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# Preventive Effect of *t,t*-Conjugated Linoleic Acid on 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Inhibition of Gap Junctional Intercellular Communication in Human Mammary Epithelial MCF-10A Cells

Md. Abdur Rakib,[†] Young S. Kim,[†] Wook J. Jang,[†] Joung S. Jang,[‡] Seok J. Kang,[§] and Yeong L. Ha*,[†]

[†]Division of Applied Life Science (BK21 Program), Graduate School, and Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea

[‡]Department of Internal Medicine, Chung-Ang University College of Medicine, Seoul 156-576, Republic of Korea

[§]Department of Marine Life Science/Institute of Marine Industry, Gyeongsang National University, Tongyeong 650-160, Republic of Korea

**ABSTRACT:** The anti-tumor promotional effects of *t*9,*t*11-conjugated linoleic acid (*t*9,*t*11-CLA) and *t*10,*t*12-CLA were evaluated on the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inhibition of gap junctional intercellular communication (GJIC) in the human mammary epithelial cell line MCF-10A. The results were compared to those obtained from *c*9,*t*11-CLA, which is a more effective anti-tumor promoter on TPA-induced GJIC inhibition in MCF-10A cells than *t*10,*c*12-CLA. Cells were treated with 20 μM *t*9,*t*11-CLA, *t*10,*t*12-CLA, or *c*9,*t*11-CLA for 24 h followed by 60 nM TPA for 1 h. Both *t*9,*t*11-CLA and *t*10,*t*12-CLA equally protected MCF-10A cells from TPA-induced inhibition of GJIC with inferior efficacy to *c*9,*t*11-CLA.The protection was due to the ameliorated phosphorylation of connexin43 via suppression of extracellular signal-regulated kinases (ERK1/2) activation. Suppression of TPA-induced reactive oxygen species (ROS) generation by *t*9,*t*11-CLA and *t*10,*t*12-CLA was less effective, relative to *c*9,*t*11-CLA. The results suggest that the anti-promotional activities of *t*9,*t*11-CLA and *t*10,*t*12-CLA are equal but less potent than *c*9,*t*11-CLA in TPA-treated MCF-10A cells. The activity might be mediated by the attenuation of ROS production in MCF-10A cells by preventing the downregulation of GJIC during the cancer promotion stage.

**KEYWORDS:** *t,t*-Conjugated linoleic acid (CLA), human mammary epithelial cell line MCF-10A, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), gap junctional intercellular communication (GJIC), connexin43 (Cx43), reactive oxygen species (ROS)

## INTRODUCTION

Gap junctional intercellular communication (GJIC), involving the exchange of small hydrophilic ions and molecules of low molecular weight (<1 kD), such as calcium ion, cyclic AMP, and inositol triphosphate, to neighboring cells through a transmembrane channel, is essential to maintain cell homeostasis in multicellular organisms.[1] Gap junctions are composed of connexin (Cx) proteins;[2] of these, connexin43 (Cx43) is a major Cx present in many tissues, including normal human mammary epithelial cells.[3] GJIC plays a critical role in cell proliferation, tissue development, and differentiation.[4] GJIC is not only dysfunctional in most cancer cells[5] but has been linked to carcinogenesis, particularly in the tumor-promotion stage.[6]

Inhibition of GJIC is induced by many chemical tumor promoters[7] that exhibit cell, tissue, or organ specificity.[8] Furthermore, growth factors and several oncogenes have been shown to inhibit GJIC,[9] while anti-tumor-promoting agents and anticancer drugs can reverse the downregulation of GJIC.[10,11] GJIC is regulated by Cx, and the closure of gap junctions is particularly mediated by phosphorylation-related conformational changes of Cx43.[12,13] Previous studies have suggested that the inhibition of GJIC that is induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) involves the mitogen-activated protein kinase (MAPK) pathway.[14,15] Oxidative stress has also been strongly implicated in tumor promotion by epigenetic mechanisms, such as the activation of protein kinases and their inhibition of GJIC.[9,16]

Conjugated linoleic acid (CLA) is a group of positional (7,9; 8,10; 9,11; 10,12; 11,13; and 12,14) and geometrical (*cis,trans*; *trans,cis*; *cis,cis*; and *trans,trans*) isomers of octadecanoic acid (C18:2) with a conjugated double-bond system. Since its discovery in fried ground beef,[17] CLA has gained attention because of its important biofunctional activities, especially for its anticarcinogenic[18] and body fat reduction[19] properties. In synthetic CLA, *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers predominate and *t*9,*t*11-CLA and *t*10,*t*12-CLA are present as minor components.[20] The anti-carcinogenic effects of different CLA isomers have been evaluated in many human cancer cell lines and animal models. The various CLA isomers exhibit different anti-proliferative potentials mediated by an apoptotic mechanism.[18,21,22] Despite the plethora of data concerning the chemopreventive properties of CLA isomers, their effects on the GJIC-related, multi-step process of carcinogenesis[23] are unclear. Previously, we reported that *c*9,*t*11-CLA is more effective than *t*10,*c*12-CLA in the prevention of TPA-induced inhibition of GJIC.[24]

