treatment with CLA also suggest that CLA has a direct influence on ErbB2/ErbB3 signaling. Recent studies utilizing MCF-7 breast cancer cells have shown that the activation of PPARγ through the 15-deoxy-Δ12,14-prostaglandin J2 ligand causes a dramatic inhibition of ErbB2 and -3 tyrosine phosphorylation induced by HRG-α and HRG-β (38). It remains to be determined whether CLA inhibits expression of these receptor transcripts and/or tyrosine phosphorylation of these receptor proteins by activating PPARγ.

CLA decreased the protein levels of the ErbB receptors in HT-29 cells in a dose-dependent manner. HRG is known to induce the formation of heterodimers between ErbB3 and -2 or between ErbB4 and -2, thereby transactivating ErbB2 (5, 39, 43). The ErbB2/ErbB3 dimer constitutes a high-affinity coreceptor for HRG (43, 44), which is capable of powerful mitogenic signaling (5). Because we observed that ErbB4 was undetectable in HT-29 cells and HRG induced phosphorylation of ErbB2 and -3 in HT-29 cells, it is reasonable to assume that the two receptors form heterodimers.

ErbB3 has been characterized as a major mediator of HRG-dependent activation of the PI3-kinase pathway (5, 44). ErbB3 is particularly well adapted to mediate PI3-kinase signaling because it contains six YXXM consensus-binding sites for p85 (16). We observed that HRG stimulated the recruitment of PI3-kinase to the ErbB3 receptor in HT-29 cells, and CLA decreased ErbB3-associated PI3-kinase protein levels and PI3-kinase activities. These decreases do not appear to be attributed to changes in the PI3-kinase protein expression but to the decrease in ErbB3 protein levels and p85 recruitment.

Akt is a downstream target of PI3-kinase and plays a central role in PI3-kinase-mediated protection against apoptosis (9). The present data show that CLA inhibited HRG-α-induced activation of Akt, which could be due to both decreased ErbB2 and -3 levels and decreased ErbB2/ErbB3 activation. The slight decrease in Akt protein levels may also have contributed to the decreased p-Akt levels. These results imply that CLA inhibits DNA synthesis and induces apoptosis of HT-29 cells by inhibiting the Akt signaling pathway. Increased expression and/or activity of ErbB receptors and downstream signaling proteins, such as Akt, are frequent events in cancer. Future studies are needed to examine the effect of Akt on HT-29 cell growth.

In conclusion, we have demonstrated that CLA negatively regulates levels of ErbB receptors and subsequent activation of Akt but not the MAPK pathway in HT-29 cells. Inhibition of ErbB receptor signaling may be one of the mechanisms by which CLA inhibits cancer cell growth and viability. Aberrant activation of ErbB receptors and Akt signaling contributes to the development of many types of cancer, and downregulation of these pathways by CLA could have important therapeutic benefits.

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proteins reconstitutes a high affinity receptor for heregulin. 