# Inhibition of Gap Junctional Intercellular Communication by the Green Tea Polyphenol (−)-Epigallocatechin Gallate in Normal Rat Liver Epithelial Cells

Nam Joo Kang,[†,‡] Kyung Mi Lee,[†] Jong Hun Kim,[‡] Bo Kyung Lee,[‡]
Jung Yeon Kwon,[‡] Ki Won Lee,*,[‡] and Hyong Joo Lee*,[†]

Department of Agricultural Biotechnology and Research Institute for Agriculture and Life Sciences,
Seoul National University, Seoul 151-921, Republic of Korea, and Department of Bioscience and
Biotechnology, Konkuk University, Seoul 143-701, Republic of Korea

(−)-Epigallocatechin gallate (EGCG), a polyphenolic compound found in green tea, is a promising chemopreventive agent against cancer due to its strong antiproliferative effects on cancer cells; however, its possible toxicity and carcinogenicity must be investigated before EGCG can be used as a dietary supplement for chemoprevention. The inhibition of gap junctional intercellular communication (GJIC) is strongly associated with carcinogenesis, particularly the tumor promotion process; thus, we investigated the effects of EGCG on GJIC in WB-F344 normal rat liver epithelial (RLE) cells. EGCG, but not (−)-epicatechin (EC), another polyphenol found in green tea, inhibited GJIC in a dose-dependent and reversible manner in RLE cells. EGCG also induced the phosphorylation of connexin 43 (Cx43), a major regulator of GJIC. The phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) was also observed in EGCG-treated RLE cells. The inhibition of GJIC and phosphorylation of Cx43 and ERK1/2 by EGCG were completely blocked by U0126, a pharmacological inhibitor of mitogen-activated protein kinase/ERK kinase. EGCG generated a larger amount of hydrogen peroxide than EC in a dose-dependent manner. Furthermore, catalase partially inhibited the EGCG-induced inhibition of GJIC and the phosphorylation of Cx43 and ERK1/2. These results indicated that EGCG inhibited GJIC mainly due to its prooxidant activity.

KEYWORDS: (−)-Epigallocatechin gallate; gap junctional intercellular communication; connexin 43; extracellular signal-regulated protein kinase

## INTRODUCTION

Accumulating evidence suggests that green tea possesses a wide range of pharmacological properties, including antioxidative, antimutagenic, anti-inflammatory, and anticarcinogenic effects. Catechins, the major polyphenols found in green tea and some foods and plants, have been shown to mediate the anticarcinogenic effect of green tea. Among the catechins present in green tea, (−)-epigallocatechin gallate (EGCG) (**Figure 1**) is believed to have considerable cancer-chemopreventive potential due to its strong antiproliferative effects on various tumor cells (*1, 2*). However, recent epidemiological studies do not support these results. In two large cohorts of both men and women, the consumption of tea containing caffeine or caffeine intake alone was shown to have no effect on the incidence of colon or rectal cancer (*3, 4*). Moreover, a prospective study found that there was no inverse association between the consumption of green tea and the risk of stomach cancer (*5*), and several epidemiological studies have indicated the presence of a significant positive correlation between green tea consumption and cancer (*6*). In addition, studies have reported the oxidative damage of isolated and cellular DNA caused by EGCG (*7*) and that green tea catechins enhanced colon carcinogenesis in rats exposed to a chemical carcinogen (*8*). A recent study found that EGCG was by far the most cytotoxic of the green tea polyphenols using isolated rat hepatocytes, and it has also been shown to be hepatotoxic *in vivo* in mice (*9, 10*). Furthermore, the intraperitoneal injection of 120 mg/kg EGCG was shown to increase the plasma alanine aminotransferase level in CD-1 mice by 4-fold after 24 h (*11*), indicating that EGCG causes major hepatic damage *in vivo* in rodent models. Because of recent reports showing the beneficial effects of green tea, various dietary supplements containing the phenolic compounds found in green tea have been developed. Thus, concerns about the safety of high doses of tea phenolic supplements must be addressed (*10*).

