Inhibition of apolipoprotein B secretion by IL-6 is mediated by EGF or an EGF-like molecule in CaCo-2 cells

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Abstract Small intestinal mucosal inflammation observed in celiac disease is associated with the local release of growth factors and various cytokines. In a previous study, we investigated the effects of various cytokines on triacylglycerol and apoB secretion by CaCo-2 cells and observed that TNF-α, IL-1β, and particularly IL-6, decreased apolipoprotein (apo) B and triacylglycerol secretion. In this study, we explored possible mechanisms to explain the inhibitory effect of IL-6 on apoB secretion. IL-6, 10 ng/mL, added to the basolateral medium of CaCo-2 cells grown on semi-permeable filters, decreased apoB secretion by 42%. Adding a blocking monoclonal antibody (mAb 528) to the EGF receptor completely prevented this effect. IL-6 decreased the amount of EGF receptor protein and the binding of iodinated EGF to its receptor by 50% and 30%, respectively. Incubation of cells with various ligands to the EGF receptor, such as EGF, TGF-α, HB-EGF, and amphiregulin, also decreased apoB secretion. Inhibition of apoB secretion by EGF was prevented by the mAb 528 or an EGF neutralizing antibody. In a dose-dependent manner, the neutralizing antibody to EGF prevented the decrease in secretion of apoB, triacylglycerol mass, and cell-surface binding of labeled EGF caused by IL-6. Similar to the effects of IL-6, EGF decreased the secretion of triacylglycerol mass and the synthesis and secretion of newly synthesized apoB. The results suggest that, in CaCo-2 cells, IL-6 causes the release of EGF or an EGF-like molecule. By binding to cell surface EGF receptors, the molecule then causes a decrease in triacylglycerol and apoB secretion.—**Murthy, S., S. N. Mathur, W. P. Bishop, and F. J. Field.** Inhibition of apolipoprotein B secretion by IL-6 is mediated by EGF or an EGF-like molecule in CaCo-2 cells. *J. Lipid Res.* 1997. **38:** 206–216.

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In inflammatory conditions of the bowel, such as occurs in celiac disease, it has been proposed that the local release of cytokines into the mucosa initiates and propagates the inflammatory response (1). IL-6 is a potent inflammatory cytokine. It is secreted by a variety of cell types in response to injury and inflammation (2), and is found in the submucosa of intestine (3–5) and in enterocytes (6) of patients with idiopathic inflammatory bowel disease and gluten-sensitive enteropathy. Synthesis and release of IL-6 are stimulated by other pro-inflammatory cytokines such as IL-1 (7, 8) and TNF-α (7, 9) both of which are detected in the mucosa under conditions of inflammation (4, 5). This suggests that IL-6 may be the "effector molecule" which mediates the biological effects of other cytokines. Intestinal epithelial cells, which possess a variety of cell surface cytokine receptors, including receptors for IL-6 (10–12), and express mRNA for several cytokines (4, 5, 13) likely contribute to the pathological and physiological derangement observed in inflammatory conditions of the gut. Recent evidence for the interaction of cytokines with intestinal epithelial cells demonstrates that IL-6 induces the synthesis of acute phase proteins by intestinal cells (11). Interaction of intestinal epithelial cells with cytokines, however, has not been examined with respect to their secretory functions. In a previous study, cultured human intestinal epithelial cells, CaCo-2, were shown to decrease their secretion of apoB and triacylglycerol in response to inflammatory cytokines, IL-1β, TNF-α, and IL-6 (14). It was postulated that even in early stages of inflammation when absorptive cells are not morphologically damaged, the absorption of lipids and likely other nutrients would be decreased secondary to a direct interaction of pro-inflammatory cytokines with enterocytes. In this study we examined possible mechanisms by which IL-6 interferes with the secretion of apoB and triacylglycerols.

Abbreviations: IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; IL-1β, interleukin 1beta; EGF, epidermal growth factor; TGF-α, transforming growth factor-alpha; HB-EGF, heparin-binding EGF-like growth factor; TLCK, Na-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride; VLDL, very low density lipoprotein; LDL, low density lipoprotein; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; BS^3, bis succinylsuberate.

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The results suggest that incubation of CaCo-2 cells with IL-6 results in the release of EGF or an EGF-like molecule, which by activation of EGF receptors causes a decrease in secretion of triacylglycerol and apoB mass.

MATERIALS AND METHODS

Materials

Recombinant human IL-6, EGF, TGF-α, amphiregulin, HB-EGF, and neutralizing polyclonal antibody to recombinant human EGF, were purchased from R & D Systems (Minneapolis, MN). Carrier-free EGF was purchased from Beckton Dickensen (Bedford, MA). Bis(sulfosuccinimidyl) suberate (BS^3) was purchased from Pierce (Rockford, Ill). Rabbit polyclonal antibody to human EGF receptor was from Oncogene Sciences (Uniondale, NY). The ECL chemiluminescent detection kit and anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase (HRP) was from Boehringer Mannheim (Indianapolis, IN). Oleic acid, BSA, Protein G-Sepharose, and GPO Trinder kit were purchased from Sigma Chemicals (St. Louis, MO). Monoclonal antibody to human apoB, and rabbit anti-human apoB polyclonal antibody conjugated to HRP were bought from Biodesign (Kennebunkport, ME). TMB microwell peroxidase substrate system containing 3, 3′, 5, 5′-tetramethyl benzidine and hydrogen peroxide was purchased from Kirkegaard and Perry (Gaithersburg, MD). Nunc 96-well immunoplates were obtained from PGC Scientific (Gaithersburg, MD). Rabbit anti-human apoB polyclonal antibody was obtained from Calbiochem (San Diego, CA). Recombinant protein A-Sepharose was purchased from Repligen (Cambridge, MA). CellTiter 96 was provided by Promega (Madison, WI). Silica gel G plates were purchased from Fisher Scientific (Batavia, IL). ApoB cDNA was purchased from American Type Culture Collection (Rockville, MD). [α-^{32}P]dCTP (6,000 Ci/mmol) was purchased from DuPont-New England Nuclear Research Products (Boston, MA). *Trans*[^{35}S]methionine (1100 Ci/mmol) and carrier-free ^{125}I (100 mCi/mL) were purchased from ICN Biomedicals Inc. (Costa Mesa, CA).

