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ARTICLE

Attenuation of 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-Induced Gap Junctional Intercellular Communication (GJIC) Inhibition in MCF-10A Cells by c9,t11-Conjugated Linoleic Acid

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The protective effect of c9,t11-conjugated linoleic acid (CLA) on the inhibition of gap junctional intercellular communication (GJIC) was examined in a human mammary epithelial cell line (MCF-10A) treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), relative to t10,c12-CLA isomer. TPA inhibited GJIC in a dose-dependent and reversible manner and was associated with connexin 43 phosphorylation. Pretreatment of 20 μM c9,t11-CLA for 24 h prior to 60 nM TPA for 1 h prevented the inhibition of GJIC by reducing the phosphorylation of connexin 43 via suppressing extracellular signal-regulated kinases (ERK1/2) activation. Reactive oxygen species (ROS) accumulation by TPA was attenuated by c9,t11-CLA. The efficacy of c9,t11-CLA in protecting inhibition of GJIC, connexin 43 phosphorylation, and ROS production was superior to that of t10,c12-CLA. These results suggest that c9,t11-CLA, including t10,c12-CLA, prevents the carcinogenesis of MCF-10A cells by protecting down-regulation of GJIC during the cancer promotion stage, and lack of their toxicities could be an excellent indicator for the chemoprevention of breast cancer.

KEYWORDS: Conjugated linoleic acid (CLA); human mammary epithelial cell line (MCF-10A); 12-*O*-tetradecanoylphorbol-13-acetate (TPA); gap junctional intercellular communication (GJIC); connexin 43 (Cx43)

INTRODUCTION

Conjugated linoleic acid (CLA) comprises positional and geometrical isomers of octadecadienoic acid (C18:2) with a conjugated double bond (*1*). Synthetic CLA consists of c9,t11-CLA and t10,c12-CLA isomers as major constituents (*2*). CLA and its individual isomers have been reported to have several health benefits in vivo, including anticarcinogenesis (*3*), antiatherogen (*4*), decreased body fat (*5*), and regulation of immune dysfunction (*6*).

There have been numerous papers regarding the anticarcinogenic effects of CLA and individual CLA isomers evaluated in many human cancer cell lines and animal models. CLA inhibits 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mouse skin carcinogenesis (*7*), benzo(*a*)pyrene (BP)-induced mouse forestomach tumorigenesis (*8*), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ)-induced rat colon cancer (*9*), and *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinogenesis (*10*). The activity of individual CLA isomers was also investigated in the osteosarcoma cell line MG-63 (*11*), human breast cancer cell line MCF-7 (*12*), colorectal adenocarcinoma cell line Caco-2 (*13*), and human prostate cancer cell line PC-3 (*14*). Given these data, each individual CLA isomer exhibited a different extent of anticarcinogenic potential.

Gap junctional intercellular communication (GJIC) is associated with carcinogenesis during the tumor promotion process (*15*). Gap junctions are intercellular membrane channels between adjacent cells that are formed by two transmembrane hemichannels containing six connexin proteins and, thus, can exchange small molecules (< 1 kDa), such as Ca^{2+}, cAMP, and inositol triphosphate, through these channels (*16*). GJIC is important in maintaining cellular homeostasis, controlled cell growth, and differentiation (*17*). GJIC is also associated with apoptosis (*18*), because tumor promoters can block apoptosis and chemopreventive agents can enhance or prevent the inhibition of apoptosis by tumor promoters, whereas they either inhibit or enhance GJIC, respectively (*19*). The activities of gap junctions are regulated by transcriptional, translational, or post-translational modifications of connexin proteins (*20*, *21*). It is evident that GJIC is dysfunctional in most cancer cells, and its inhibition is strongly related to carcinogenesis, especially tumor promotion (*22*, *23*). Although CLA and its individual isomers are potent anticancer agents that have been reported to have growth suppressive activity of cancer

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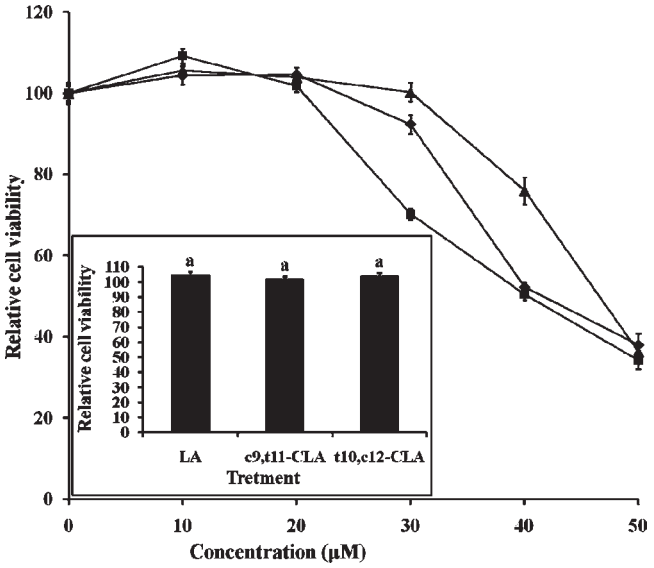

Figure 1. Cytotoxicity of c9,t11-CLA in MCF-10A cells. Cells were treated with 0, 10, 20, 30, 40, and 50 μM concentrations of (■) c9,t11-CLA, (◆) t10,c12-CLA, and (▲) LA for 24 h on MCF-10A cells. Cell viability was determined by MTT assay. Values shown are of the relative cell viability against the untreated control cells. (Inset) Relative cell viability at 20 μM concentration of c9,t11-CLA, t10,c12-CLA, and LA with no statistical difference at $p < 0.05$.

cells both in vitro and in vivo systems via the induction of apoptosis, their effects on the GJIC-related process of carcinogenesis, particularly in tumor promotion stage, have not yet been studied. It was therefore important to investigate the effect of CLA isomers on the enhancement of GJIC in the tumor promotion stage.