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The anti-carcinogenic effect of a mixture of *t,t*-CLA or *t*9,*t*11-CLA has been evaluated in many different cancer cell lines and using models of chemical-induced animal carcinogenesis. *t,t*-CLA significantly affects proliferation of MG-63 osteocarcoma cells[21] and MCF-7 breast cancer cells,[22] relative to *c*9,*t*11-CLA and *t*10,*c*12-CLA. In addition, *t*9,*t*11-CLA significantly inhibits the growth of Caco-2 colon cancer cells[25] and exerts strong anti-cancer activity in azoxymethane-induced colonic aberrant crypt foci in rats.[26] Therefore, the preventive effect of *t*9,*t*11-CLA and *t*10,*t*12-CLA on TPA-induced inhibition of GJIC in non-tumorigenic human mammary epithelial MCF-10A cells after treatment with TPA requires investigation.

The present study was performed to evaluate the anti-carcinogenic activity of *t,t*-CLA (*t*9,*t*11-CLA and *t*10,*t*12-CLA) in the TPA-induced inhibition of GJIC in MCF-10A cells, relative to *c*9,*t*11-CLA, which is the CLA isomer that strongly ameliorates the inhibition of TPA-induced GJIC. The present data indicate that *t*9,*t*11-CLA and *t*10,*t*12-CLA prevent TPA-induced inhibition of GJIC in MCF-10A cells but less than *c*9,*t*11-CLA.

**Table 1. Cytotoxic Effect of *t*9,*t*11-CLA and *t*10,*t*12-CLA Isomers on MCF-10A Cells**

| concentration ($\mu$M) | treatment[a] | | | |
|---|---|---|---|---|
| | LA | *c*9,*t*11-CLA | *t*9,*t*11-CLA | *t*10,*t*12-CLA |
| 0 | 100.0 ± 0.0 c | 100.0 ± 0.0 b | 100.0 ± 0.0 b | 100.0 ± 0.0 b |
| 10 | 112.1 ± 2.9[b,c] a | 105.5 ± 3.4 a | 110.7 ± 1.6 a | 109.2 ± 1.9 a |
| 20 | 105.5 ± 1.6 b | 101.1 ± 2.1 b | 103.9 ± 1.4 b | 102.2 ± 2.5 b |
| 30 | 90.2 ± 0.5 d | 76.6 ± 1.8 c | 81.1 ± 5.9 c | 85.8 ± 5.0 c |
| 40 | 59.2 ± 2.1 e | 49.1 ± 0.8 d | 55.8 ± 2.8 d | 51.8 ± 1.1 d |

[a] Cells were treated with 0, 10, 20, 30, or 40 $\mu$M concentrations of *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, and LA for 24 h. The cell viability was measured by the MTT assay. [b] Values are the relative cell viability against control cells (0 $\mu$M). [c] Values are the mean ± SD of triplication. Means with different lowercase letters are significantly different at $p < 0.05$ by Duncan's multiple range test.

## MATERIALS AND METHODS

**Reagents.** Dulbecco's modified Eagle's medium (DMEM)/F12 and penicillin−streptomycin were obtained from Gibco BRL (Rockville, MD). Heat-inactivated horse serum, hydrocortisone, recombinant human epidermal growth factor (EGF), insulin, phenylmethylsulfonyl fluoride (PMSF), sodium selenite, L-ascorbic acid, α-tocopherol phosphate, Lucifer yellow dilithium salt (LY), linoleic acid (LA, 99%), 2′,7′-dihydrodichlorofluorescein diacetate (DCFH-DA), *N*-(2-Hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), and TPA were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA), phosphate-buffered saline (PBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Amresco (Solon, OH). Radio-immunoprecipitation assay (RIPA) buffer and rabbit polyclonal antibodies for extracellular signal-regulated kinases (ERK1/2), pERK1/2, and anti-Cx43 were purchased from Cell Signaling Technology (Danvers, CO). Monoclonal anti-$\beta$-actin was purchased from Sigma-Aldrich. Goat anti-rabbit IgG and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used were of analytical grade.