Cell culture

CaCo-2 cells were cultured on T-75 flasks (Corning Glassworks, Corning, NY) in DMEM (GIBCO, Grand Island, NY) with 4.5 g/L glucose, and supplemented with 20% FBS (Hyclone Laboratories, Logan, UT), 4 mM glutamine, 50 μg/mL penicillin, 50 μg/mL streptomycin, and 1% nonessential amino acids. Once the flasks reached 80% confluency, the cells were split and seeded onto polycarbonate micropore membranes (0.4 μm pore size, 6.5 mm diameter) inserted into Transwells (Costar, Cambridge, MA) at a density of 0.2 × 10^5 cells/well. For experiments in which triacylglycerol mass and cell surface ^{125}I-labeled EGF binding were measured, cells were sub-cultured in 24.5-mm diameter Transwells. Cells were fed every other day and were used 14 days after seeding.

On the day of the experiment, cells and the basal chamber were washed twice with M199, and cytokines were added to the basal chamber in serum-free HBSS/1 M HEPES (HBSS) or M199/1 M HEPES (M199) containing 0.1% BSA. Control cells received HBSS/0.1% BSA or M199/0.1% BSA alone. All cells received M199 in the apical chamber. Incubations were carried out for 18 h at 37°C in an atmosphere of 95% compressed air/5% CO_2.

Cell viability/proliferation

Cell viability and proliferation were assessed by the activity of mitochondrial dehydrogenase by using the CellTiter 96 assay kit as described previously (14). Compared to the absorbance of the dye released from control cells (1.00), the relative absorbances of the dye released from cells incubated with IL-6 and EGF were 1.34 ± 0.08 and 1.00 ± 0.07, respectively.

Estimation of apoB mass

ApoB mass was determined by sandwich ELISA as previously described (14). The presence of the treatments in the media did not interfere with the estimation of apoB mass by ELISA.

Measurement of apoB synthesis and secretion

After an overnight incubation with the treatments, cells were washed and incubated with methionine-free M199 in the continued presence of the treatments for 1 h. One hundred μCi of [^{35}S]methionine was added to each well in the apical chamber. After 4 h of pulse, cells were harvested in RIPA buffer (10 mM sodium phosphate, pH 7.5, 100 mM sodium chloride, 1% (v/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate, 0.5% (w/v) sodium deoxycholate, 21 μM leupeptin, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM dithiothreitol, 20 mM methionine, and 1 mM cysteine) and precleared with protein A-Sepharose. Basolateral media and the pre-cleared cell lysates were incubated overnight at 4°C with rabbit anti-human apoB antisera (1:250 dilution). The antigen–antibody complexes were precipitated by incubating with protein A-Sepharose for 1 h at room temperature on a shaker followed by a brief high speed centrifugation. The immune complexes were washed extensively and dissociated from protein

A-Sepharose by adding 30 μL 2× Laemmli sample buffer and 10 μL 0.2 M glycine buffer (pH 2). Proteins were separated by SDS PAGE on 8% porous gels as described by Doucet, Murphy, and Tuana (15). Gels were fixed in 7% acetic acid/5% methanol solution and enhanced in 1 M sodium salicylate solution. After drying, the gels were exposed to X-ray films for 2 h. Bands corresponding to apoB-100 and B-48 were cut from the gels and counted by liquid scintillation counting.

Estimation of apoB mRNA abundance

Total RNA was isolated using TRI Reagent (Sigma Chemical Co., St. Louis, MO) in a modification of the single-step method originally described by Chomczynski and Sacchi (16). Northern blots were run with 20 μg of total RNA and probed for apoB mRNA as previously described (14). ApoB mRNA was quantified by densitometry using Sigma Gel software (Jandel Scientific, San Rafael, CA).

LDL degradation

Human LDL was iodinated with ^{125}I according to the method of McFarlane (17). Ninety eight percent of the label was precipitated by trichloroacetic acid. ^{125}I-labeled LDL (5 μg, 0.2 μCi) was added per well in the basolateral chamber along with the treatments. Glutaraldehyde-fixed cells served as controls. To account for nonspecific degradation of LDL, cells were incubated with 50-fold excess of unlabeled LDL in the presence of labeled LDL. After 3, 6, and 18 h of incubation, the amount of ^{125}I-labeled LDL that was degraded was estimated according to the method of Goldstein, Basu, and Brown (18).

Binding of ^{125}I-labeled EGF to the EGF receptor

EGF was iodinated using IODO-BEADS iodination reagent, Pierce Chemical Co. (Rockford, IL). After treatment with IL-6 for 18 h, binding of ^{125}I-labeled EGF to the EGF receptor was estimated as described previously (19). Cells were washed with M199 and incubated for 2 h at 4°C with 0–100 ng/mL of iodinated EGF (0.1 μCi/ng) in M199/0.1% BSA in the basal wells. M199/0.1% BSA was added to the apical chambers. To estimate nonspecific binding, cells were incubated with 2 mg/mL of cold EGF in the presence of 25 ng/mL of (^{125}I)-labeled EGF. After extensive washing with ice-cold M199, EGF receptors were cross-linked to the radiolabeled ligand by incubating cells for 2 h at 4°C with BS^3 (1.3 mg/mL) in HBSS. Cells were rinsed and scraped in 25 mM Tris (pH 7.5), 125 mM sodium chloride, containing 250 mM sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 2 mM benzamidine, and 21 μM leupeptin. Cell scrapings were centrifuged at 3000 rpm for 10 min and the pellet was taken up in 30 μL of 10 mM Tris (pH 7.4), 125 mM sodium chloride containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 21 μM leupeptin, 2 mM benzamidine, 2 μM Pepstatin A, 25 μM TLCK, and 3 μM aprotinin and 30 μL of 2× Laemmli sample buffer, and vortexed vigorously to lyse the cells. The cell lysates were boiled for 5 min and centrifuged at 14,000 rpm for 5 min. The supernatants were applied onto 8% polyacrylamide/SDS gels and separated by electrophoresis at 150 V. Gels were fixed in 7% acetic acid/5% methanol, dried, and exposed overnight to X-ray films. Bands corresponding to EGF receptor cross-linked to EGF were cut from the gels and counted in a gamma counter. The specificity of the binding of iodinated EGF to its receptor in CaCo-2 cells was determined by Bishop and Wen (19), by demonstrating that the binding of iodinated EGF to cell surfaces is abolished in the presence of the blocking antibody to the EGF receptor.