The purpose of the present study was to evaluate the protective effects of c9,t11-CLA and t10,c12-CLA isomers on 12-*O*- tetradecanoylphorbol-13-acetate (TPA)-induced inhibition of GJIC in the MCF-10A cell line, nontumorigenic human epithelial mammary cells, and their underlying molecular mechanisms. Here, we report that TPA inhibits GJIC in MCF-10A cells and that the effect is attributable to the phosphorylation of connexin 43 (Cx43), which is mediated by the protein kinase C (PKC) activated mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinases (ERK) signaling pathway. Both CLA isomers protect the inhibition of GJIC by inactivating the phosphorylation of ERK1/2, but the efficacy of c9,t11-CLA is superior to that of t10,c12-CLA.

MATERIALS AND METHODS

Materials. Human mammary epithelial cell line (MCF-10A) was purchased from the American Type Culture Collection (Manassas, VA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffer saline (PBS), and bovine serum albumin (BSA) were purchased from Amresco (Solon, OH). Heat-inactivated horse serum, insulin, hydrocortisone, recombinant human epidermal growth factor (EGF), phenylmethanesulfonyl fluoride (PMSF), Lucifer Yellow dilithium salt (LY), TPA, 2′,7′-dihydrodichlorofluorescein diacetate (DCFH-DA), MEK1 inhibitor PD98059, PKC inhibitor GF109203X, and linoleic acid (LA) were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/F-12 and penicillin−streptomycin were obtained from Gibco BRL (Rockville, MD). Radioimmune precipitation assay (RIPA) buffer and rabbit polyclonal antibodies for ERK1/2 and pERK1/2, and anti-connexin 43 were obtained from Cell Signaling Technology (Danvers, CO). Monoclonal anti-β-actin was purchased from Sigma-Aldrich. Goat anti-rabbit IgG HRP and goat anti-mouse IgG HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals used in this study were of analytical grade.

Preparations of CLA Isomers and Fatty Acid−Albumin Complexes. Synthetic CLA was prepared from LA by alkaline isomerization (*24*). The c9,t11-CLA and t10,c12-CLA isomers were isolated from synthetic CLA methyl ester by low-temperature crystallization at −68 and −71 °C in conjunction with urea treatment (*24*). Each CLA isomer or LA was complexed with fatty acid-free BSA according to the method described by van Greevenbroek et al. (*25*). The purity of CLA isomer samples was found to be 94.5% for c9,t11-CLA and 98.5% for t10, c12-CLA when analyzed by gas chromatography (*24*).

Cell Culture. MCF-10A cells were cultured in DMEM/F12 medium supplemented with 5% heat-inactivated horse serum, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF, 2 mM L-glutamine, and 100 μg/mL penicillin−streptomycin mixture as previously described (*26*). Cells were grown in culture dishes (Nunc, Rochester, NY) to 80% confluence and trypsinized with 0.25% trypsin containing 2 mM EDTA to disperse cells. The cells were subsequently collected by centrifugation (1000*g*, 10 min) and then resuspended in fresh culture medium and dispensed into new cell culture dishes. Cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Bioassay of Cell Viability. The cytotoxicity of CLA isomers on MCF-10A cells was measured by MTT assay (*27*). Briefly, 4×10^4 cells/well in 24-well plates were cultured and incubated in serum-free DMEM/F12 medium supplemented with 5 μg/mL of transferrin, 5 ng/mL of selenium, and 0.1 mg/mL of BSA for 24 h. Cells were treated with CLA isomers in DMEM/F12 medium supplemented with 50 ng/mL ascorbic acid and 20 ng/mL α-tocopherol phosphate for 24 h, followed by exposure to 100 μL of MTT solution (5 mg of MTT/mL of PBS) for 4 h. MTT solution was removed, and 200 μL of DMSO was added to each well and mixed to dissolve the MTT formazan crystals formed by viable cells. One hundred microliters of the dissolved solution was transferred to a 96-well plate, and absorbance was measured at 570 nm using a microplate reader (Anthos 2020, Anthos Labtech Instruments, Wals, Austria). Cytotoxicity was calculated by absorbance reduction of samples compared with untreated control (*27*).

Determination of GJIC. GJIC of MCF-10A cells was measured by the scrape loading/dye transfer technique (SL/DT) (*28*). Briefly, cells were cultured in 35 mm cell culture dishes (Nunc) and incubated in serum-free medium (DMEM/F12 supplemented with 5 μg/mL transferrin, 5 ng/mL selenium, and 0.1 mg/mL BSA) for 24 h. The cells were treated with 20 μM c9,t11-CLA, t10,c12-CLA, and LA and various concentrations of enzyme inhibitors (GF109203X and PD98059) with or without 60 nM TPA in DMEM/F12 medium supplemented with 50 ng/mL ascorbic acid and 20 ng/mL α-tocopherol phosphate. Cells were washed twice with 2 mL of PBS, followed by the addition of 0.05% LY and scraping with a surgical steel scalpel at low light intensities. After 3 min, the cells were washed four times with 2 mL of PBS and fixed with 4% paraformaldehyde. The number of dye-receiving cells on the line perpendicular to the scrape lines was counted under an inverted fluorescence microscope equipped with DPC controller software (IX70, Olympus, Okaya, Japan).

Western Blot Analysis. Cells treated with 20 μM c9,t11-CLA, t10, c12-CLA, and LA and 0.1 μM GF109203X and 0.5 μM PD98059 with or without 60 nM TPA were washed with PBS and lysed at 4 °C by shaking for 15 min followed by homogenization with RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin and protease inhibitors]. After centrifugation at 13000*g* for 15 min, the supernatant was collected and stored at −70 °C until use. The protein concentration was determined using the Bradford reagent (Bio-Rad Co., Hercules, CA). Proteins were separated using 12.5% SDS−polyacrylamide gel electrophoresis (*29*) and Western blot analysis of Cx43, ERK1/2, and pERK1/2 was performed according to the manufacturer's instructions. Protein bands were detected using an enhanced chemiluminescence (ECL) detection kit (Thermo Scientific, Rockford, IL).

