

## **Research Communication**

# **Effects of Epidermal Growth Factor and Linoleic Acid on Lipid Contents in Human Intestinal C2BBE1 Cells**

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### **Summary**

Epidermal growth factor (EGF) was reported to regulate triacyl glycerol synthesis in various cells. Linoleic acid and its metabolites were thought to modulate the signal transduction of growth factors. This study determined whether linoleic acid regulated the effect of EGF on lipid contents in human intestinal C2BBE1 cells. Confluent cells were incubated with serum-free medium (control), EGF (45 ng/mL), linoleic acid (42  $\mu$ g/mL), or combined EGF (45 ng/mL) and linoleic acid (42  $\mu$ g/mL) for 48 h. The results showed EGF and linoleic acid significantly increased intracellular cholesterol and triglyceride levels compared with the control and combined groups. EGF was a more potent stimulator for triacyl glycerol synthesis in C2BBE1 cells than linoleic acid. However, intracellular cholesterol and triglyceride levels did not differ between the control and combined groups. The secretion of cholesterol and triglyceride into the medium by C2BBE1 cells did not differ among four groups. Both EGF and linoleic acid strongly stimulated the expression of EGF receptor mRNA in C2BBE1 cells at 48 h compared with the control and combined groups. Therefore, EGF and linoleic acid increased triacyl glycerol synthesis in C2BBE1 cells through stimulating the expression of EGF receptor mRNA. The effect of EGF and linoleic acid on this lipogenesis was reversed in the presence of both EGF and linoleic acid by downregulating the expression of EGF receptor mRNA.

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### **INTRODUCTION**

There is evidence that linoleic acid and its metabolites modulate the signal transduction of growth factors, such as epidermal growth factor (EGF), insulin, and insulin-like growth factor-1, in vitro (1–8). Previous studies showed that linoleic acid and its eicosanoid metabolites enhanced and sustained the mitogenic activity of EGF in fibroblasts and mammary epithelial cells (1–6). Additionally, EGF-stimulated biosynthesis of 13-hydroxyoctadecadienoic acid, the major lipoxygenase product, in Syrian hamster embryo fibroblasts was blocked by the inhibitors of EGF receptor tyrosine kinase activity, suggesting that EGF induction of the linoleic acid pathway was linked to the activation of the EGF receptor (3).

Epidermal growth factor, distributed widely in a variety of body fluid in the mammals (9), is secreted by the salivary glands, gastric mucosa, and duodenum (10). It was reported that EGF regulated the secretion and formation of lipids in various cells (11–13). The secretion of triglycerides in human colon adenocarcinoma Caco-2 cells was stimulated by EGF (11). However, EGF decreased the intracellular accumulation of triglycerides in hamster auricular sebocytes (12). The stimulation of triacyl glycerol synthesis by insulin in primary cultures of adult rat hepatocytes was also suppressed by EGF through the inhibition of malic enzyme activity (13).

The C2BBE1 cell line, cloned from human colon adenocarcinoma Caco-2 cells, has high homology in morphology compared with Caco-2 cells that was often used as an experimental model for the synthesis, absorption, or transport of macronutrients, minerals, vitamins, or drugs in the intestine (14, 15). It is unclear whether linoleic acid also serves a regulatory function in the action of EGF on lipogenesis in the intestinal cells. Therefore, the purpose of this study investigated the effects of EGF

and linoleic acid on lipid levels and EGF receptor expression in human intestinal C2BBE1 cells.