**Preparation of CLA Isomers.** The *c*9,*t*11-CLA and *t*10,*c*12-CLA isomers were isolated from synthetic CLA methyl ester.[27] *c*9,*t*11-CLA and *t*10,*c*12-CLA were converted to *t*9,*t*11-CLA and *t*10,*t*12-CLA, respectively, by iodine treatment, using ultraviolet light followed by low-temperature crystallization at −80 °C.[28] The purity of the CLA isomers was 93.6, 90.5, and 94.5% for *t*9,*t*11-CLA, *t*10,*t*12-CLA, and *c*9,*t*11-CLA, respectively, when analyzed by gas chromatography.[27] The CLA isomers and LA were complexed with fatty-acid-free BSA, as previously described.[29]

**Cell Culture and Sample Treatment.** MCF-10A cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in DMEM/F12 supplemented with 5% heat-inactivated horse serum, 10 $\mu$g/mL insulin, 0.5 $\mu$g/mL hydrocortisone, 20 ng/mL EGF, 2 mM L-glutamine, and 100 $\mu$g/mL penicillin−streptomycin mixture at 37 °C in a humidified incubator containing 5% $CO_2$ and 95% air, as previously described.[24] In brief, cells grown to 80% confluence were dispersed by treatment with 0.25% trypsin−2 mM ethylenediaminetetraacetic acid (EDTA). The dispersed cells were collected by centrifugation (1000*g* for 10 min) and then incubated in cell culture dishes with DMEM/F12 containing 5% heat-inactivated horse serum for 24 h. These cells were grown to 80% confluence in DMEM/F12 medium supplemented with 5 $\mu$g/mL transferrin, 5 ng/mL sodium selenite, and 0.1 mg/mL BSA and were used for *t,t*-CLA experiments. CLA treatment used DMEM/F12 supplemented with 50 ng/mL L-ascorbic acid and 20 ng/mL α-tocopherol phosphate.

**Cytotoxicity Assay.** The cytotoxic effect of CLA isomers on MCF-10A cells was measured by an established MTT-based assay.[30] Briefly, $1 \times 10^4$ cells were seeded in wells of 96-well plates. After 24 h of incubation in serum-free DMEM/F12, the cells were then treated with different concentrations of CLA isomers and LA for 24 h. The cells were exposed to MTT solution (5 mg/mL in PBS) for 4 h. The MTT solution was removed, and 200 $\mu$L of dimethylsulfoxide was added to each well and mixed to dissolve the MTT formazan crystals formed by viable cells. The optical density of each well was measured at 570 nm using an Anthos 2020 microplate reader (Anthos labTech Instruments, Wals, Austria).

**GJIC Assay.** GJIC was measured by the scrape loading/dye transfer (SL/DT) technique.[24] Briefly, $1 \times 10^5$ cells were added to a 35 mm diameter cell culture dish (Nunc, Rochester, NY) and incubated in serum-free medium for 24 h. The cells were then incubated with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA for 24 h prior to 1 h of stimulation with 60 nM TPA. After incubation, cells were washed twice with 2 mL of PBS. LY (0.05%) was added to the washed cells, and scraping with a surgical steel scalpel at low light intensity was performed. After 3 min of incubation, the cells were washed 4 times with 2 mL of PBS. After the final wash, the cells were fixed with 4% paraformaldehyde. The dye-communicating cells perpendicular to the scraped lines were counted under an IX70 inverted fluorescence microscope equipped with DPC controller software (Olympus, Okaya, Japan).

**Western Blot Analysis.** Western blot analysis of Cx43, ERK1/2, and pERK1/2 was adapted as previously described.[24] Briefly, $1 \times 10^6$ MCF-10A cells were cultured in serum-free medium in a 100 mm diameter culture dish (Nunc) and treated with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA for 24 h prior to 1 h exposure of 60 nM TPA. Proteins were extracted from the harvested cells with RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $Na_2EDTA$, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM $\beta$-glycerophosphate, 1 mM $Na_3VO_4$, 1 $\mu$g/mL leupeptin, and 1 mM PMSF]. The protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA). The proteins in the cell lysate were separated by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred to a polyvinylidene fluoride membrane. Each blotted membrane was blocked with 0.1% Tween-20 Tris-buffered saline (TBST) containing 5% BSA and then probed with specific antibodies according to the instructions of