Detection of Intracellular Reactive Oxygen Species (ROS). The ROS-sensitive fluorescent probe DCFH-DA was used to assess the generation of intracellular ROS (*30*). MCF-10A cells, grown to 80% confluence on glass coverslips (Lab Tek Chamber slide), were washed with PBS and incubated with 20 μM c9,t11-CLA, t10,c12-CLA, and LA for 24 h, prior to exposure to 60 nM TPA for 1 h. Cells were washed with PBS, and 50 μM DCFH-DA in DMSO was added to the cultures for 30 min

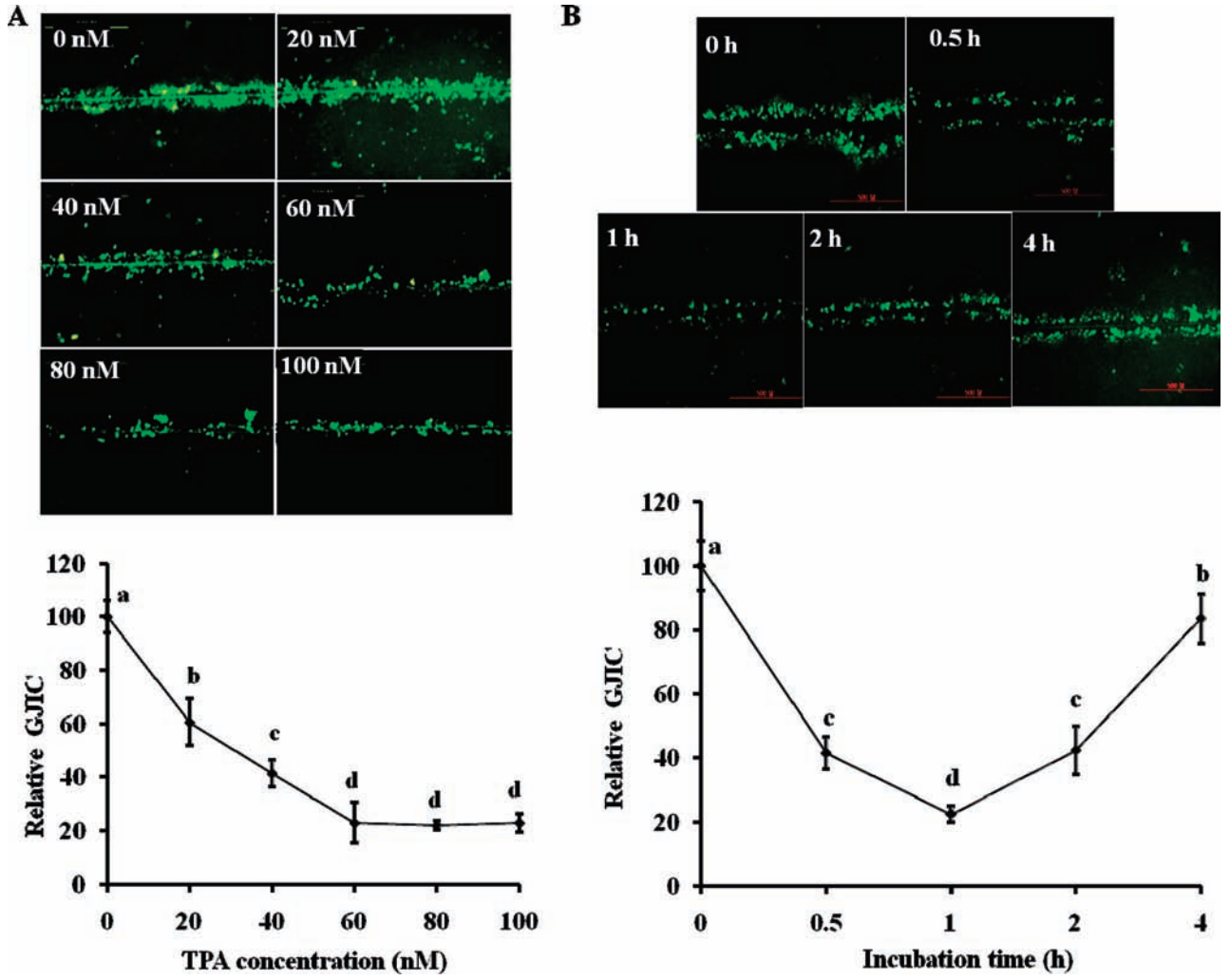

Figure 2. Dose- and time-dependent effect of TPA on GJIC in MCF-10A cells: (**A**) dose-dependent effect of TPA on GJIC (cells were exposed to 0, 20, 40, 60, 80, and 100 nM TPA for 1 h); (**B**) time-dependent effect of TPA on GJIC (cells were exposed to 60 nM TPA for 0, 0.5, 1, 2, and 4 h). The number of Lucifer Yellow dye communicating cells represented by a bright green color was counted under an inverted fluorescence microscope. The values represent the mean ± SD ($n = 3$). Means with different lower case letters were significantly different at $p < 0.05$ by Duncan's multiple-range test.

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, pH 7.4). Cells were then analyzed for ROS positive cells under a confocal microscope (FV-1000, Olympus) equipped with an argon laser with excitation and emission wavelengths of 485 and 530 nm, respectively.

Statistical Analysis. Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Duncan's multiple-range test for comparisons of group means using SPSS for Windows, version 11 (SPSS, Inc.). The value $p < 0.05$ was considered to be statistically significant.

RESULTS

Cytotoxicity of CLA Isomers on MCF-10A Cells. To determine the appropriate concentrations of CLA isomers in MCF-10A cells, cytotoxicity was evaluated by MTT assay. The noncytotoxic concentrations of c9,t11-CLA, t10,c12-CLA, and LA were determined in cells treated for 24 h. The optimal noncytotoxic dose of c9,t11-CLA, t10,c12-CLA, and LA was found to be 20 μM (**Figure 1**). Hence, we performed the subsequent experiments using 20 μM concentration for all of the CLA isomers and LA.