Western blotting of the EGF receptor

After incubation with IL-6, cells were scraped and lysed as described above for the binding experiments. Cell lysates were boiled for 5 min with 2× Laemmli sample buffer and separated by SDS/PAGE on 8% polyacrylamide gels. The proteins were electroblotted onto PVDF membranes at 15 V overnight and blocked for an hour at room temperature in TBS (10 mM Tris/100 mM sodium chloride, pH 7.4) containing 3% non-fat milk powder and 0.1% Tween-20. The membranes were incubated for an hour with 5 μg/mL of rabbit anti-human EGF receptor antibody diluted in 1% non-fat dry milk in TBS. After washing in TBS/0.1% Tween-20, the membranes were incubated for 1 h with anti-rabbit IgG-HRP in TBS/1% non-fat dry milk. After extensive washing with TBS/0.1% Tween-20 and a final rinse with TBS, the membranes were incubated with HRP chemiluminescent substrates contained in the ECL kit, wrapped in Saran wrap and then exposed to X-ray film. Band densities were scanned on Hewlett-Packard ScanJet IIcx/T scanner, Hewlett-Packard (Greely, CO) and quantified with the computer-assisted program, Sigma Gel, (Jandel Scientific, San Rafael, CA).

EGF receptor blocking antibody

Murine hybridoma cells that secrete mAb 528 (ATCC, Rockville, MD) were grown in serum-free HB-101 medium. The secreted antibody was purified from the medium by protein A-agarose column chromatography using the Immunopure System (Pierce, Rockford, IL) and then concentrated by centrifugal ultrafiltration through a 10,000 molecular weight cut-off Centricon concentrator (Amicon, Danvers, MA). This antibody blocks EGF binding to the human EGF receptor but has no agonist activity (20).

TABLE 1. Effect of IL-6 in the presence or absence of EGF receptor blocking antibody on the secretion of apoB mass

Treatment	ApoB
	ng/well
Control	458 ± 8
IL-6	263 ± 7[a]
IL-6 + EGF receptor antibody	490 ± 22
EGF receptor antibody	438 ± 10

CaCo-2 cells were incubated for 18 h with HBSS/0.1% BSA containing one of the following treatments: 10 ng/mL IL-6, 10 ng/mL IL-6 and 5 μg/mL EGF receptor blocking antibody, or 5 μg/mL the blocking antibody alone. Control cells received HBSS/0.1% BSA. All treatments were added to the basolateral wells while the apical wells received M199. ApoB mass in the basolateral media was analyzed by sandwich ELISA as described in Methods. Data are presented as mean ± SEM of 6 wells.

[a] $P < 0.01$ vs. control.

Chemical analyses

Total protein content in cells was determined by the method of Lowry et al. (21). Triacylglycerol mass in cells was measured using the GPO Trinder kit (Sigma Chemicals, MO) as described previously (22).

Statistical analyses of data were performed by ANOVA, Tukey's *t*-test, and Student's *t* test (23).

RESULTS

EGF receptor and apoB secretion

It is recognized that members of the EGF family bind to the EGF receptor (24) and promote wound healing in the gut (25–27). To address whether the EGF receptor was involved in the regulation of apoB secretion by IL-6 (14), CaCo-2 cells, grown on semi-permeable filters separating an upper and lower well, were incubated for 18 h with 10 ng/mL IL-6 added to the lower well, a concentration that has been previously reported in actively inflamed intestinal mucosa (3). In some of the wells, a specific blocking antibody to the EGF receptor was added at the same time as IL-6. After the incubation the amount of apoB secreted into the basolateral medium (lower well) was estimated (**Table 1**). Compared to control cells, cells incubated with IL-6 secreted 43% less apoB. The concomitant addition of 5 μg/mL of EGF receptor blocking antibody, however, completely prevented the decrease in secretion of apoB caused by IL-6. EGF receptor blocking antibody alone had no effect on apoB secretion.

Activation of EGF receptor by IL-6

As the blocking antibody to the EGF receptor competitively binds to the EGF receptor, preventing receptor tyrosine phosphorylation and receptor activation (20), we next addressed whether IL-6 activates the EGF receptor. Receptor activation as a result of ligand binding has been observed to cause a decrease in receptor affinity for ligands and a decrease in receptor protein leading to down-regulation of receptor binding (28, 29). We, therefore, estimated EGF receptor binding to EGF in the presence of IL-6 in CaCo-2 cells. CaCo-2 cells were incubated with IL-6 for 18 h. Iodinated EGF was then added at 4°C to the basolateral surfaces of cells in increasing concentrations and the binding of labeled EGF to cell surfaces was estimated after cross-linking of the receptor to its ligand and separation of proteins by SDS/PAGE. **Figure 1A** shows an autoradiogram of the