## EXPERIMENTAL PROCEDURES

### Cell Line and Culture

Human intestinal C2BBE1 cells (CCRC 60182, Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, R.O.C.) were grown in 90% Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum, 10  $\mu\text{g}/\text{mL}$  transferrin, and 1 mM sodium pyruvate at 37 °C in a humidified CO<sub>2</sub> incubator (95% air and 5% CO<sub>2</sub>). Upon approximately 80–90% confluency, the cells were subcultured using 0.25% trypsin and 0.02% EDTA. Prior to the addition of the treatment, the cells were grown to 100% confluency, overgrown for another 7 days, and synchronized by incubating in the basal medium (100% DMEM) for 24 h. The cells were then incubated with recombinant human EGF (45 ng/mL; 7.5 nM) (16), linoleic acid (42  $\mu\text{g}/\text{mL}$ ; 150  $\mu\text{M}$ ) (17) in physiological concentrations, or combined EGF (45 ng/mL) and linoleic acid (42  $\mu\text{g}/\text{mL}$ ) for 48 h. Conjugated 2 moles of  $\alpha$ -linoleic acid with 1 mole of bovine serum albumin (BSA) (Sigma L9530, Sigma-Aldrich, Inc., St. Louis, MO) was added to the medium to increase its water solubility. Each treatment was also added an equal amount (100 mg/mL) of BSA.

The cells and conditioned media were collected by triplet at 48 h. The cells were washed three times with phosphate-buffered saline (PBS), resuspended in PBS containing 100  $\mu\text{L}/10^7$  cells protease inhibitors [4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, transepoxysuccinyl-L-leucylamido (4-guanidino) butane, bestatin, leupeptin, and aprotinin, Sigma P8340, Sigma-Aldrich, Inc.], and ultrasonicated for 5–10 s to break the membrane for further analyses.

### Endogenous EGF Secretion

Prior to the addition of the treatment, endogenous EGF secretion into the conditioned medium was measured at 450 nm (18) by a commercial EGF immunoassay kit (Quantikine, DEQ00, Research and Diagnostics Systems, Inc., Minneapolis, MN). The serum-free conditioned medium (200  $\mu\text{L}$ ) was incubated with EGF antibody coated in a 96-well plate for 2 h at room temperature, washed 3 times with wash buffer, and then incubated with 200  $\mu\text{L}$  polyclonal EGF antibody conjugated to horseradish peroxidase for 1 h. After several washes, samples were incubated with 200  $\mu\text{L}$  substrate (tetramethylbenzidine:H<sub>2</sub>O<sub>2</sub> = 1:1) for 20 min. The reaction was terminated by 50  $\mu\text{L}$  of 1 M sulfuric acid. EGF levels were determined at 450 nm and corrected at 540 nm using an ELISA reader (Multiskan RC, Labsystems, Helsinki, Finland).

### Lipid Analyses

Total cholesterol and triglyceride levels in C2BBE1 cells and the conditioned medium were measured spectrophotometrically

at 490 nm (U2000 double-beam spectrophotometer, Hitachi, Tokyo, Japan) by cholesterol oxidase/*p*-aminophenazone (CHOD-PAP) and glycerol-3-phosphate oxidase/*p*-aminophenazone (GPO-PAP) methods, respectively, using commercial kits (Randox Laboratories Ltd., Antrim, UK). Total lipid levels were measured spectrophotometrically at 540 nm using a commercial kit (Randox Laboratories Ltd.). Cholesterol and triglyceride levels were expressed as the percentage of total lipids.

### RNA Extraction and Quantitation

The cells (10<sup>7</sup>) were lysed by 1 mL TRIZOL reagent (Life Technologies GIBCO BRL, Taipei, Taiwan, R.O.C.), and mixed with 200  $\mu\text{L}$  chloroform. After centrifugation at 12,000  $\times g$  for 15 min at 4 °C, RNA was separated in the upper aqueous phase. The aqueous phase containing RNA was mixed with 500  $\mu\text{L}$  of cold 100% isopropanol for 10 min. The RNA pellet was then washed with 1 mL of cold 75% ethanol, air dried, and rehydrated in 10 to 20  $\mu\text{L}$  of 0.1% diethylpyrocarbonate-water. The concentration and purity of total cellular RNA were determined spectrophotometrically by the absorbance at 260 nm and by the ratio of the absorbance at 260 nm to that at 280 nm, respectively.

### Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The level of EGF receptor mRNA was determined by RT-PCR. Total cellular RNA (5  $\mu\text{g}$ ) as a template mixed with 0.5  $\mu\text{g}$  oligo(dT)<sub>12–18</sub> was heated at 70 °C for 10 min, and chilled on ice at least for 1 min. The RNA and primer mixture was incubated with 2  $\mu\text{L}$  of 10  $\times$  PCR buffer, 2  $\mu\text{L}$  of 25 mM MgCl<sub>2</sub>, 1  $\mu\text{L}$  of 10 mM dNTP mix, and 2  $\mu\text{L}$  of 0.1 M dithiothreitol at 42 °C for 5 min, and then incubated with 200 units of SUPERSCRIPT II reverse transcriptase (Life Technologies GIBCO BRL) at 42 °C for 50 min. The reaction was terminated at 70 °C for 15 min. The mixture was incubated with RNase H at 37 °C for 20 min prior to the amplification of the target cDNA. The cDNA (2  $\mu\text{L}$ ) was incubated with 1  $\times$  PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 1.25 units Taq DNA polymerase (Life Technologies GIBCO BRL), and 0.2  $\mu\text{M}$  primer of EGF receptor or  $\beta$ -actin (Life Technologies GIBCO BRL) at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s for 40 cycles. The 20-mer sequence of EGF receptor primer was 5'-ACCAGAGTGATGTCTGGAGC-3' for sense strand and 5'-GATGAGGTACTCGTCGGCAT-3' for antisense strand. The 20-mer sequence of  $\beta$ -actin primer was 5'-CGGATGTCCACGTCACACTT-3' for sense strand and 5'-GTTGCTATCCAGGCTGTGCT-3' for antisense strand. The PCR product for EGF receptor and  $\beta$ -actin was 367 and 469 bp, respectively. The amplified products were separated by 2% agarose gel electrophoresis, and visualized by staining with ethidium bromide under ultraviolet light. The bands were quantitated by an image analysis system (Gel analysis system, EverGene Biotechnology, Taipei, Taiwan, R.O.C.) and Phoretix 1D Lite software (Phoretix International Ltd., Newcastle upon Tyne, UK).

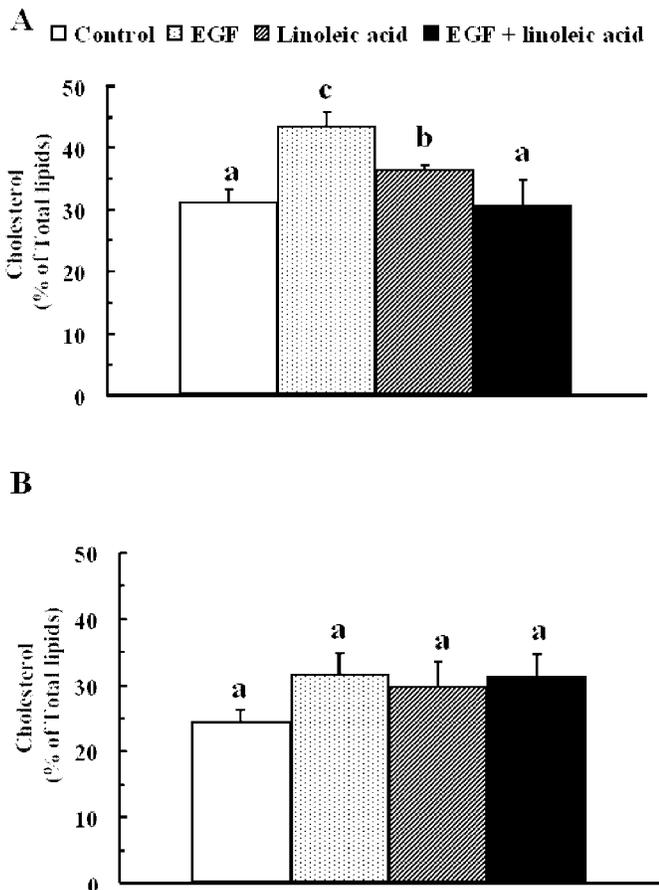
### Statistical Analysis

Data are expressed as the mean  $\pm$  SD. All data were analyzed by Statistical Analysis System (SAS) software (v6.12, SAS Institute Inc., Cary, NC). Two-way ANOVA and Fisher's least significant difference test were used to determine the effect of EGF and linoleic acid on lipid levels at different times. Statistical significance was assigned at the 0.05 level.