Protection of the TPA-Induced GJIC Inhibition in MCF-10A Cells by c9,t11-CLA. We first investigated the effect of TPA on GJIC using various doses (0, 20, 40, 60, 80, and 100 nM TPA) for 1 h by the scrape-loading dye transfer technique (*28*). TPA significantly induced the inhibition of GJIC in a dose-dependent manner ($p < 0.05$). At a concentration of ≥60 nM no further significant inhibition of GJIC was observed (**Figure 2A**), suggesting that 60 nM TPA is the optimal dose for the inhibition of GJIC in MCF-10A cells. Time (0, 0.5, 1, 2, and 4 h)-dependent analysis using 60 nM TPA revealed that the TPA-induced inhibition of GJIC was reversible in the cells (**Figure 2B**). Compared to the level of inhibition at 0 h, 60 nM TPA inhibited GJIC by 58.6, 77.6, 57.7, and 16.6% at 0.5, 1, 2, and 4 h after treatment ($p < 0.05$), respectively (**Figure 2B**). Prolonged exposure to 60 nM TPA for >2 h resulted in reoccurrence of cell GJIC, and after 4 h of continuous exposure, GJIC was recovered to 83.4% compared to the normal cell level. These results indicate that 60 nM TPA was found to be the most effective concentration in the inhibition of GJIC in MCF-10A cells for 1 h and that its inhibition was reversible.

The protective effect of CLA isomers on the TPA (60 nM)-induced inhibition of GJIC in MCF-10A cells for 1 h was assessed using the scrape-loading dye transfer technique (**Figure 3A**). Untreated normal MCF-10A cells showed the active state of GJIC. After exposure to 60 nM TPA for 1 h, 80.3% inhibition of GJIC was observed compared to untreated normal cells. Pretreatment of 20 μM c9,t11-CLA, t10,c12-CLA, and LA for 24 h before being exposed to 60 nM TPA for 1 h resulted in a significant protection of GJIC inhibition in MCF-10A cells.

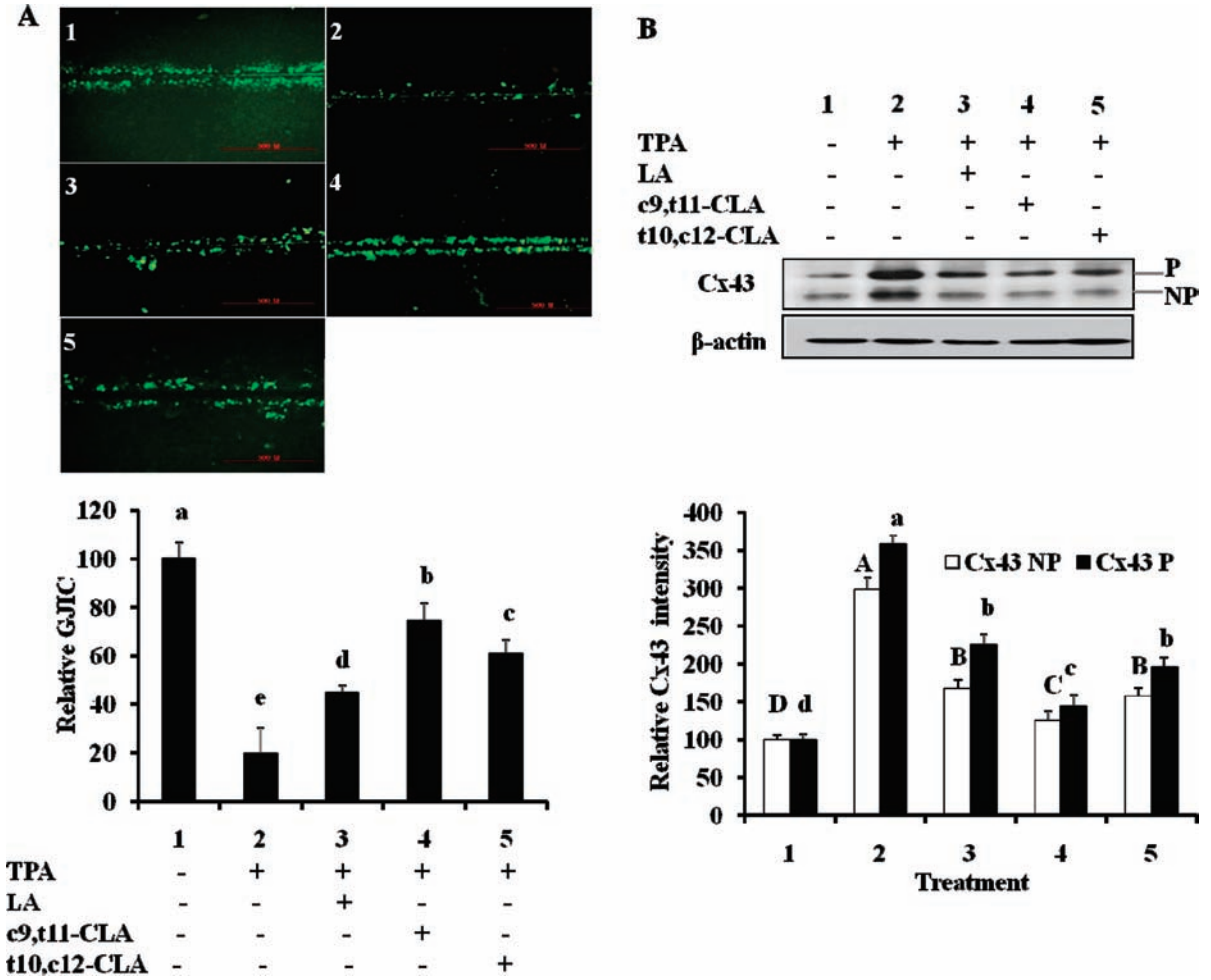

Figure 3. Protective effect of c9,t11-CLA in TPA-induced inhibition of GJIC and Cx43 phosphorylation in MCF-10A cells: (**A**) GJIC measured using the SL/DT method (the number of Lucifer Yellow dye communicating cells represented by a bright green color was counted under an inverted fluorescence microscope); (**B**) Western blot analyses of Cx43 phosphorylation in MCF-10A cells. Cells were pretreated with c9,t11-CLA, t10,c12-CLA, and LA at 20 μM concentration for 24 h before being exposed to 60 nM TPA for 1 h.All treatments show two distinct Cx43 bands, nonphosphorylated (NP) and phosphorylated (P). The band intensities relative to untreated normal cell were quantified. Values are means ± SD (n = 3). Means with different lower case and upper case letters are significantly different at $p < 0.05$ by Duncan's multiple-range test.