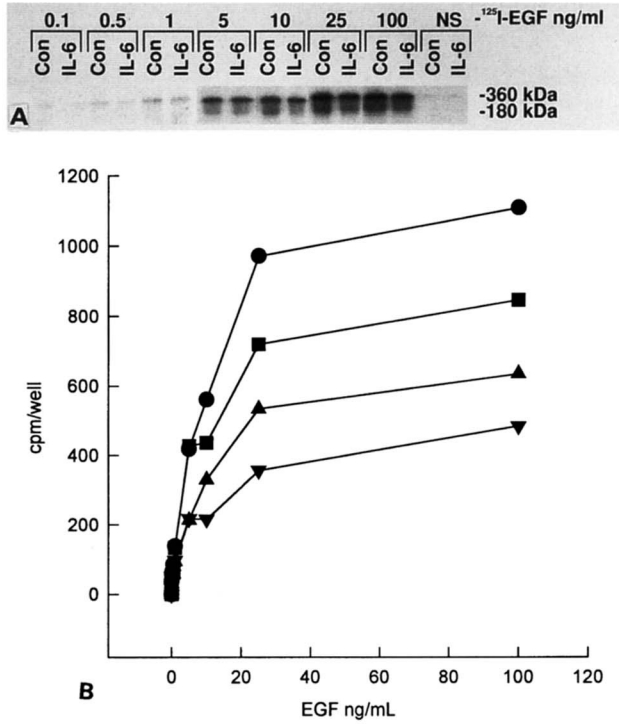

Fig. 1. Effect of IL-6 on ^{125}I-labeled EGF binding to cell surface EGF receptors. CaCo-2 cells were incubated for 18 h with 10 ng/mL of IL-6 in HBSS/0.1% BSA. Control cells received HBSS/0.1% BSA alone. At the end of the overnight incubation, cells were washed thoroughly with ice-cold M199 and the basolateral media were replaced with M199/0.1% BSA containing varying concentrations of ^{125}I-labeled EGF (0.01–100 ng/mL) (0.1 μCi/ng EGF). M199/0.1% BSA was added to the apical media. After 2.5 h incubation at 4°C, cells were washed thoroughly to remove unbound labeled EGF and incubated for an additional 2 h at 4°C with 1.3 mg/mL BS^3 to cross-link bound radiolabeled ligand to its receptor. Cells were scraped, lysed, and cell associated proteins were separated by SDS-PAGE as described in Methods. A: A representative autoradiogram of the gel demonstrating the 180 kDa EGF receptor-EGF complex and the 360 kDa dimerized receptor-ligand complex. B: Bands corresponding to the 180 kDa EGF receptor complexed to EGF as well as the 360 kDa dimerized receptor-ligand complex were cut from the gel and counted in a gamma counter. Data are presented as mean ± SEM of 6 wells. Control 360 kDa (●); IL-6 360 kDa (■); Control 180 kDa (▲); IL-6 180 kDa (▼).

separation on a polyacrylamide gel of labeled EGF cross-linked to its receptor. The EGF receptor–EGF complex migrates at approximately 180 kDa. The band at approximately 360 kDa represents the dimerized receptor–ligand complex as this band is abolished by the addition of the EGF receptor blocking antibody to cells (19). As shown in the last two lanes (NS), in the presence of excess unlabeled EGF, nonspecific binding of labeled EGF to cell surfaces is minimal. The data shown in Fig. 1B demonstrates the effects of IL-6 on the binding of EGF to its receptor on CaCo-2 cell surfaces. At each of the concentrations tested, cells incubated with IL-6 bound significantly less labeled EGF to their surfaces than control cells. Maximal binding was observed at 100 ng/mL, a concentration at which a 30% decrease in cell-surface receptor binding to iodinated EGF was observed in cells incubated with IL-6.

A decrease in EGF binding to cells incubated with IL-6 was associated with a 50% decrease in total receptor mass in cells as estimated by immunoblotting (data not shown). Thus IL-6 decreases EGF receptor binding and mass.

EGF receptor ligands and apoB secretion

Members of the EGF family bind to and activate the EGF receptor (24). It was postulated that IL-6 might induce the release of one or more of these receptor ligands which, in turn, activate the receptor causing a decrease in apoB secretion. To determine whether known ligands for the EGF receptor could decrease apoB secretion, cells were incubated with EGF, TGF-α, HB-EGF, or amphiregulin at concentrations observed to occur in the gastrointestinal tract (25). After the incubation, the amount of apoB secreted into the basolateral medium was estimated (**Fig. 2**). Compared to controls, apoB secretion was significantly decreased by the four different ligands. The EGF-mediated decrease in apoB secretion was completely prevented in the presence of the EGF receptor blocking antibody, suggesting that EGF receptor activation may play an important role in decreasing apoB secretion.

Effect of EGF neutralizing antibody

To address whether EGF was the effector molecule released by cells incubated with IL-6, we attempted to measure EGF in the basolateral medium after incubation with IL-6. After concentrating the conditioned media from cells incubated with IL-6, separating the proteins on polyacrylamide gels, and running an immunoblot with anti-EGF antibodies, we were unable to detect the presence of EGF. Alternatively, therefore, we used a neutralizing antibody to EGF to determine whether we could prevent the effects of IL-6. Cells were incubated for 18 h with IL-6, or IL-6 and an anti-EGF

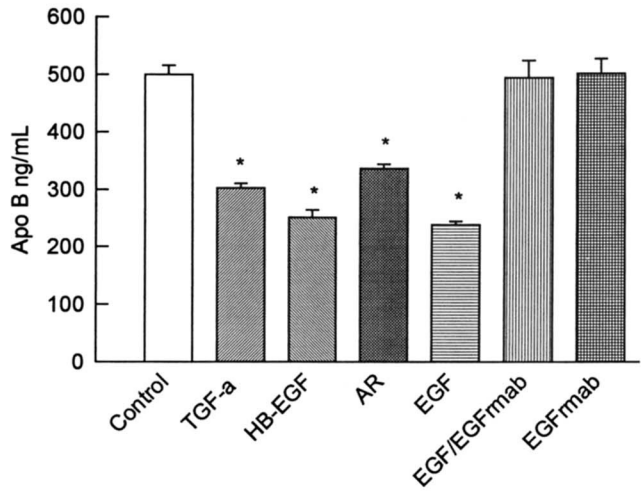

Fig. 2. Effect of various EGF receptor ligands on apoB secretion. CaCo-2 cells were incubated for 18 h with HBSS/0.1% BSA containing 0.5 ng/mL TGF-α, 5 ng/mL HB-EGF, or 50 ng/mL amphiregulin (AR), 0.5 ng/mL EGF, 0.5 ng/mL EGF with 5 μg/mL of EGF receptor blocking monoclonal antibody (EGFrmab), or 5 μg/mL of EGFrmab alone. Control cells received HBSS/0.1% BSA alone. The treatments were added to the basolateral chambers. Apical wells received M199. ApoB mass in the basolateral media was estimated by sandwich ELISA as described in Methods. Data from two experiments are presented as mean ± SEM of 6 wells. $*P < 0.01$ vs. control.

neutralizing antibody. Cell surface receptor binding of iodinated EGF was then estimated (**Table 2**). As shown in Table 2, IL-6 significantly decreased EGF binding by 22%. In the presence of the neutralizing antibody, however, binding of EGF to its receptor was similar to controls. The anti-EGF neutralizing antibody alone had no effect. These results suggest that IL-6 causes EGF receptor activation and down-regulation. This effect is likely secondary to the secretion of EGF itself or a protein that is recognized by the neutralizing antibody.