* Corresponding author. (K.W.L.) Tel: +82-2-2049-6178. Fax: +82-2-3436-6178. E-mail: kiwon@konkuk.ac.kr. (H.J.L.) Tel: +82-2-880-4853. Fax: +82-2-873-5095. E-mail: leehyjo@snu.ac.kr.
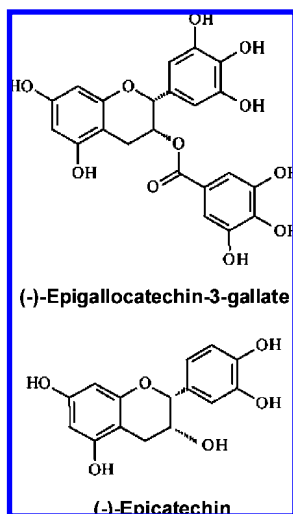† Seoul National University.
‡ Konkuk University.

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**Figure 1.** Chemical structures of (−)-epigallocatechin gallate (EGCG) and (−)-epicatechin (EC).

Gap junctional intercellular communication (GJIC) is essential for maintaining the homeostatic balance by modulating cellular proliferation and differentiation in multicellular organisms (*12*). Multiple lines of evidence indicate that the inhibition of GJIC is a carcinogenic process. Most normal cells exhibit functional GJIC, while most cancer cells exhibit dysfunctional GJIC (*13*). The molecular mechanism responsible for the inhibition of GJIC has been suggested to involve conformational changes in gap junctions due to the phosphorylation of connexin 43 (Cx43), a major component of gap junction channels. The activation of mitogen-activated protein kinases (MAPKs) has also been shown to be related to the inhibition of GJIC via the phosphorylation of Cx43 in WB-F34 rat liver epithelial (RLE) cells. In particular, extracellular signal-regulated kinase (ERK) plays a key role in GJIC inhibition in several cell lines, including RLE cells. The disruption of GJIC might play a role in the actions of various toxic chemicals that exhibit cell, tissue, or organ specificity (*14*). A consistent finding is that tumor promoters inhibit GJIC (*15−17*), while antitumor-promoting agents and anticancer drugs can reverse the downregulation of GJIC (*6, 18, 19*). Recent reports suggest that the carcinogenicity of oxidative stress is also attributable to the inhibition of GJIC (*20, 21*). In the present study, we investigated the possible toxicological and carcinogenic effects of the green tea polyphenolic phytochemicals EGCG and (−)-epicatechin (EC) using a GJIC model.

## MATERIALS AND METHODS

**Chemicals.** EGCG, EC, hydrogen peroxide ($H_2O_2$), catalase (bovine liver, EC 1.11.1.6), dimethyl sulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical (St. Louis, MO). All other chemicals used were of analytical grade (Fisher, Springfield, NJ). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamate, penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY).

**Sample Preparation.** Sample stocks were prepared by dissolving EGCG and EC in DMSO. For use in our experiments, the stocks were diluted with double-distilled water (ddH$_2$O).

**Cell Culture.** WB-F344 normal RLE cells were kindly provided by Dr. J. E. Trosko (Michigan State University, East Lansing, MI). The cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and penicillin/streptomycin in a humidified incubator maintained at 37 °C and supplied with 5% $CO_2$ and 95% air.

**Bioassay for GJIC.** The level of GJIC was measured by the scrape-loading/dye-transfer technique as described previously (*22*). Briefly, RLE cells were treated with EGCG, EC, or $H_2O_2$ at various concentra-
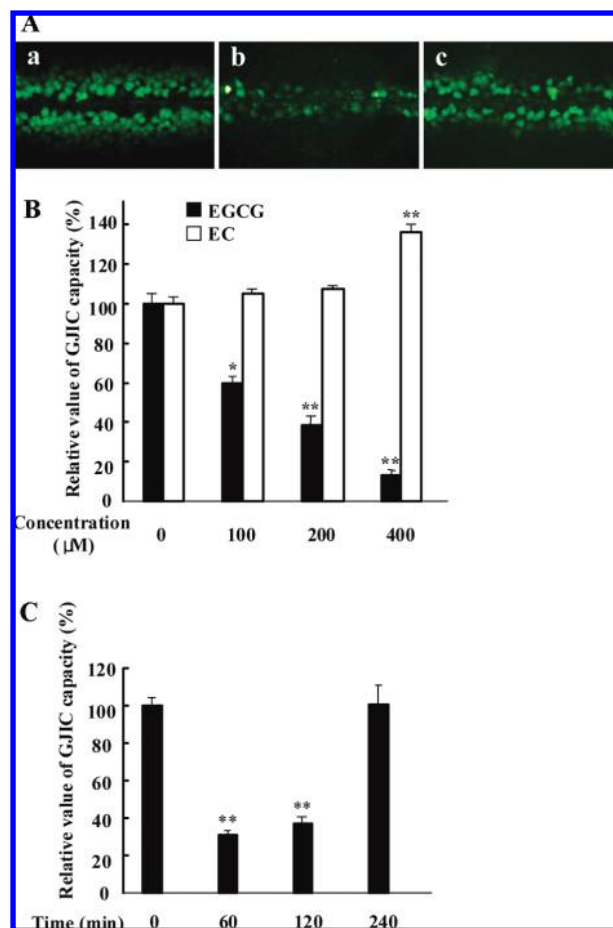