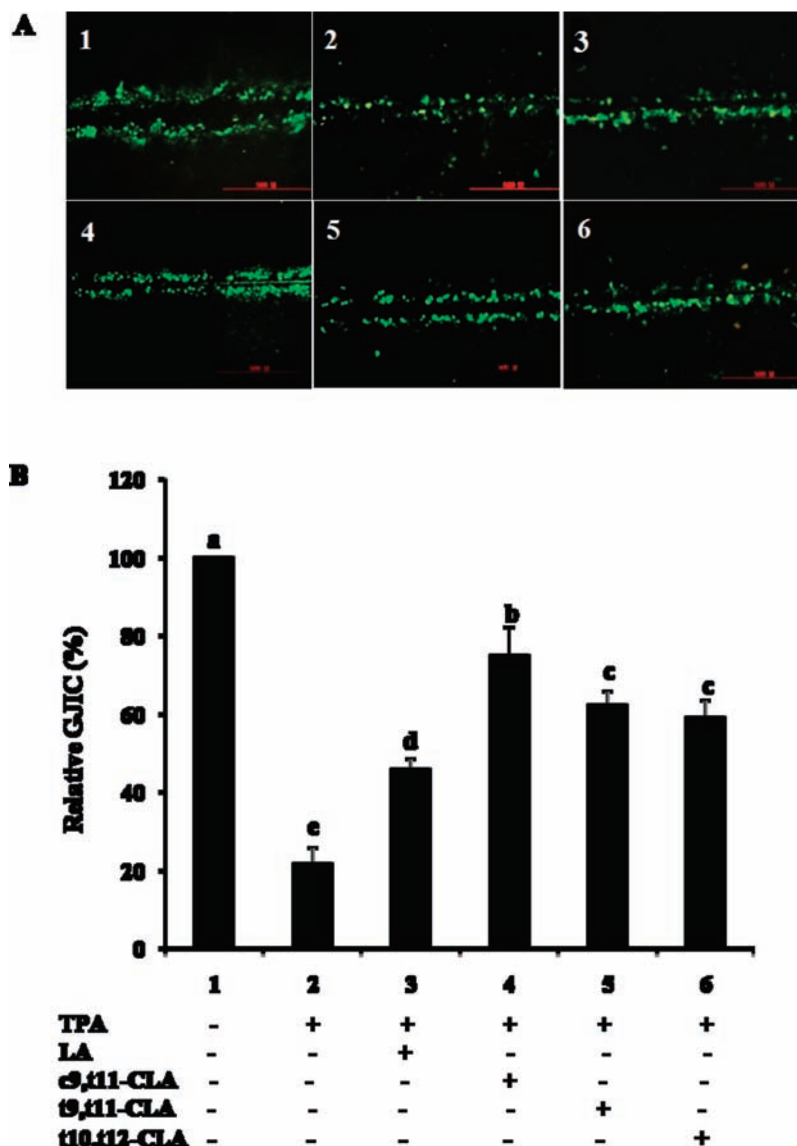


**Figure 1.** Effects of *t*9,*t*11-CLA and *t*10,*t*12-CLA on TPA-induced inhibition of GJIC in MCF-10A cells. MCF-10A cells were treated with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA for 24 h followed by 60 nM TPA for 1 h. GJIC was assessed using the SL/DT method. (A) Representative image of the following treatments: 1, untreated normal cells; 2, TPA only; 3, TPA and LA; 4, TPA and *c*9,*t*11-CLA; 5, TPA and *t*9,*t*11-CLA; and 6, TPA and *t*10,*t*12-CLA. The number of Lucifer yellow dye communicating cells was counted under an inverted fluorescence microscope. (B) Each value represents the mean ± SD of triplication. Means with different lowercase letters are significantly different at $p < 0.05$ by Duncan's multiple range test.

the manufacturer. Protein bands were visualized by an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL).

**Measurement of Intracellular Reactive Oxygen Species (ROS).** Intracellular ROS in MCF-10A cells was determined by DCFH-DA dye as previously described.[24] Cells ($1 \times 10^4$) were grown on glass coverslips (Lab Tek Chamber slide; Nunc) for 24 h in serum-free medium, washed with PBS, and incubated with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA for 24 h prior to 1 h of stimulation with 60 nM TPA. Cells were washed with PBS prior to the addition of 50 $\mu$M DCFH-DA and incubated for 30 min at 37 °C in the dark. After incubation, cells were washed with Locke's buffer [154 mM NaCl, 25 mM KCl, 2.3 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 8.6 mM HEPES, and 5.6 mM glucose at pH 7.4]. Cells were then analyzed for fluorescent images and enumeration of ROS-positive cells using a confocal microscope (FV-1000, Olympus, Japan) equipped with an argon laser, with an excitation and emission wavelength of 485 and 530 nm, respectively.

**Statistical Analysis.** Data are presented as the mean ± standard deviation (SD). Analysis was performed by one-way analysis of variation (ANOVA) followed by Duncan's multiple range test. $p < 0.05$ was considered as statistically significant.

## RESULTS

**Cytotoxicity of *t*,*t*-CLA Isomers on MCF-10A Cells.** Prior to the performance of further experiments, nontoxic levels of *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, and LA were determined on MCF-10A cells. No cytotoxicity was evident in the presence of up to 20 $\mu$M concentrations of each isomer and LA (Table 1). Subsequent experiments used 20 $\mu$M of the CLA isomers and LA.