As both IL-6 and EGF decrease apoB secretion, we next addressed whether the inhibitory effect of IL-6 on apoB secretion was due to release of EGF. Cells were again incubated with IL-6 in the presence or absence of the neutralizing antibody to EGF, and the amount of apoB secreted into the basolateral medium was estimated (**Table 3**). The neutralizing antibody to EGF blocked the inhibitory effects of IL-6 on apoB secretion in a dose-dependent manner.

To address the specificity of this effect for EGF, cells were incubated with EGF, TGF-α, HB-EGF, or amphiregulin in the presence or absence of the anti-EGF neutralizing antibody. After the incubation, apoB mass secreted into the basolateral medium was measured. The results are shown in **Table 4.** The anti-EGF neutralizing antibody prevented the inhibitory effect of EGF on apoB secretion, but did not prevent the decrease in apoB secretion observed with TGF-α, HB-EGF, or am-

TABLE 2. Effect of IL-6 in the presence or absence of anti-EGF neutralizing antibody on cell surface EGF receptor binding of ^{125}I-labeled EGF

Treatment	^{125}I Bound to EGF Receptor
	cpm/well
Control	8859 ± 415
IL-6	6946 ± 249[a]
IL-6 + anti-EGF neutralizing antibody	8810 ± 176
Anti-EGF neutralizing antibody	8979 ± 76

CaCo-2 cells were incubated for 18 h with HBSS/0.1% BSA containing 10 ng/mL of IL-6 plus or minus 5 μg/mL of anti-EGF neutralizing antibody, or 5 μg/mL of the neutralizing antibody alone. Control cells were incubated with HBSS/0.1% BSA. The treatments were provided in the basal chambers, and M199 was added to the apical chambers. After 18 h, cells were washed with ice-cold M199 and incubated for 2.5 h at 4°C with cold M199/0.1% BSA containing 100 ng/mL of iodinated EGF (0.1 μCi/ng EGF) in the basal wells. M199/0.1% BSA was added to the apical wells. At the end of the incubation, excess unbound labeled EGF was thoroughly washed off from cell surfaces and the bound labeled EGF was cross-linked to cell surfaces by incubating cells for an additional 2 h at 4°C with 1.3 mg/mL of BS[3]. Cell lysates were separated on 8% polyacrylamide gels as described in Methods. Bands corresponding to the EGF receptor complexed to EGF as well as the higher molecular weight dimerized EGF receptor-ligand complex were excised from the gels and counted by gamma counting. Data are represented as mean ± SEM of 4 wells.

[a] $P < 0.01$ vs. control.

phiregulin. Thus, the effect of the anti-EGF neutralizing antibody is specific for EGF, implying that EGF and activation of the EGF receptor pathway are likely responsible for the inhibitory effects of IL-6 on apoB secretion.

Effect of EGF on apoB and triacylglycerol secretion

The inhibition of apoB secretion by EGF was further characterized. As shown in **Fig. 3A,** EGF decreased the basolateral secretion of apoB mass in a dose-dependent fashion. The inhibitory effect of EGF on apoB secretion was evident as early as 3 h and increased with the time of the incubation (Fig. 3B). The regulation of apoB secretion by EGF was not associated with changes in cell number or proliferation.

TABLE 3. Effect of IL-6 in the presence or absence of anti-EGF neutralizing antibody on the secretion of apoB mass

Treatment	ApoB
	ng/well
Control	508 ± 27
IL-6	305 ± 8[b]
IL-6 + 0.1 μg/mL anti-EGF neutralizing antibody	400 ± 10[a]
IL-6 + 1 μg/mL anti-EGF neutralizing antibody	388 ± 16[a]
IL-6 + 5 μg/mL anti-EGF neutralizing antibody	454 ± 5
IL-6 + 10 μg/mL anti-EGF neutralizing antibody	454 ± 24
5 μg/mL anti-EGF neutralizing antibody	484 ± 30

CaCo-2 cells were incubated for 18 h with HBSS/0.1% BSA containing 10 ng/mL IL-6 with or without 0.1–10 μg/mL anti-EGF neutralizing polyclonal antibody, or 5 μg/mL anti-EGF neutralizing antibody alone. Control cells were incubated with HBSS/0.1% BSA. All treatments were added to the basolateral chambers, whereas the apical chambers received M199. At the end of incubation, apoB mass in the basolateral media was analyzed as described in Methods. Data from two representative experiments are shown as mean ± SEM, n = 6.

[a] $P < 0.05$; [b] $P < 0.01$ vs. control.

TABLE 4. Effect of EGF receptor ligands on the secretion of apoB in the presence of anti-EGF neutralizing antibody

Treatment	ApoB
	ng/well
Control	484 ± 41
Anti-EGF neutralizing antibody	462 ± 29
EGF	204 ± 27[b]
EGF + anti-EGF neutralizing antibody	449 ± 25
TGF-α	293 ± 1[a]
TGF-α + anti-EGF neutralizing antibody	293 ± 13[a]
HB-EGF	243 ± 10[b]
HB-EGF + anti-EGF neutralizing antibody	289 ± 13[a]
Amphiregulin (AR)	326 ± 17[a]
AR + anti-EGF neutralizing antibody	332 ± 22[a]

CaCo-2 cells were incubated as described for Fig. 2 with the same concentrations of EGF, TGF-α, HB-EGF, and amphiregulin in the presence or absence of 5 μg/mL of anti-EGF neutralizing antibody, or with 5 μg/mL of anti-EGF neutralizing antibody alone. Control cells received HBSS/0.1% BSA. The accumulation of apoB in the basolateral media was assayed by sandwich ELISA as described in Methods. Data are represented as mean ± SEM of 6 wells.