**Figure 2.** Time- and dose-dependent effects of EGCG and EC on GJIC in WB-F344 rat liver epithelial (RLE) cells. (**A**) Comparison of the effects of EGCG and EC on the inhibition of GJIC in RLE cells. The images are representative of the following groups: (**a**) untreated control, (**b**) 200 $\mu$M EGCG, and (**c**) 200 $\mu$M EC. (**B**) Quantitative comparison of the effects of EGCG and EC at various concentrations on the inhibition of GJIC. The relative recovery rate was determined by counting the number of communicating cells. The values are the means ± standard deviation (SD) from at least three independent experiments. The asterisks indicate significant decreases in the number of communicating cells in the group treated with EGCG or EC compared to the untreated controls (*, $p < 0.05$; **, $p < 0.01$). (**C**) EGCG induces the inhibition of GJIC in a reversible manner. The relative recovery rate was determined by counting the number of communicating cells. The values are the means ± SD from at least three independent experiments. The asterisks indicate significant decreases in the number of communicating cells in the group treated with EGCG compared to the untreated controls (**, $p < 0.01$).

tions for 1 h. When cotreatments with U0126 or catalase were needed, they were treated on cells 30 min before EGCG treatment. The assay was conducted using noncytotoxic doses of the samples. Following incubation, the cells were washed twice with 2 mL of phosphate buffer solution (PBS). Lucifer yellow was added to the washed cells, and three scrapes were made using a scalpel with a surgical steel blade at a low light intensity. Each scrape was performed so as to ensure that it traversed a large group of confluent cells. After an incubation period of 3 min, the cells were washed four times with 2 mL of PBS then fixed in 2 mL of 4% formalin. The number of communicating cells as indicated by the dye was counted under an inverted fluorescence microscope (IX70; Olympus, Okaya, Japan).

**Western Blotting.** Western blotting for Cx43, ERK1/2, and phospho-ERK1/2 was performed as described previously (*21, 23*). Proteins were extracted with 20% sodium dodecyl sulfate (SDS) containing 1 mM phenylmethylsulfonyl fluoride (a protease inhibitor), 10 mM iodoacetamide, 1 mM leupeptin, 1 mM antipain, 0.1 mM sodium orthovana-
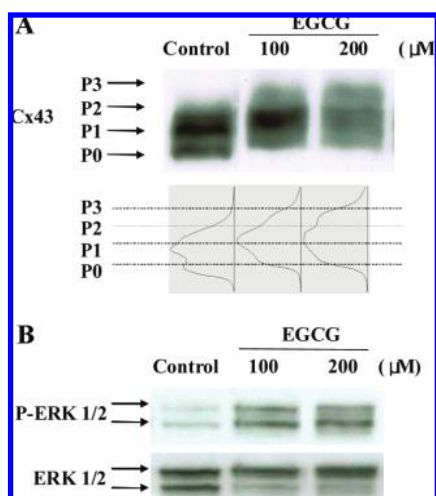
**Figure 3.** EGCG-induced phosphorylation of connexin 43 (Cx43) and extracellular signal-regulated protein kinase 1/2 (ERK1/2) in RLE cells. (**A**) EGCG induces the phosphorylation of Cx43. RLE cells were stimulated with 100 or 200 $\mu$M EGCG for 30 min. Cell lysates were then prepared and immunoblotted with anti-Cx43 antibodies. (**B**) EGCG induces the phosphorylation of ERK1/2. RLE cells were stimulated with 100 or 200 $\mu$M EGCG for 15 min. Cell lysates were then prepared and immunoblotted with antiphospho-ERK1/2 or -ERK1/2 antibodies.