***t*,*t*-CLA-Mediated Protection of TPA-Induced GJIC Inhibition in MCF-10A Cells.** The optimal condition for the induction

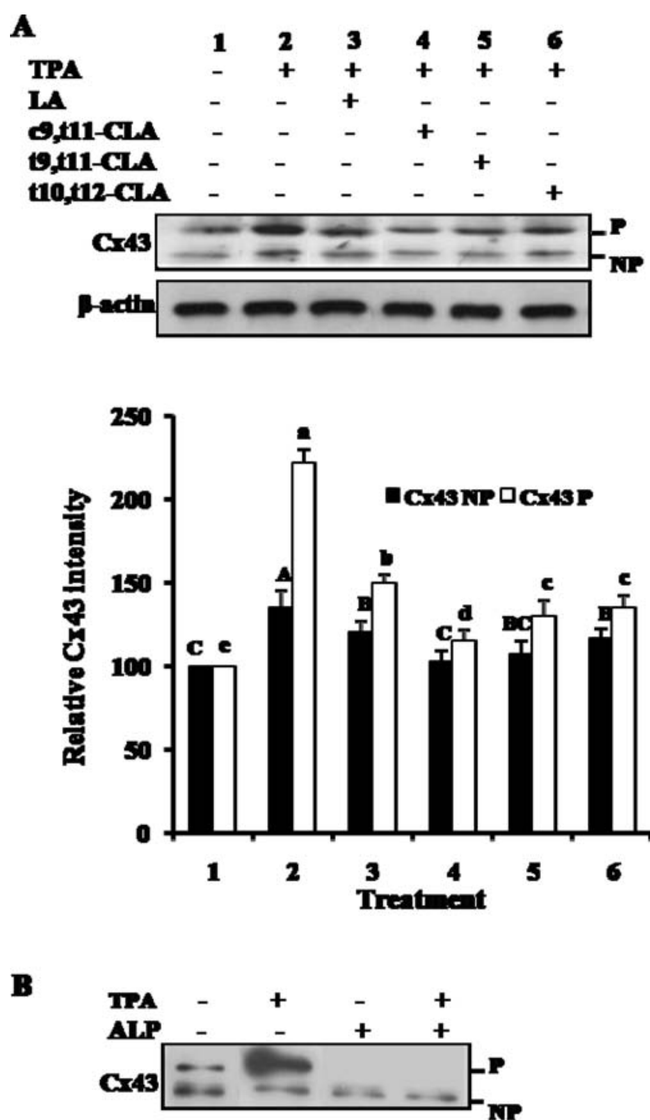


**Figure 2.** Effects of *t*9,*t*11-CLA and *t*11,*t*12-CLA on the TPA-induced Cx43 phosphorylation. (A) Western blot analyses of Cx43 phosphorylation in MCF-10A cells. Cells were treated with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA for 24 h followed by 60 nM TPA for 1 h. All treatments show two distinct Cx43 bands representing non-phosphorylated (NP) and phosphorylated (P) proteins. Intensities of each Cx43 band were quantified relative to untreated normal cells. Each value represents the mean ± SD of triplication. Means with different lowercase and capital letters are significantly different at $p < 0.05$ by Duncan's multiple range test. (B) Western blot analysis for dephosphorylation of the phosphorylated Cx43. Cells were stimulated with or without 60 nM TPA for 1 h. Protein lysate was treated with or without 40 units of alkaline phosphatase (ALP) (Roche Diagnostics, Indianapolis, IN) for 1 h at 37 °C before SDS−PAGE.

of GJIC inhibition in MCF-10A cells is 60 nM TPA for 1 h.[24] The effects of *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA are summarized in Figure 1. Untreated MCF-10A cells exhibited active GJIC. Treatment of cells with 60 nM TPA for 1 h resulted in a significant inhibition of GJIC (up to 78.2% relative to untreated normal cells). In contrast, treatment of cells with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA for 24 h followed by 60 nM TPA for 1 h resulted in a significant prevention of GJIC inhibition by TPA. In particular, *t*9,*t*11-CLA and *t*10,*t*12-CLA were inferior in preventing GJIC inhibition, as compared to *c*9,*t*11-CLA.

***t*,*t*-CLA-Mediated Suppression of TPA-Induced Cx43 Phosphorylation and ERK1/2 Activation in MCF-10A Cells.** The extent of phosphorylation of Cx43 is directly correlated to functional GJIC. TPA-mediated inhibition of GJIC in MCF-10A cells correlates with the phosphorylation of Cx43 and ERK. To investigate the mechanism by which *t*9,*t*11-CLA, and *t*10,*t*12-CLA isomers prevented the downregulation of GJIC, the levels and phosphorylation patterns of Cx43 were assessed by western blot analysis. Two distinct bands of Cx43 representing the non-phosphorylated (NP) and phosphorylated (P) states were detected in untreated MCF-10A cells (Figure 2A). To identify NP and P bands of Cx43, protein lysate was treated with calf intestinal alkaline phosphatase. This enzyme treatment resulted in the disappearance of the upper band but did not affect the lower band intensity on western blot (Figure 2B), indicating that the upper band represents the phosphorylated Cx43.