[a] $P < 0.05$; [b] $P < 0.01$ vs. control.

Previously, we demonstrated that IL-6 also decreased triacylglycerol secretion by CaCo-2 cells (14). To address whether EGF was also responsible for this effect, cells were incubated with IL-6 or IL-6 and anti-EGF neutralizing antibody in the presence of 1 mM oleic acid to drive triacylglycerol-rich lipoprotein synthesis and secretion (30). These results are shown in **Table 5.** IL-6 decreased triacylglycerol secretion by 39%. In the presence of increasing concentrations of the neutralizing antibody, however, the decrease in triacylglycerol secretion was partially prevented and at 10 ng/mL the secretion was similar to controls.

The effect of EGF itself on triacylglycerol secretion was also determined. Compared to control cells, in the presence of 1 mM oleic acid, EGF, at 5 ng/mL, a concentration likely to occur in the gut (25), inhibited the basolateral secretion of triacylglycerol mass by 41% (19.75 μg/mg protein versus 11.71 μg/mg protein). Cellular triacylglycerol mass was not altered by EGF.

A decrease in the synthesis and secretion of newly synthesized apoB by IL-6 can be prevented by the anti-EGF neutralizing antibody (data not shown). To address whether EGF also interferes with the synthesis and secretion of newly synthesized apoB, CaCo-2 cells were incubated for 18 h with 5 ng/mL EGF. Cells were then incubated for 4 h, in the continued presence of the treatments, with [^{35}S]methionine. The incorporation of label into cellular apoB and apoB secreted into the ba-

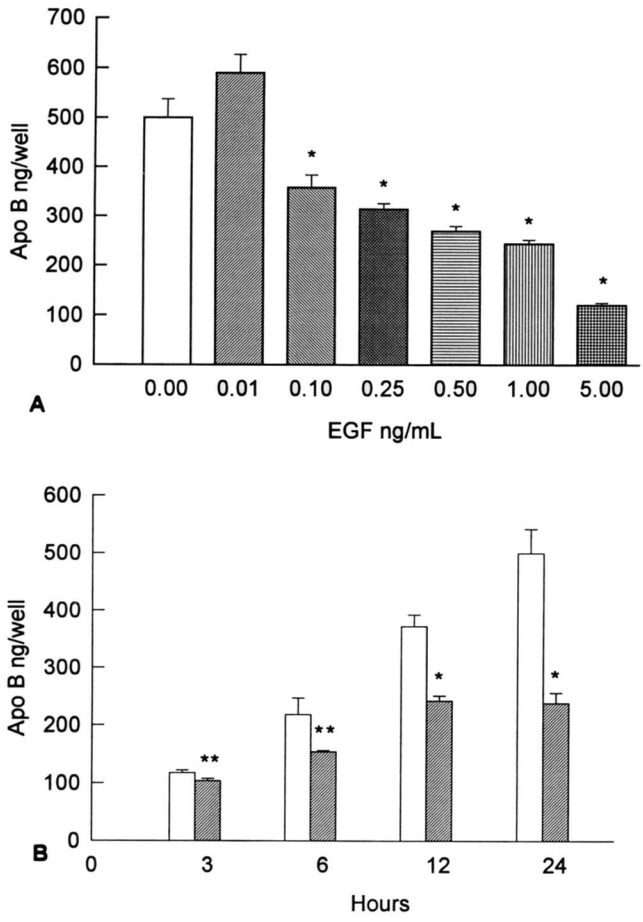

Fig. 3. Concentration and time-dependent effects of EGF on apoB secretion. CaCo-2 cells were incubated for 18 h with HBSS/0.1% BSA containing 0–5 ng/mL of EGF (A), or for 3–24 h with HBSS/0.1% BSA containing 5 ng/mL of EGF (hatched bars) or HBSS/0.1% BSA alone (open bars) (B). All treatments were provided as described for Table 1. ApoB mass in the basolateral media was estimated by sandwich ELISA as described in Methods. The data are presented as mean ± SEM of 6 wells and represent two separate experiments. $^{**}P < 0.05$, and $^{*}P < 0.01$ vs. control.

solateral medium was estimated after immunoprecipitation and polyacrylamide gel electrophoresis. **Figure 4** shows these results. ApoB synthesis was calculated by estimating the total amount of radiolabeled protein immunoprecipitated from both cells and media. Similar to our previous observations with IL-6, compared to control cells, cells incubated with EGF synthesized and secreted less apoB-100. In response to EGF, the fraction of newly synthesized apoB-100 secreted was dramatically reduced compared to control cells (14% versus 47%, respectively). The synthesis and secretion of apoB-48, however, was unaltered by EGF. EGF did not alter total protein synthesis compared to control cells (14.63 ± 0.6 million cpm/well in control cells and 14.96 ± 1.51 cpm/well in EGF-treated cells after a 4-h pulse).

To determine whether changes in apoB secretion observed in response to IL-6 and EGF could be secondary to lipoprotein re-uptake after secretion, ^{125}I-labeled LDL was added to the basolateral medium of cells exposed to cytokines. After 18 h of incubation, less than 0.02% of added LDL was degraded by control cells or cells incubated with IL-6 or EGF. The results suggest, therefore, that the accumulation of apoB in the basolateral media reflects changes in secretion and not changes in post-secretory re-uptake.

As observed previously (14), the abundance of apoB mRNA in cells incubated with IL-6 was decreased by 50% in comparison to control cells. In cells incubated with EGF, however, the levels of apoB mRNA were unaltered.