date, and 5 mM sodium fluoride. The protein content was determined using a DC assay kit (Bio-Rad, Hercules, CA), and the proteins were separated by 12.5% SDS−PAGE. The proteins were then transferred to nitrocellulose membranes at 100 V/350 mA for 1 h. All antibodies were used according to the manufacturer's instructions, and the protein bands were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Measurement of $H_2O_2$.** The concentration of $H_2O_2$ was determined by the oxidation of ferrous iron in xylenol orange (the FOX assay) according to a previously described procedure (*24*). Samples were prepared by desolving EGCG and EC in the absence or presence of catalase in DMEM containing 10% FBS. Twenty microliters of each sample were mixed with 180 $\mu$L of FOX working reagent (100 $\mu$M xylenol orange, 4.4 mM butylated hydroxytoluene, 250 $\mu$M $Fe(NH_4)_2(SO_4)_2$, and 25 mM $H_2SO_4$ in 90% [v/v] methanol) for 1 h at 37 °C, and the absorbance at 560 nm was measured using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA). The $H_2O_2$ concentration was calculated from a standard curve with authentic $H_2O_2$.

**Statistical Analysis.** The data are expressed as the mean ± SD; Student's *t*-test was used for single statistical comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

## RESULTS

**EGCG, but Not EC, Inhibits GJIC in a Dose-Dependent and Reversible Manner in RLE Cells.** We first investigated the effect of EGCG on GJIC in RLE cells. EGCG inhibited GJIC in a dose-dependent manner in RLE cells, whereas EC had no effect on GJIC up to 200 $\mu$M and enhanced GJIC at 400 $\mu$M up to 136% (**Figure 2A** and **B**). Compared to the data collected at 0 min, 200 $\mu$M EGCG inhibited GJIC by 30 and 40% after 60 and 120 min, respectively; however, GJIC was completely restored 240 min after treatment, indicating that the EGCG-induced inhibition of GJIC is reversible (**Figure 2C**). The amount of DMSO used to dissolve the EGCG and EC stocks had no effect on GJIC (data not shown).

**EGCG Induces the Phosphorylation of Cx43 and ERK1/2 in RLE Cells.** We next studied the effects of EGCG on Cx43 and ERK1/2 in RLE cells, which is responsible for the inhibition of GJIC. We found that EGCG induced the phosphorylation of
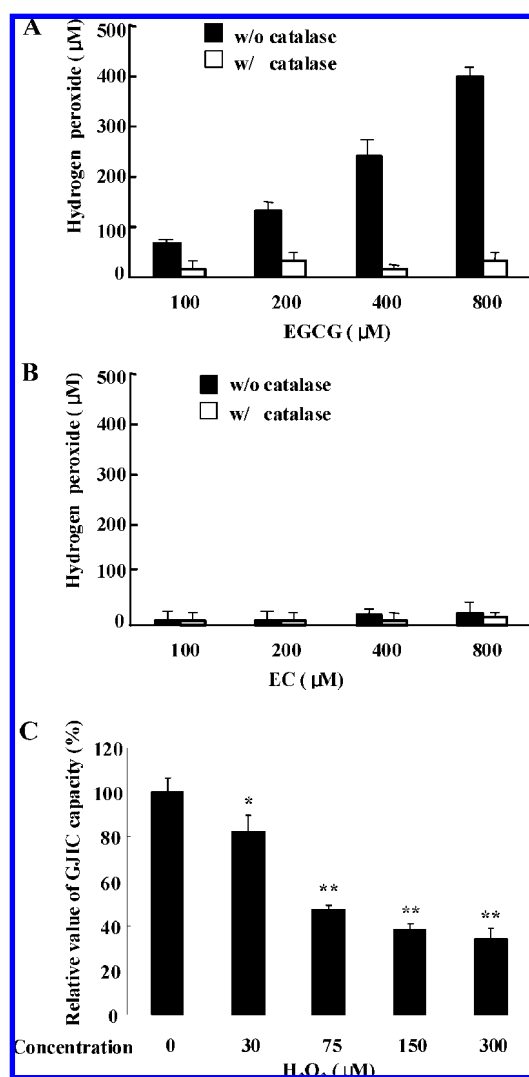