Table 1. Effects of PKC Inhibitor GF109203X and MEK1 Inhibitor PD98059 on GJIC of TPA-Induced MCF-10A Cells Treated with c9,t11-CLA

	concentration[a] (μM)		
treatment[b]	c9,t11-CLA−20 GF109203X−10 PD98059−50	c9,t11-CLA−20 GF109203X−1 PD98059−5	c9,t11-CLA−20 GF109203X−0.1 PD98059−0.5
1. normal	100 ± 8.6[c] b	100 ± 5.6 a	100 ± 4.0 a
2. TPA	26.1 ± 9.0 e	27.0 ± 6.1 e	27.2 ± 1.2 d
3. TPA + c9,t11-CLA	76.3 ± 6.9 d	72.8 ± 3.7 bc	75.0 ± 4.9 b
4. TPA + GF109203X	104.3 ± 8.2 a	69.9 ± 7.0 c	49.1 ± 3.5 c
5. TPA + PD98059	86.5 ± 6.3 c	62.3 ± 7.3 d	50.9 ± 5.1 c
6. TPA + c9,t11-CLA + GF109203X	100.6 ± 6.1 ab	75.2 ± 9.6 b	74.5 ± 3.2 b
7. TPA + c9,t11-CLA + PD98059	76.6 ± 7.0 d	73.7 ± 8.2 bc	72.7 ± 4.0 b

[a] The number following c9,t11-CLA, GF109203X, and PD98059 represents the concentration. [b] Cells were treated with c9,t11-CLA for 24 h and with GF109203X and PD98059 for 1 h before being exposed to 60 nM TPA for 1 h. [c] The relative GJIC was determined by counting the number of communicating cells as indicated by Lucifer Yellow dye under an inverted fluorescence microscope. Values were means ± SD (n = 3). Means with different lower case letters are significantly different at $p < 0.05$ by Duncan's multiple-range test.

In particular, 20 μM c9,t11-CLA retained 74.5% of GJIC compared to the normal cells, and this isomer was found to be superior in GJIC recovery to t10,c12-CLA and LA.

Suppression of TPA-Induced Cx43 Phosphorylation in MCF-10A Cells by c9,t11-CLA. Normal human mammary epithelial cells have been shown to contain Cx43 and Cx26. Cx43 is predominantly expressed in myoepithelial cells, luminal cells of the ducts, and the secretory epithelium and myoepithelium cell interface (*31*, *32*), and its phosphorylation is responsible for GJIC inhibition; hence, we measured the degree of Cx43 phosphorylation. Two distinct bands [nonphosphorylated state (NP) and phosphorylated state (P)] of Cx43 were detected in untreated normal MCF-10A cells, but in response to TPA exposure, a significant induction of Cx43 phosphorylation was observed

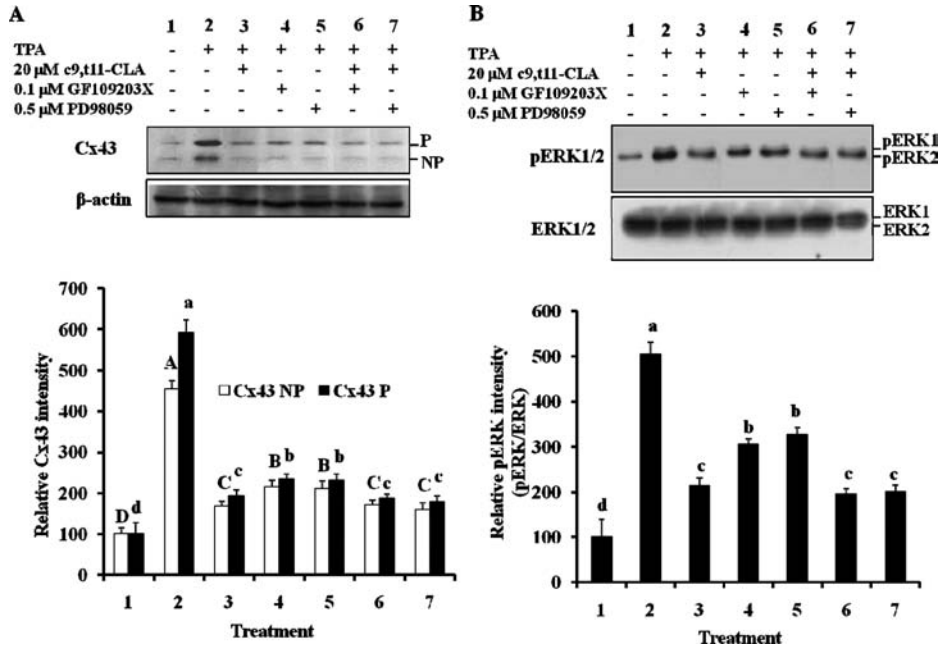

Figure 4. Effect of the PKC inhibitor GF109203X and MEK1 inhibitor PD98059 with and without c9,t11-CLA on TPA-induced Cx43 phosphorylation and ERK1/2 phosphorylation in MCF-10A cells: Western blot analyses of (**A**) Cx43 protein and (**B**) phosphorylated ERK1/2 and total ERK1/2. Cells were pretreated with c9,t11-CLA at 20 μM concentration for 24 h and with 0.1 μM GF109203X and 0.5 μM PD98059 for 1 h before being exposed to 60 nM TPA for 1 h. The band intensities relative to the untreated normal cell were quantified. Values are means ± SD ($n = 3$). Means with different lower case and upper case letters are significantly different at $p < 0.05$ by Duncan's multiple-range test.

as shown in **Figure 3B**. Meanwhile, the treatment of c9,t11-CLA, t10,c12-CLA, and LA significantly decreased ($p < 0.05$) the phosphorylation of Cx43 compared to that of TPA exposure only. Protective efficacy of Cx43 phosphorylation by c9,t11-CLA was significantly higher ($p < 0.05$) in comparison to that by t10, c12-CLA and LA. These results indicate that phosphorylation of Cx43 might be involved in GJIC inhibition of MCF-10A cells by TPA and that c9,t11-CLA prevents the phosphorylation of Cx43.