Densitometric analysis of the Cx43 protein revealed a significant induction of Cx43 phosphorylation in response to stimulation with 60 nM TPA for 1 h. Meanwhile, treatment with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA significantly ($p < 0.05$) decreased Cx43 phosphorylation relative to 60 nM TPA exposure only (Figure 2A). However, the protective efficacy of Cx43 phosphorylation by *t*9,*t*11-CLA and *t*10,*t*12-CLA isomers was significantly lower ($p < 0.05$) compared to that of *c*9,*t*11-CLA.

To investigate the molecular mechanism by which *t*9,*t*11-CLA and *t*10,*t*12-CLA isomers prevented TPA-induced inhibition of GJIC, the phosphorylation status of ERK1/2 was measured. Treatment with 60 nM TPA for 1 h induced ERK1/2 phosphorylation (panels A and B of Figure 3). However, treatment with 20 $\mu$M *t*9,*t*11-CLA, *t*10,*t*12-CLA, *c*9,*t*11-CLA, or LA significantly ($p < 0.05$) prevented the phosphorylation of ERK1/2. *t*9,*t*11-CLA and *t*10,*t*12-CLA were less effective in blocking the phosphorylation of ERK1/2, as compared to *c*9,*t*11-CLA (panels A and B of Figure 3).

**Attenuation of TPA-Induced Accumulation of Intracellular ROS Generation in MCF-10A Cells by *t*,*t*-CLA.** Accumulation of excessive ROS induces inhibition of GJIC, which is a prominent factor for carcinogenesis in normal cells.[9,16] In the present study, a DCFH-DA assay was used to investigate the generation of intracellular ROS in MCF-10A cells treated with TPA (panels A and B of Figure 4). The fluorescent intensity significantly ($p < 0.05$) increased in the TPA-treated cells in comparison to untreated normal cells, suggesting the generation of ROS. Meanwhile, pretreatment of *c*9,*t*11-CLA, *t*9,*t*11-CLA, and *t*10,*t*12-CLA significantly ($p < 0.05$) protected from the TPA-induced accumulation of intracellular ROS. The efficacy of *t*9,*t*11-CLA and *t*10,*t*12-CLA in protecting from intracellular ROS generation was inferior to that of *c*9,*t*11-CLA.

## DISCUSSION

The loss of gap junctions or impairment of their permeability have been observed in many neoplastic cells and cells treated with growth-promoting carcinogens and other agents.[31] A reversible disruption of GJIC plays a role during the tumor-promotion phase of carcinogenesis, and a stable downregulation of GJIC leads to the conversion of a premalignant cell to an invasive and metastatic cancer cell.[32] CLA and its isomers play a role of anticarcinogenic actions in tumor-initiation and tumor-promotion

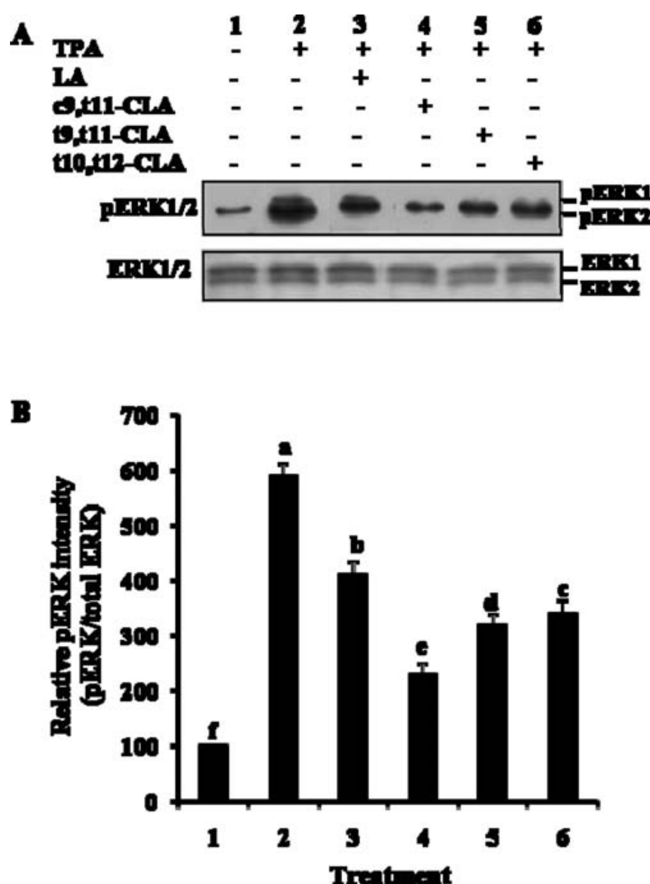


Figure 3. Effects of t9,t11-CLA and t10,t12-CLA on TPA-induced phosphorylation of ERK1/2. MCF-10A cells were treated with 20 μM t9,t11-CLA, t10,t12-CLA, c9,t11-CLA, or LA for 24 h followed by 60 nM TPA for 1 h. (A) Western blot analyses of phosphorylated ERK1/2 (pERK1/2) and total ERK1/2. (B) Band intensities relative to untreated normal cells were quantified. Each value represents the mean ± SD of triplication. Means with different lowercase letters are significantly different at $p < 0.05$ by Duncan's multiple range test.