**Figure 4.** Inhibitory effects of hydrogen peroxdide ($H_2O_2$) generated by EGCG on GJIC. Production of $H_2O_2$ by EGCG (**A**) or EC (**B**) in the presence or absence of catalase. The values are the means ± SD from at least six independent experiments. The asterisks indicate the significant difference of $H_2O_2$-generation between EGCG and EC (**, $p < 0.01$). (**C**) Inhibitory effects of $H_2O_2$ on GJIC. The relative recovery rate was determined by counting the number of communicating cells. The values are the means ± SD from at least three independent experiments. The asterisks indicate significant decreases in the number of communicating cells in the group treated with EGCG compared to the untreated controls (*, $p < 0.05$; **, $p < 0.01$).

Cx43 but did not affect its expression (**Figure 3A**). Three major bands (P0, P1, and P2) were detected in the untreated cells, and mobility shifts from P0 or P1 to P2 or P3 (with a higher molecular weight) indicated the phosphorylation of Cx43. The cells treated with EGCG exhibited shifts to P2 based on the relative band intensity. We found that EGCG strongly induced the phosphorylation of ERK1/2 in RLE cells (**Figure 3B**), indicating that ERK1/2 signaling is involved in the EGCG-induced phosphorylation of Cx43, which inhibits GJIC.

**EGCG Possesses Prooxidant Activity.** We examined the possible prooxidant activity of EGCG. EGCG, but not EC, produced $H_2O_2$ in a dose-dependent manner (**Figure 4A** and **B**). At 100 and 200 $\mu$M, EGCG produced 67 and 133 $\mu$M $H_2O_2$, respectively, within 1 h, while at 200 $\mu$M, EC produced only 9 $\mu$M $H_2O_2$ under the same conditions. The production of $H_2O_2$ was confirmed by treatment with catalase, which effectively
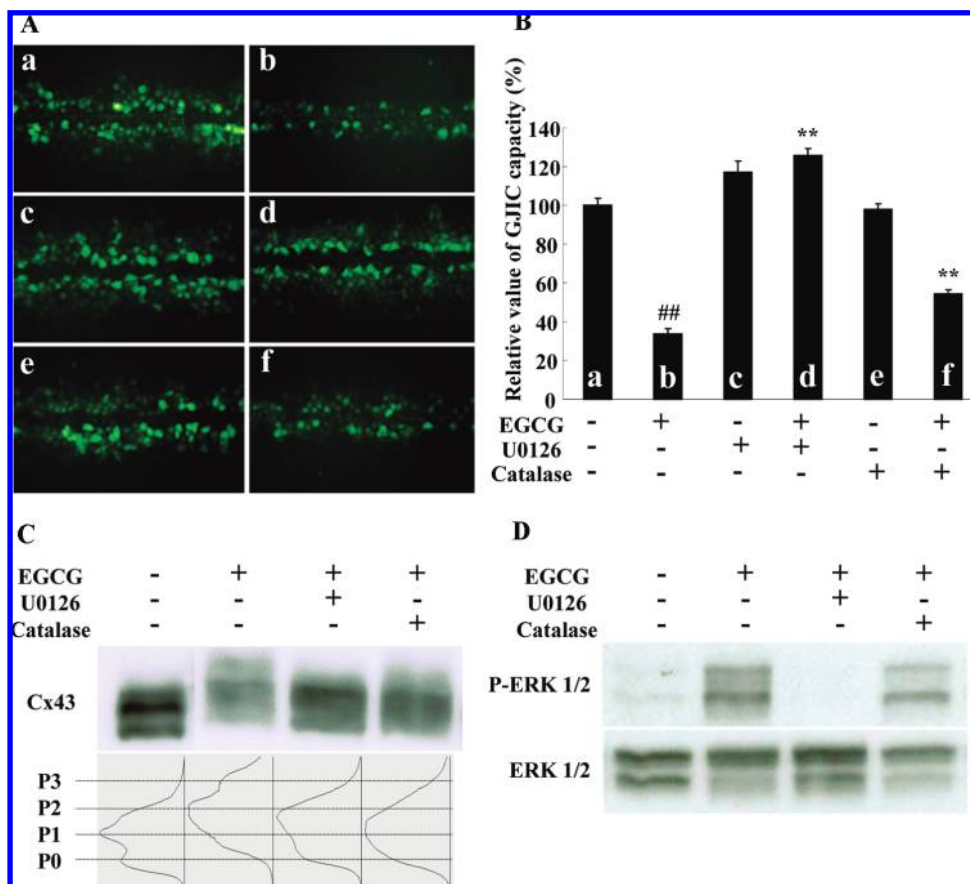
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**Figure 5.** Effects of U0126 and catalase on the EGCG-induced inhibition of GJIC in RLE cells. (**a**) Untreated control, (**b**) 200 $\mu$M EGCG, (**c**) 10 $\mu$M U0126, (**d**) 200 $\mu$M EGCG and 10 $\mu$M U0126, (**e**) 100 U of catalase, and (**f**) 200 $\mu$M EGCG and 100 U of catalase. (**A**) Protective effects of U0126 and catalase on the EGCG-induced inhibition of GJIC. Representative images are shown for each group. (**B**) Quantitative analysis of the protective effects of U0126 and catalase at various concentrations on the EGCG-induced inhibition of GJIC. The values are the means $\pm$ SD from at least three independent experiments. ## $p < 0.01$ vs untreated control (**a**); ** $p < 0.01$ vs EGCG treatment (**b**). Protective effects of U0126 and catalase on the EGCG-induced phosphorylation of Cx43 (**C**) and ERK1/2 (**D**). RLE cells were pretreated with 10 $\mu$M U0126 or 100 U of catalase for 30 min prior to 30 min of stimulation with 200 $\mu$M EGCG. Cell lysates were then prepared and immunoblotted with anti-Cx43, -phospho-ERK1/2, or -ERK1/2 antibodies.