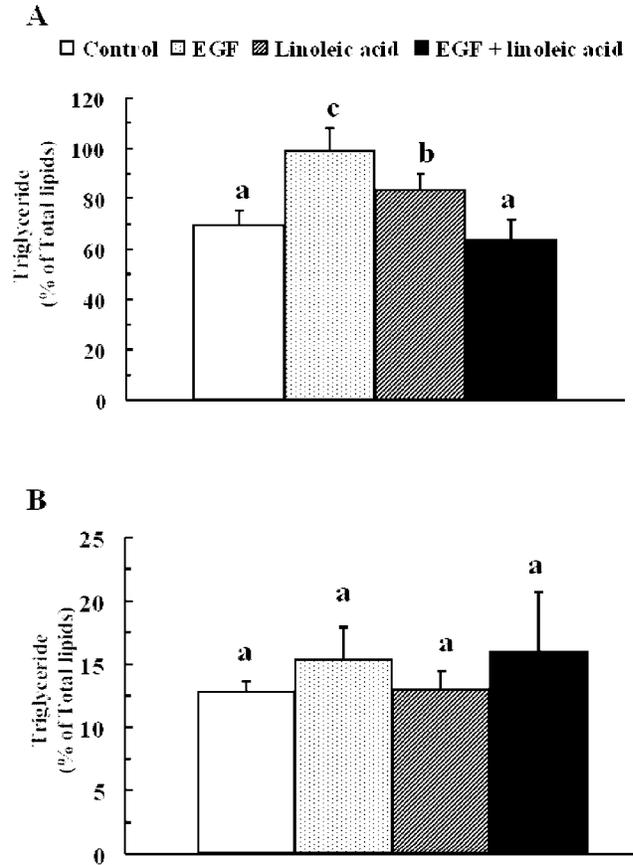
## RESULTS

### Endogenous EGF Secretion and Lipid Levels

Endogenous EGF secretion into the conditioned medium of C2BBE1 cells was  $0.12 \pm 0.01$  pg/mL. Because the secretion of cholesterol into the medium by C2BBE1 cells was undetectable in all groups at 24 h, lipid levels were measured after incubation for 48 h. Intracellular levels of cholesterol (Fig. 1A)



**Figure 1.** Intracellular (A) and secreted (B) cholesterol levels in C2BBE1 cells after the addition of 45 ng/mL recombinant human EGF, 42  $\mu$ g/mL linoleic acid, or combined EGF (45 ng/mL) and linoleic acid (42  $\mu$ g/mL) for 48 h. Without EGF or linoleic acid was the control group. Data are means  $\pm$  SD ( $n = 3$ ). Means not sharing a letter differ significantly ( $P < 0.05$ ) by Fisher's least significant difference test.