TABLE 5. Effect of IL-6 in the presence or absence of anti-EGF neutralizing antibody on the secretion of triacylglycerol mass

Treatment	Triacylglycerol Mass
	μg/ mg protein
Control	27 ± 1
IL-6	16 ± 0.5[b]
IL-6 + 0.1 μg/mL anti-EGF neutralizing antibody	18 ± 1[b]
IL-6 + 1 μg/mL anti-EGF neutralizing antibody	20 ± 1[b]
IL-6 + 5 μg/mL anti-EGF neutralizing antibody	21 ± 1[b]
IL-6 + 10 μg/mL anti-EGF neutralizing antibody	24 ± 2[a]

CaCo-2 cells were incubated for 18 h with 10 ng/mL of IL-6 with or without 0.1–10 μg/mL anti-EGF neutralizing antibody in M199/0/1% BSA added to the basal chambers. Control cells received M199/0.1% BSA. M199 containing 1 mM oleic acid complexed to 0.25 mM BSA was added to the apical wells. Lipids in basolateral media were extracted with chloroform–methanol 2:1 (v/v) and triacylglycerol mass was determined as described in Methods. Mean ± SEM of 6 wells is presented.

$^{a}P < 0.05$; $^{b}P < 0.01$ vs. control.

DISCUSSION

In a previous study performed in CaCo-2 cells, we examined the effects of pro-inflammatory cytokines, TNF-α, IL-1β and IL-6, on the synthesis and secretion of triacylglycerols and apoB (14). Individually, each of these cytokines inhibited apoB and lipid transport. IL-6, however, appeared to be the most potent of the cytokines in interfering with triacylglycerol and apoB secretion. In the present study, we addressed a possible mechanism by which IL-6 might mediate the regulation of lipid transport.

In addition to its recognized role in the immune-mediated removal of infectious pathogens, IL-6 is also believed to be involved in promoting tissue repair after infection (31). Likewise, in response to injury in intestinal epithelial cells, EGF or related proteins that bind to the EGF receptor are thought to play a role in epithelial cell restitution (25–27). Involvement of the EGF recep-

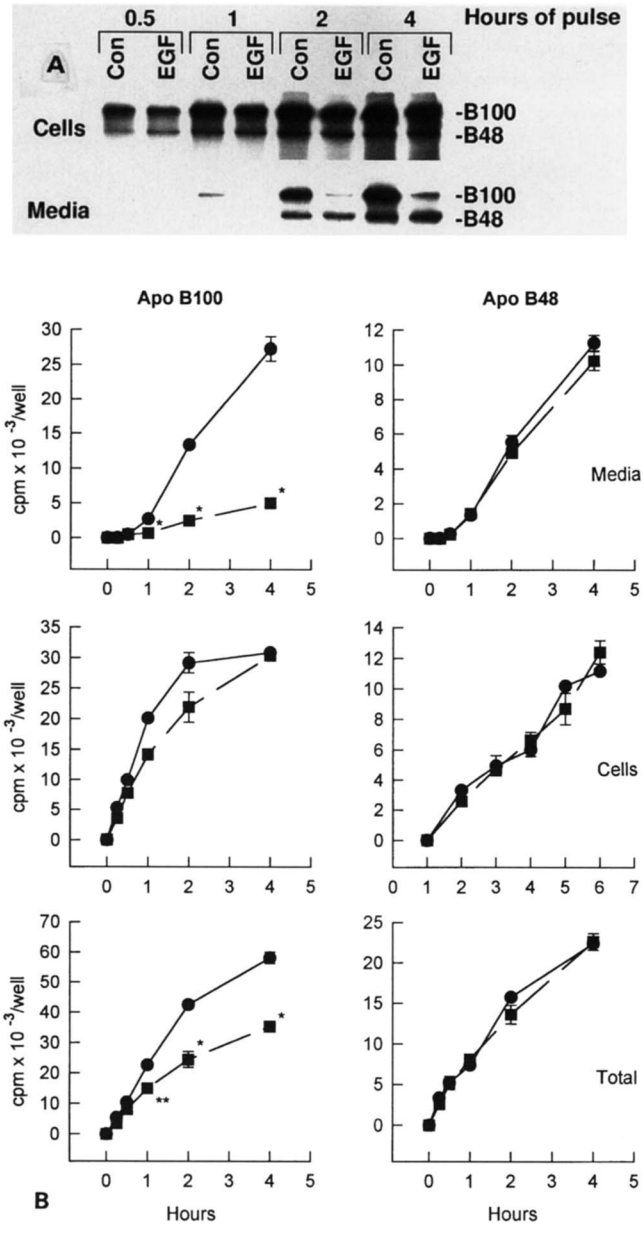

Fig. 4. Effect of EGF on the rate of synthesis and secretion of apoB. CaCo-2 cells were incubated for 18 h with M199/0.1% BSA containing 5 ng/mL EGF. Control cells were incubated with M199/0.1% BSA alone. The treatments were added to basolateral wells, whereas M199 was added to the apical wells. After 1 h of incubation with methionine-free M199 containing the respective treatments, 100 μCi of [^{35}S]methionine was added per well to the apical wells in the continued presence of the treatments. At the indicated times, apoB was immunoprecipitated from basolateral media and cell lysates and separated by PAGE as described in the Methods. A: An autoradiogram of a representative gel demonstrating the migration of apoB-100 and B-48 is shown. B: Bands representing apoB-100 and B-48 were cut from the gel and counted by liquid scintillation counting. Results from one representative experiment out of three are expressed as mean ± SEM of 3 wells. $^{**}P < 0.05$, and $^{*}P < 0.01$ vs. control.

tor pathway in cytokine-mediated intestinal cell proliferation has been previously observed (32, 33). Because IL-6 (3–5) and EGF (34–36) are both found in inflamed intestinal mucosa, we questioned whether EGF may act as a mediator for the effects of IL-6 on lipid transport. We addressed this possibility in several ways. Preventing EGF receptor occupancy or interfering with the ability of EGF to bind to its receptor negated the inhibitory effects of IL-6 on apoB and triacylglycerol secretion. In addition, EGF and other molecules known to bind to the EGF receptor all decreased apoB secretion, similar to the effects of IL-6. Moreover, IL-6 activated CaCo-2 cell EGF receptors. The combined results of these experiments suggest that EGF or a related protein is released into the basolateral medium of CaCo-2 cells incubated with IL-6. The ligand then binds to the EGF receptor resulting in a decrease in triacylglycerol and apoB secretion. Despite the use of immunoassay techniques, we were unable to measure the release of EGF into the basolateral medium, which, unfortunately, has been the experience of other investigators as well (37). We cannot conclude with certainty, therefore, that the ligand being released by CaCo-2 cells is EGF itself. What can be concluded, however, is that the ligand released in response to IL-6 binds to the EGF receptor to mediate its effect.