reduced the amount of $H_2O_2$ generated by EGCG. The amount of DMSO used to dissolve the EGCG and EC stocks had no effect on our FOX assay results (data not shown). We next measured the inhibitory effects of $H_2O_2$ on GJIC (**Figure 4C**). $H_2O_2$ inhibited GJIC in a dose-dependent manner, indicating that $H_2O_2$ produced by EGCG correlates with the GJIC-inhibitory effect of EGCG.

**Catalase and a MEK Inhibitor May Be Used to Prevent the EGCG-Induced Inhibition of GJIC in RLE Cells.** To determine whether the EGCG-induced inhibition of GJIC is mediated by the phosphorylation of ERK1/2 and the production of $H_2O_2$, we examined the effects of U0126 (a pharmacological inhibitor of MEK, the upstream kinase of ERK1/2) and catalase on the EGCG-induced inhibition of GJIC in RLE cells. Both U0126 and catalase protected against the EGCG-induced inhibition of GJIC, but catalase, compared with U0126, partially protected the GJIC (**Figure 5A** and **B**). The EGCG-induced phosphorylation of Cx43 (**Figure 5C**) and ERK1/2 (**Figure 5D**) was also inhibited by treatment with U0126 or catalase, but again the inhibition by catalase was incomplete. Thus, high doses of EGCG directly induce the inhibition of GJIC through ERK1/2 phosphorylation via the compound's prooxidant activity.

## DISCUSSION

GJIC plays an important role in the normal development of multicellular organisms. Specifically, it helps create and integrate extracellular phenotypes and functions, such as growth control and multiple types of gene expression patterns, in normal cells. Without GJIC, the higher-order phenotypes and functions that exist during different stages of cell development might be impaired. Certain chemicals and genetic factors that downregulate the function of gap junctions were reported to induce a wide range of abnormal developmental and functional processes, suggesting the vital role of gap junctions in normal development and function (*25*). During the epigenetic induction of carcinogenesis by chemical toxicants, the lack of GJIC plays an important role at both the promotion and progression stages.