Role of ERK and PKC in the TPA-Induced Inhibition of GJIC and Cx43 Phosphorylation. GF109203X, a PKC inhibitor, and PD98059, a specific inhibitor of MEK1 that directly activates MAP kinase ERK1/2 when no specific inhibitor of ERK1/2 is available, were applied to elucidate the possible role of CLA isomers in the signaling pathway involved in TPA-induced inhibition of GJIC (**Table 1**). The GJIC by 60 nM TPA treatment was approximately 27% relative to normal cells, whereas c9, t11-CLA protected the inhibition of GJIC to 72.8%. Meanwhile, the GJIC appeared to be 69.9 and 62.3%, respectively, by 1 μM GF109203X and 5 μM PD98059; no difference in the reversion was seen between inhibitors. Thus, we reduced the concentration of the two inhibitors to 0.1 μM GF109203X and 0.5 μM PD98059. At lower concentrations of inhibitors, a >20% suppressive effect of c9,t11-CLA was seen, but no difference in the suppression was observed between the two inhibitors. Meanwhile, higher concentrations of these inhibitors, 10 μM GF109203X and 50 μM PD98059 with 20 μM c9,t11-CLA, exhibited 100.6 and 76.6% recoveries of GJIC inhibition by TPA, respectively, suggesting that PKC is the upstream control site and MEK/ERK is the downstream control site. These results suggest that c9,t11-CLA blocked TPA signals transferred to ERK1/2 through PKC in MCF-10A cells.

It was confirmed that c9,t11-CLA reduced TPA-induced Cx43 phosphorylation with or without use of kinase inhibitors (**Figure 4A**). TPA treatment significantly elevated the phosphorylation level of Cx43 relative to normal cells, but the phosphorylation was significantly reduced ($p < 0.05$) by c9,t11-CLA treatment. Meanwhile, Cx43 phosphorylation was significantly lowered by c9,t11-CLA compared with that by 0.1 μM GF109203X and 0.5 μM PD98059, but that by c9,t11-CLA was not significantly different from that by the treatment with 0.5 μM PD98059 plus c9,t11-CLA or 0.1 μM GF109203X plus c9, t11-CLA combination. These results revealed that c9,t11-CLA inhibits the signal for TPA-induced Cx43 phosphorylation through PKC and ERK1/2. Likewise, the TPA-induced activation of MAP kinase ERK1/2 was counteracted ($p < 0.05$) by 0.5 μM PD98059 and 0.1 μM GF109203X, and c9,t11-CLA inhibited the phosphorylation of ERK1/2 (**Figure 4B**). Collectively, these results suggest that the TPA-induced inhibition of GJIC is mediated by the phosphorylation of Cx43 through the PKC-activated MEK/ERK signaling pathway and that c9,t11-CLA reduces the phosphorylation of Cx43 in MCF-10A cells.

Reduction of TPA-Induced Oxidative Stress by c9,t11-CLA. ROS induces inhibition of GJIC, which contributes to carcinogenesis of normal cells (*33*−*35*). Hence, in the present study, the production of ROS in TPA-treated MCF-10A cells was determined to evaluate the effect of CLA isomers on the GJIC. ROS is monitored by using the cell-permeable probe DCFH-DA. When nonpolar and nonfluorescent DCFH-DA dye is added to cells, it diffuses across the cell membrane and is hydrolyzed by intracellular esterases to liberate nonfluorescent 2′,7′-dichlorofluorescein (DCFH), which upon reaction with ROS forms its highly fluorescent dichlorofluorecein (DCF). DCF fluorescence was assayed by confocal microscope. As shown in **Figure 5**, the fluorescent intensity was increased in the TPA-treated cells in comparison with untreated cells, suggesting generation of ROS in TPA-treated cells. CLA isomers (c9,t11-CLA and t10,c12-CLA) reduced the TPA-induced accumulation of intracellular ROS level in MCF-10A cells. The exposure of 60 nM TPA for 1 h significantly increased ($p < 0.05$) the intracellular ROS level in MCF-10A cells, and this was significantly ($p < 0.05$) attenuated by c9,t11-CLA and t10,c12-CLA isomers when preincubated for 24 h before being exposed to 60 nM TPA for 1 h. The efficacy of c9,t11-CLA was