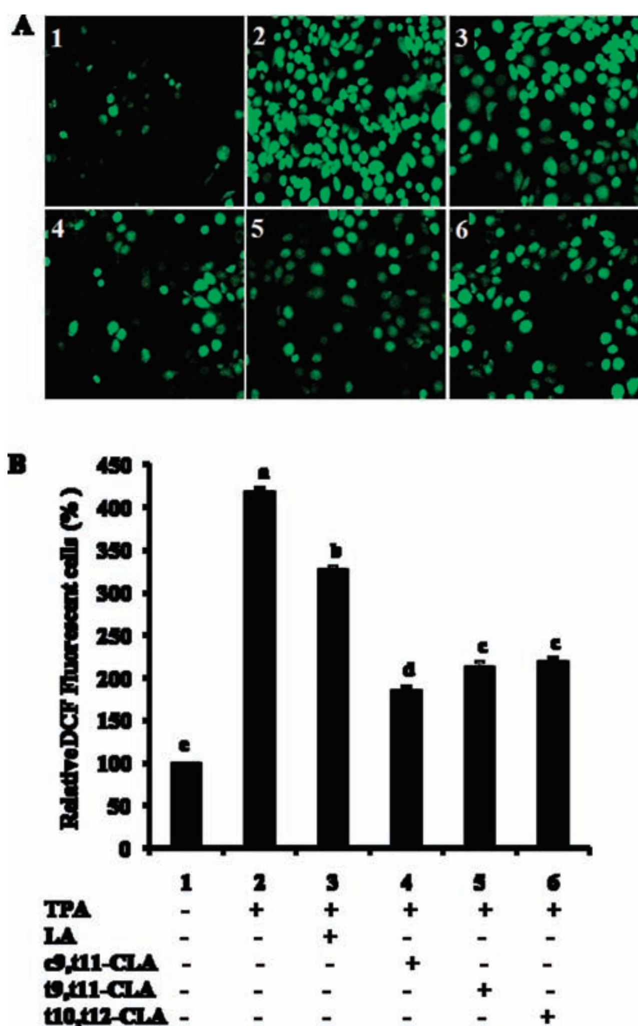


Figure 4. Effects of t9,t11-CLA and t10,t12-CLA on TPA-induced ROS generation in MCF-10A cells. MCF-10A cells were treated with 20 μM t9,t11-CLA, t10,t12-CLA, c9,t11-CLA, or LA for 24 h followed by 60 nM TPA for 1 h. (A) Representative image of the following treatments: 1, untreated control cells; 2, TPA; 3, TPA and LA; 4, TPA and c9,t11-CLA; 5, TPA and t9,t11-CLA; and 6, TPA and t10,t12-CLA. (B) Relative DCF fluorescent ROS-positive cells against untreated normal cells. Each value represents the mean ± SD of triplication. Means with different lowercase letters are significantly different at $p < 0.05$ by Duncan's multiple range test.

stages.[33,34] Currently, we reported that the c9,t11-CLA isomer acts as an anti-tumor promoter by attenuating GJIC inhibition in MCF-10A cells treated with TPA.[24] In the present study, t9,t11-CLA and t10,t12-CLA acted as potent anti-tumor promoters in MCF-10A cells treated with TPA but their efficacies were inferior to that of c9,t11-CLA isomers.

TPA is a potent tumor promoter and is extensively used for the mechanistic study of tumor promotion in cells. TPA increases Cx43 phosphorylation and concomitantly causes the inhibition of GJIC in a number of cell types.[35,36] TPA inhibited GJIC in MCF-10A cells, and t9,t11-CLA and t10,t12-CLA prevented the TPA-induced inhibition of GJIC. In MCF-10A cells, GJIC depends upon the extent of phosphorylation of Cx43.[24] In the present study, t9,t11-CLA and t10,t12-CLA suppressed the TPA-induced phosphorylation of Cx43, suggesting that t,t-CLA maintains GJIC by regulating gap junction channels in MCF-10A cells.