In the present study, EGCG, but not EC, inhibited GJIC in the absence of $H_2O_2$. Differences in structure between EGCG and EC (a hydroxyl group on the B-ring and a gallic acid moiety) are associated with the differential effects of the compounds on the regulation of GJIC. The inhibition of GJIC by EGCG was reversible, which suggests that it influences the promotion stage of carcinogenesis. A recent study demonstrated that 40 $\mu$M EC enhanced GJIC 1.5-fold after 24 h (*26*); similarly, our results showed that 400 $\mu$M EC enhanced GJIC 1.36-fold after 1 h. Although the antioxidant activity of EGCG is stronger than that of EC, only EGCG over 100 $\mu$M inhibited GJIC in RLE cells, which shows that antioxidant activity and its effects on GJIC do not always act in the same direction. However, at 180 $\mu$M, EGCG was reported to protect against the dimethylnitrosamine-induced inhibition of GJIC in Mardin-Darby

canine kidney cells (*27*). Another report showed that EGCG at 5−25 enhanced GJIC inhibited by DDT in RLE cells (*28*). Previously, we showed that EGCG at 1 or 5 $\mu$g/mL protected against the $H_2O_2$-induced inhibition of GJIC in RLE cells, while at higher concentrations, 20 $\mu$g/mL or more, EGCG inhibited GJIC in the absence of $H_2O_2$ (*29*). These contradictions suggest that the influence of various chemicals on GJIC differs depending on the presence of an inducer and on the concentration and cell type used.

In the present study, we investigated the mechanism of the EGCG-induced inhibition of GJIC in RLE cells. We found that EGCG, but not EC, induced the phosphorylation of Cx43 and ERK1/2. Activation of the ERK signal has been previously reported to have a role in EGCG-induced cellular response (*30*). U0126 fully restored the EGCG-induced inhibition of GJIC and blocked the EGCG-induced phosphorylation of Cx43 and ERK1/2 indicating that the MEK/ERK1/2 pathway plays a key role in the EGCG-induced inhibition of GJIC. Quinones such as menadione inhibit GJIC through the phosphorylation of Cx43 mediated by epidermal growth factor receptor (EGFR) (*31, 32*). Given that EGCG can be converted into a quinone in the presence of metal ions (*7*), the EGCG-induced inhibition of GJIC may be mediated by EGFR (and PKC), which acts upstream of ERK1/2. However, we do not exclude the possibility that other signaling pathways such as p38 MAP kinase are also involved in EGCG-induced inhibition of GJIC.

EGCG generated significant amounts of $H_2O_2$ in a dose-dependent manner, and the concentration was sufficient to inhibit GJIC. Several studies have suggested that the carcinogenicity of reactive oxygen species (ROS) is involved in the inhibition of GJIC, and hence, the chemopreventive effects of these compounds can be tested by investigating where they restore the $H_2O_2$-induced inhibition of GJIC (*20, 33*). $H_2O_2$, however, causes the DNA damage that is associated with tumor initiation, but $H_2O_2$ has also been reported to promote cancer through the inhibition of GJIC (*16, 34*). The $H_2O_2$-induced inhibition of GJIC is mediated by the phosphorylation of Cx43 via the activation of MAPKs, particularly ERK1/2 (*35, 36*). The EGCG-induced inhibition of GJIC was partially reduced by the addition of catalase, which differs somewhat from our previously reported data concerning the effects of catalase on the $H_2O_2$-induced inhibition of GJIC (*37*). These results indicate that the EGCG-induced inhibition of GJIC is partly related to the production of $H_2O_2$ in media, but other factors may be linked to the EGCG-induced inhibition of GJIC. This may be due to the effects of ROS besides $H_2O_2$ because the FOX assay cannot detect every type of ROS. The possibility exists that EGCG generates significant amounts of ROS.

Phenolic substances are generally recognized as antioxidants, but they can exert prooxidant activities under certain conditions, such as in the presence of transition metal ions or alkalis. A recent study has shown that the concentration of EGCG detected in the liver of mice has a linear relationship to the dose of administration even with the high concentrations (*38*). Our findings suggest the necessity for safety standards for dietary supplements made from isolated phenolic compounds.

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