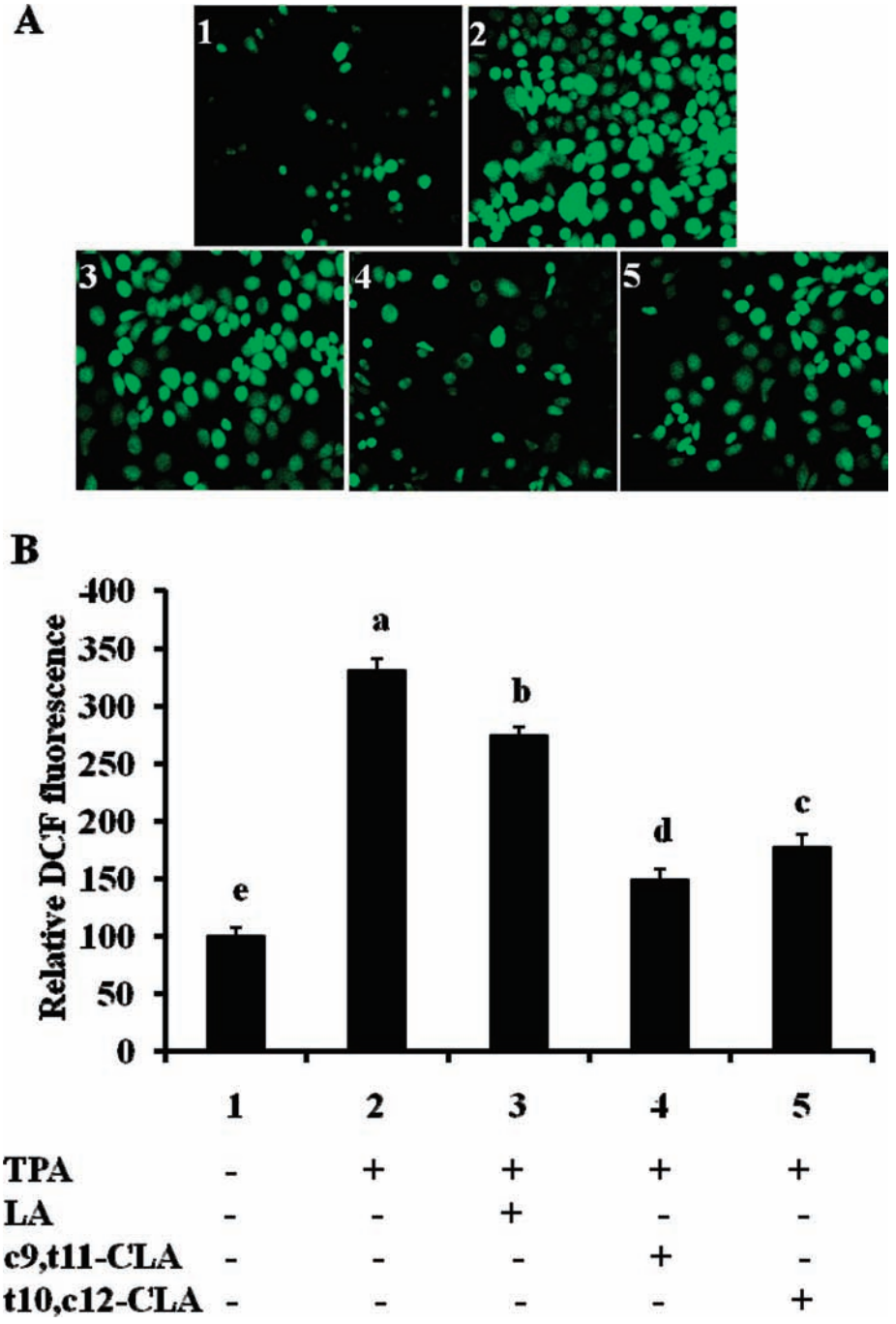

Figure 5. Effects of c9,t11-CLA on TPA-induced intracellular ROS generation in MCF-10A cells: (**A**) representative images of different treatment cells with ROS; (**B**) relative intracellular ROS generation in MCF-10A cells. Cells were treated with c9,t11-CLA, t10,c12-CLA, and LA at 20 μM concentration for 24 h before being exposed to 60 nM TPA for 1 h. Values are means $\pm$ SD ($n = 3$). Means with different lower case letters are significantly different at $p < 0.05$ by Duncan's multiple-range test.

superior to that of t10,c12-CLA and LA as well. We thus assume the protective effect of c9,t11-CLA on TPA-induced GJIC inhibition is in part mediated by the attenuation of intracellular ROS.

DISCUSSION

The present study has clearly revealed that c9,t11-CLA suppressed the TPA-induced GJIC inhibition in MCF-10A cells. This effect of the CLA isomer was attributed to the inhibition of Cx43 phosphorylation by PKC-mediated ERK1/2 activation (**Figure 6**). The exact mechanistic action by which c9,t11-CLA reverses the inhibition of GJIC by TPA is not clear, but cellular ROS attenuated by c9,t11-CLA and the configuration of c9, t11-CLA might be at least partly attributed to the enhanced GJIC in MCF-10A cells by TPA.

Tumor promoters such as TPA and hydrogen peroxide (H_2O_2) almost immediately inhibit GJIC in vitro systems (*23*, *28*, *36*). The phosphorylation of Cx43, a phosphoprotein, is associated with both reduced channel functionality and down-regulation of GJIC (*37*, *38*). Several exogenous and endogenous chemicals have been shown to regulate GJIC by altering the Cx43 phosphorylation state (*39*, *40*). In agreement with these previous papers, the present study has demonstrated that TPA-induced inhibition of GJIC was dose-dependent and reversible in MCF-10A cells (**Figure 2**), and its inhibition was prevented by c9,

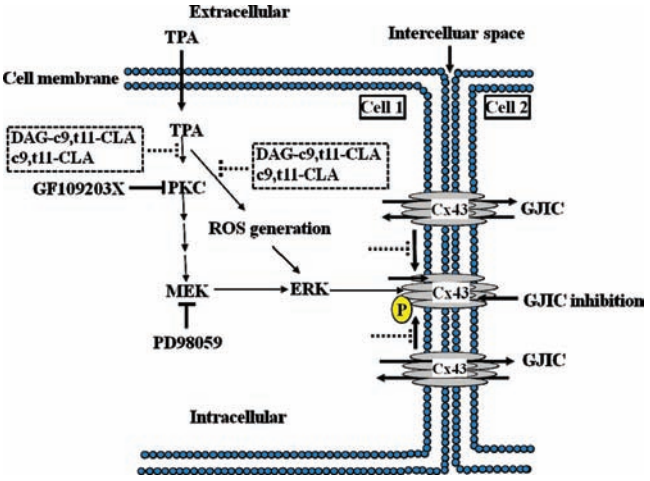

Figure 6. Hypothetical mechanism of the attenuation of TPA-induced GJIC inhibition in MCF-10A cells by c9,t11-CLA. TPA inhibits GJIC by Cx43 phosphorylation through PKC-activated MEK−ERK kinase pathway and/or ROS generation. DAG having c9,t11-CLA and/or free form of c9,t11-CLA attenuate the activation of PKC and/or the production of ROS, resulting in the suppression of TPA effects. GF109203X, PKC inhibitor, PD98059, and MEK1 inhibitor similarly inhibit the phosphorylation for the reduction of TPA effects.

t11-CLA isomer (**Figure 3A**). These results, therefore, are in accordance with the anticarcinogenic action of c9,t11-CLA isomer in tumor cells and animal models (*11*, *12*).