EGF is a likely candidate, however, as it is ubiquitous within the intestinal tract, being secreted by salivary glands, Brunner's glands, and the pancreas (25). Moreover, its expression in the intestinal mucosa is increased after injury (34–36). Increased amounts of EGF have been found in cells surrounding ulcer margins and in mucosal biopsies from patients with peptic ulcers or Crohn's disease (34). Moreover, the administration of EGF to animals in which ulcers and/or colitis were experimentally induced resulted in improved wound healing (38–40).

The predominant biological functions of EGF on the small intestinal enterocyte are mediated by basolateral EGF receptors and include stimulation of DNA synthesis and growth (41). In addition, EGF is thought to protect the epithelial lining of the mucosa from injury by inhibiting gastric acid secretion in the stomach (41), increasing the synthesis of mucopolysaccharides, prostaglandins, and extracellular matrix components (25), and by enhancing mobility of enterocytes over denuded surfaces (37). Other effects of EGF such as modulation of small intestinal transport processes might also play a role in the restoration of epithelial cell function after injury. EGF has been observed to increase the uptake of simple sugars such as glucose (42, 43), galactose (44), and of electrolytes (43), and glycine (44) from the small intestines of rats. Little is known, however, regarding the effects of EGF on secretory processes of the enterocyte. As is the case with normal intestinal cells (45),

CaCo-2 cells express receptors for EGF predominantly on their basolateral surfaces (19). Our data would suggest that EGF or a related protein might bind to these receptors and alter normal lipid transport by the gut. In fact, it would make good sense that in response to an inflammatory reaction and the local release of IL-6, the secretion of intestinal EGF might protect the organism by diverting metabolic processes away from transport and secretion of nutrients and, instead, providing a stimulus for cellular restitution.

Not all the effects we previously observed with IL-6 on triacylglycerol and apoB secretion were similarly reproducible by EGF. As observed with IL-6, EGF also decreased the synthesis and fractional secretion of apoB. Unlike IL-6, however, which decreased the secretion of both newly synthesized apoB-100 and B-48, EGF did not alter the secretion of apoB-48 while at the same time interfering with apoB-100 secretion. In addition, EGF did not alter apoB mRNA levels. We (46, 47) and others (48) have observed modest differences in how a cell processes apoB-48 versus apoB-100. For example, in CaCo-2 cells, apoB-48 is degraded at rates that differ from those of apoB-100. Presently, we are not certain whether these differences observed in cellular processing of apoB-48 and apoB-100 have physiological relevance or whether the differences are related to the experimental design or cell system used.

In the only other studies investigating the regulation of intestinal apoB secretion by EGF, Levy and his colleagues (49, 50) found that EGF increased the secretion of apoB-48 in lipoproteins of $d<0.97$ g/mL but decreased apoB-100-containing lipoproteins of $d<1.006$ g/mL. These studies were performed in fetal explant cultures incubated for 48 h with concentrations of EGF (25–100 ng/mL) that were considerably higher than those used in the present study. Moreover, contrary to the recognized mitogenic effect of EGF, these investigators found a decrease in protein synthesis in explants incubated with the growth factor. In the present study, we found no alteration in cellular proliferation or protein synthesis using more physiological concentrations of EGF. Blake, Vidmar, and Melchior (51) found that EGF decreased apoB-100 secretion in primary hepatocyte cultures. The combined data would strongly suggest, however, that EGF does alter lipoprotein transport from both liver and intestine.

In CaCo-2 cells, apoB secretion is dependent upon cell maturity. Cells that are not confluent nor fully differentiated secrete very little apoB into the medium (52). Editing of apoB message is also limited in undifferentiated cells (53, 54). Soon after confluency, however, when cells are taking on morphological and biochemical characteristics of mature intestinal absorptive cells, both apoB secretion and editing increase, reaching a plateau 14–21 days after confluency (52–54). EGF, because of its known mitogenic potential, could have altered lipoprotein secretion in CaCo-2 cells by influencing cell proliferation and or cell maturity. This was not the case, however, in the present study. With the concentrations of EGF used, neither cell proliferation nor total protein synthesis was altered. Moreover, in results not shown, IL-6 and EGF had no effects on the activities of alkaline phosphatase or on the morphological characteristics of CaCo-2 cells that are used as markers for cell maturity.

Cellular protein phosphorylation regulates a variety of cellular processes including secretion. In a previous report on CaCo-2 cells, we demonstrated that apoB secretion was regulated by altering the phosphorylation state of the cell (47). In that study, evidence was provided for the phosphorylation of apoB-48 but not for apoB-100. The data suggested, however, that the secretion of apoB was not dependent upon its phosphorylation state but was likely related to complex metabolic processes occurring within cells when several intracellular proteins remained phosphorylated. The EGF receptor is a tyrosine kinase and when activated results in the phosphorylation of numerous intracellular proteins. It would be expected, therefore, that in response to IL-6, which we have shown activates the EGF receptor in CaCo-2 cells, the protein phosphorylation state of the cell would be altered. Whether this presumed change in cellular phosphorylation results in a decrease in apoB secretion is presently being pursued.

It is appreciated that malabsorption can occur in the early stages of celiac disease when intraepithelial lymphocytes are present but before gross changes in the morphology of the small intestine are recognized (55). We would postulate that the release of cytokines, particularly IL-6, from these early inflammatory cells, either in autocrine or paracrine fashion, might contribute to the malabsorption seen in these individuals by interfering with the transport of lipids by the small intestinal enterocyte.

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