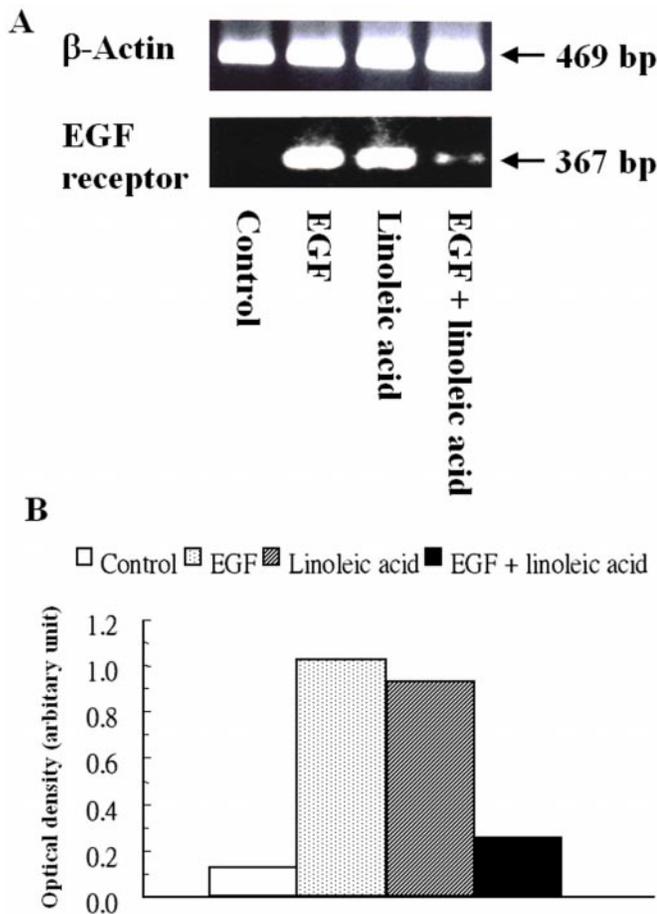


**Figure 2.** Intracellular (A) and secreted (B) triglyceride levels in C2BBE1 cells after the addition of 45 ng/mL recombinant human EGF, 42  $\mu$ g/mL linoleic acid, or combined EGF (45 ng/mL) and linoleic acid (42  $\mu$ g/mL) for 48 h. Without EGF or linoleic acid was the control group. Data are means  $\pm$  SD ( $n = 3$ ). Means not sharing a letter differ significantly ( $P < 0.05$ ) by Fisher's least significant difference test.

and triglyceride (Fig. 2A) in the EGF and linoleic acid groups significantly increased ( $P < 0.05$ ) compared with the control and combined groups at 48 h. The EGF group had significantly higher intracellular levels of cholesterol (Fig. 1A) and triglyceride (Fig. 2A) than the linoleic acid group ( $P < 0.05$ ). However, intracellular cholesterol and triglyceride levels did not differ between the control and combined groups. Additionally, the secretion of cholesterol (Fig. 1B) and triglyceride (Fig. 2B) into the medium by C2BBE1 cells did not differ in all groups.

### EGF Receptor mRNA Expression

The level of EGF receptor mRNA determined by RT-PCR was undetectable in the control group at 48 h (Fig. 3A). The expression of EGF receptor mRNA was strongly stimulated to 7- to 8-fold of the control in the EGF and linoleic acid groups (Fig. 3B). However, the level of EGF receptor mRNA was only twofold of the control in the combined group.



**Figure 3.** The expression of EGF receptor mRNA determined by RT-PCR (A) and quantitated by the optical density ratio of EGF receptor to  $\beta$ -actin (B) in C2BBE1 cells after the addition of 45 ng/mL recombinant human EGF, 42  $\mu$ g/mL linoleic acid, or combined EGF (45 ng/mL) and linoleic acid (42  $\mu$ g/mL) for 48 h in C2BBE1 cells. Without EGF or linoleic acid was the control group. Samples were obtained from three independent experiments ( $n = 3$ ), and pooled together for analysis. The purified total cellular RNA (5  $\mu$ g) isolated from freshly harvested C2BBE1 cells was used as a template for reverse transcription by SUPERSCRIPT II reverse transcriptase. The synthesized cDNA was then amplified by PCR. The amplified products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The 469- and 367-bp positions represent  $\beta$ -actin and EGF receptor cDNA, respectively.