TPA exerts most of its effect on cells through activation of PKC (*41*) and has been shown to induce phosphorylation of Cx43 in association with down-regulation of GJIC (*37*). PKC has also been shown to activate a MAP kinase (ERK1/2) pathway through activation of *Raf*, demonstrating cross-talk between PKC and MAP kinase signaling pathway (*42*, *43*). These imply the possibility that Cx43 phosphorylation as well as inhibition of GJIC by the PKC activator, TPA, could be mediated through MAP kinase. This hypothesis was also proven in MCF-10A cells in the present study, by the experiments using the PKC inhibitor GF109203X, which is capable of preventing the TPA-induced inhibition of GJIC, and the MEK1 inhibitor PD98059, which is effective in counteracting the effect of TPA on GJIC. Our data showed that MEK1 inhibitor was as effective as the PKC inhibitor to suppress the inhibition of GJIC (**Table 1**) and inhibited Cx43 phosphorylation (**Figure 4A**), demonstrating that the inhibition of GJIC by TPA could be the result of PKC-activated ERKs in MCF-10A cells. Our data are correlated with previous reports that the inhibition of GJIC and induction of Cx43 phosphorylation by TPA was attributed to PKC-activated ERKs in WB-F344 rat liver epithelial cells (*44*) and in IAR20 rat liver epithelial cells (*45*). Yet there is evidence that PKC can be directly involved in the phosphorylation of Cx43 in viable cells and is responsible for the loss of GJIC (*46*). Hence, it is not clear whether TPA mediates its affects through Cx43 phosphorylation of MCF-10A cells through the activated ERK1/2 or directly affects PKC phosphorylation of Cx43 or possibly acts via both pathways. Further studies will be needed to clarify this issue.

TPA causes the formation of superoxide (O_2^-) and H_2O_2 in cells (*47*), and both ROS can produce other intracellular ROS by way of the Fenton reaction (*48*). Several pieces of evidence indicate that both in vivo and in vitro studies have shown that ROS also play a significant role in the promotional stage of carcinogenesis (*23*, *49*) by involvement in signal transduction pathways (*50*). Currently, it is evident that ROS induced the inhibition of GJIC, which contributes to carcinogenesis of normal cells (*33*−*35*), and antioxidants such as vitamin C (*51*), germanium oxide (*29*), and resveratrol (*33*, *52*) could prevent or recover the inhibition of GJIC induced by cancer promoters such as TPA. In the present study, the GJIC mediated through Cx43 phosphorylation in MCF-10A cells by TPA was reversed by c9,t11-CLA (**Figure 3**), which could be attributed to the suppression of ROS generation (**Figure 5**). There is one study that reported that intracellular ROS reduction by c9,t11-CLA was seen in a dendritic cell line. Bergamo et al. (*53*) has shown that c9,t11-CLA reduced lipopolysaccharide- or gliadin-induced ROS generation by antioxidant defenses and activation of cytoprotective enzymes (phase 2) in dendritic cells (DCs). Some antioxidants including EGCG or other tea polyphenols suppressed tumor promotion through the prevention of the inhibition of GJIC by a chemical tumor promoter (*54*) and through the reduction of ROS generation by induction of phase 2 enzymes (*55*). Our data suggest that ROS attenuated by c9,t11-CLA is directly and/or indirectly involved in signal transduction of TPA to the activation of ERK1/2 for the phosphorylation of Cx43. Further studies need to be performed to investigate this hypothesis.

The efficacy of TPA-induced GJIC suppression in MCF-10A cells by c9,t11-CLA was superior to that by t10,c12-CLA (**Figure 3A**), which is positively related to their inhibiting ability of Cx43 phosphorylation (**Figure 3B**) and cellular ROS production in MCF-10A cells (**Figure 5**). No direct comparative data are currently available in the literature, but this efficacy might be partly attributed to the molecular configuration of c9,t11-CLA, which is greatly bent at the carbon 9 and carbon 10 double bond, relative to that of t10,c12-CLA. For the activation of PKC, diacylglycerol (DAG), derived from plasma membrane phospholipids, must be bound to a DAG binding site of the C1 domain of PKC (*56*). Biochemical and cellular studies in PKC revealed that C1A and C1B domains are nonequivalent as lipid binding motifs and that individual C1 domains have unique patterns of ligand recognition, in some cases by subtle structural differences (*56*). From these results, we presume that due to structural differences DAG containing c9,t11-CLA exhibited lower affinity for the DAG binding site of PKC than DAG having t10,c12-CLA, resulting in an enhanced ability of a down-regulated GJIC in TPA-treated MCF-10A cells by the suppression of Cx43 phosphorylation through the reduction of ROS production. However, our data are not agreement with previous papers which demonstrated that the anticarcinogenic action of t10,c12-CLA is even greater than that of c9,t11-CLA in chemical-induced rat mammary tumorigenesis (*8*) and various types of cancer cell lines (*9*−*12*). This could be due to the fact that inhibition of GJIC is only one of several factors involved in carcinogenesis, but this warrants further study for clarification.

We were able to demonstrate that c9,t11-CLA prevented TPA-induced GJIC inhibition in MCF-10A cells through the reduction of the Cx43 phosphorylation level via the PKC-activated ERK pathway. The mechanistic action by which c9,t11-CLA recovered the inhibition of GJIC in MCF10A cells induced by TPA is unclear, but c9,t11-CLA blocked the MEK−ERK signaling pathway and attenuated the intracellular ROS concentration produced by TPA. We suggest that the CLA isomer might have a cancer preventive effect during the promotion phase by the prevention of the down-regulation of GJIC.

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