How t,t-CLA isomers affect the inhibition of GJIC is not yet clearly understood, but they might downregulate TPA-induced signal transduction. TPA exerts most of its effect on cells through the activation of protein kinase C (PKC)[37] and induces phosphorylation of Cx43 in association with the downregulation of GJIC.[38] PKC activates the MAPK pathway via RAF kinase.[39] These series of studies suggest that t,t-CLA isomers might act as an anti-tumor promoter by downregulating the phosphorylation of MAPK and ERK1/2 kinases. As shown in Figures 2 and 3, both the t9,t11-CLA and t10,c12-CLA isomers, which are equally active, inhibited Cx43 phosphorylation and ERK1/2 phosphorylation in MCF-10A cells treated with TPA. Interestingly, the efficacy of these t,t-CLA isomers was inferior to that of c9,t11-CLA. Our previous study demonstrated that phosphorylations of Cx43 and ERK1/2 are important events, mediating TPA-induced inhibition of GJIC in MCF-10A cells.[24] Pharmacological inhibitors for PKC and ERK1/2 recovered up to 100 and 77% of the TPA-mediated GJIC inhibition, respectively, suggesting that PKC is the upstream control site and MEK/ERK is the downstream control site and that the TPA signal is transferred to ERK1/2 through PKC in MCF-10A cells. In particular, phosphorylation of ERK was reported to play a key role in the inhibition of GJIC *in vitro*.[15,40] In the present study, t9,t11-CLA

and t10,t12-CLA isomers, similar to c9,t11-CLA, prevented the TPA-induced phosphorylation of Cx43 and ERK1/2 in MCF-10A cells. Moreover, apart from c9,t11-CLA, both t9,t11-CLA and t10,t12-CLA could block TPA-induced phosphorylation of ERK1/2 and Cx43, thereby protecting GJIC in MCF-10A cells. These data suggest that t9,t11-CLA and t10,t12-CLA mediate their effect by preventing activation of the ERK1/2−Cx43 signaling pathway.

How CLA isomers prevent the inhibition of GJIC is also unclear. Attenuation of intracellular ROS generation by CLA isomers might be attributed to the enhanced GJIC in MCF-10A cells. The differential attenuation of intracellular ROS generation and the configurations of the CLA isomers might be attributed to the efficacy of the prevention of the downregulation of GJIC induced by TPA in MCF-10A cells. Recent reports suggest that ROS is attributable to the inhibition of GJIC, which leads to carcinogenesis in normal cells.[9,16] Some chemopreventive natural antioxidants, such as quercetin,[40] resveratrol,[41] and cocoa polyphenol,[42] prevent the downregulation of GJIC by preventing accumulation of ROS. In the present study, the inhibition of GJIC mediated through Cx43 phosphorylation in MCF-10A cells by TPA was reduced by t9,t11-CLA and t10,t12-CLA (Figure 2A), which might have reflected the attenuation of TPA-induced ROS generation (Figure 4). It has been reported that c9,t11-CLA reduces lipopolysaccharide- or gliadin-induced ROS generation by antioxidant defenses and activation of cytoprotective enzymes in dendritic cells.[43] Further studies could provide clarification.

Higher potent anti-carcinogenic activity has been shown by the mixture of t,t-CLA or the individual t9,t11-CLA isomer than the c9,t11-CLA isomer in different fully transformed cell lines through their apoptotic capabilities.[21,22,25,26] In contrast, the present study demonstrated that t9,t11-CLA and t10,t12-CLA have inferior efficacy, as compared to c9,t11-CLA, in the attenuation of GJIC inhibition in the tumor-promotion stage of carcinogenesis in MCF-10A cells. This might be due to differential interaction of different CLA isomers with MCF-10A cells especially in the tumor-promotion phase and the growth inhibition by apoptotic potentialities in different cancer cell lines.

Superior efficacy of c9,t11-CLA in preventing the downregulation of GJIC in MCF-10A cells was related to their ability to prevent Cx43 phosphorylation and ROS generation (Figures 2 and 4). The differential efficacy of CLA isomers might be partly attributed to their structural differences. PKC activation domains C1A and C1B have different diacylglycerol (DAG) recognition abilities because of structural differences.[44] Structural differences in DAG containing t9,t11-CLA, t10,t12-CLA, and c9,t11-CLA might produce different binding affinities for the DAG-binding site of PKC.

In conclusion, t9,t11-CLA and t10,t12-CLA protect MCF-10A cells from TPA-induced inhibition of GJIC with inferior efficacy to c9,t11-CLA by preventing the phosphorylation of Cx43 through the suppression of ERK1/2 activation. Thus, CLA isomers including t9,t11-CLA, t10,t12-CLA, and c9,t11-CLA might protect from cancer during the tumor-promotion phase by preventing the downregulation of GJIC.

## AUTHOR INFORMATION

### Corresponding Author

*Telephone: +82-55-751-5471. Fax: +82-55-757-0178. E-mail: ylha@gnu.ac.kr.

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