## DISCUSSION

Endogenous EGF secretion was much lower than exogenous EGF concentration, indicating that the influences of EGF resulted from the addition of EGF. Our data showed that both EGF and linoleic acid stimulated triacyl glycerol synthesis in C2BBE1 cells. A previous study found that EGF could enhance cholesterol synthesis via the stimulation of HMG CoA reductase

activity (19). Additionally, the incubation with EGF at a concentration of 1 nM (6 ng/mL) at least for 4 days significantly increased intracellular triglyceride content in mouse differentiated adipocytes (3T3-L1 cells) by stimulating acyl-CoA synthetase activity, which is the rate-limiting enzyme for adipogenesis (20). Because confluent Caco-2 monolayers primarily express intestinal fatty acid-binding protein for the uptake of long chain fatty acids (14), exogenous linoleic acid can serve as a substrate for lipid synthesis. Similarly, van Greevenbroek et al. demonstrated that the addition of  $^{14}$ C-linoleic acid was efficiently incorporated into triglycerides by Caco-2 cells (17). Our results showed neither EGF nor linoleic acid significantly affect the secretion of cholesterol and triglyceride into the medium by C2BBE1 cells. However, Levy et al. found that the addition of EGF (25, 50, and 100 ng/mL) significantly enhanced the release of triglyceride-rich lipoprotein by 25–40% in human fetal intestinal explants (21). More triglyceride was secreted into the medium during incubation with 1.0 mM linoleic acid than with 1.0 mM palmitic acid in Caco-2 cells (17).

Combined EGF and linoleic acid treatment did not affect intracellular levels and the secretion of cholesterol and triglyceride in C2BBE1 cells at 48 h. Contrast to the increases in intracellular cholesterol and triglyceride levels by EGF or linoleic acid treatment alone, it is suggested that the addition of linoleic acid may inhibit the action of EGF on lipid contents in C2BBE1 cells.

Our data demonstrated that both EGF and linoleic acid strongly stimulated the expression of EGF receptor mRNA in C2BBE1 cells at 48 h by RT-PCR analysis. Similar to our results, the addition of EGF increased the expression, the binding capacity, and tyrosine phosphorylation of the EGF receptor in confluent rat intestinal (IEC-6) cells (22). Previous studies found that 13(S)-hydroperoxyoctadecadienoic acid, a specific lipoxigenase metabolite of linoleic acid, stimulated EGF-dependent mitogenesis and upregulation of EGF-dependent tyrosine phosphorylation by inhibiting the dephosphorylation of the EGF receptor in Syrian hamster embryo fibroblasts (23, 24). It is speculated that the stimulation of EGF receptor expression by linoleic acid is regulated by the metabolite of linoleic acid. However, combined EGF and linoleic acid obviously decreased the expression of EGF receptor mRNA compared with EGF or linoleic acid alone, which was consistent with the findings of intracellular lipid levels. It is suggested that both EGF and linoleic acid may stimulate lipogenesis in C2BBE1 cells through modulating the expression of EGF receptor mRNA. The expression of EGF receptor mRNA was downregulated in the presence of both EGF and linoleic acid, resulting in an inhibition of lipogenesis in C2BBE1 cells. However, EGF was found to stimulate the conversion of exogenous linoleic acid to 13(S)-hydroxyoctadecadienoic acid in Syrian hamster embryo fibroblasts through the activation of the EGF receptor tyrosine kinase (25). Additionally, the proliferation of mammary epithelial cells induced by EGF was enhanced and sustained by linoleic acid and its eicosanoid metabolites (3). These results suggest that combined EGF and linoleic acid may have a synergistic effect

on EGF-dependent signal transduction through the amplification of signals from the EGF receptor, which conflicted with the inhibitory effect of linoleic acid on EGF-modulated lipid contents in C2BBE1 cells. Therefore, it is presumed that linoleic acid and/or its metabolites as signal transducers up- or downregulate the expression of the EGF receptor in different cell types.

In conclusion, EGF and linoleic acid increased intracellular cholesterol and triglyceride levels in C2BBE1 cells through stimulating the expression of EGF receptor mRNA. However, combined EGF and linoleic acid reversed the action on lipogenesis in C2BBE1 cells through downregulating the expression of EGF receptor mRNA.

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