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# Down-regulation of connexin43 gap junction by serum deprivation in human endothelial cells was improved by (−)-Epigallocatechin gallate via ERK MAP kinase pathway

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ABSTRACT

Intercellular communication through gap junctions (GJIC) plays an essential role in maintaining the functional integrity of vascular endothelium. Despite emerging evidence suggests that (−)-Epigallocatechin gallate (EGCG) may improve endothelial function. However, its effect on Cx43 gap junction in endothelial cells remains unexplored. Here we investigated the effect of EGCG on connexin43 (Cx43) gap junction in endothelial cells. The levels of Cx43 protein in human umbilical vein endothelial cells (HUVECs) cultured under serum-deprivation 48 h decreased about 50%, accompanied by decreased GJIC. This reduction can be reversed by treatments with EGCG. In addition, EGCG activated ERK, P38, and JNK mitogen-activated protein kinases (MAPKs), which were supposed to participate in the regulation of Cx43. A MEK inhibitor PD98059, but not SB203580 (a p38 kinase inhibitor) or SP600125 (a JNK kinase inhibitor), abolished the effects of EGCG on Cx43 expression and GJIC. Moreover, although both Akt and eNOS phosphorylation were time-dependently augmented by EGCG, neither PI3K inhibitor LY294002 nor eNOS inhibitor L-NAME blocked the effects of EGCG on Cx43 gap junctions. Thus, EGCG attenuated Cx43 down-regulation and impaired GJIC induced by serum deprivation, ERK MAPK Signal transduction pathway appears to be involved in these processes.



## 1. Introduction

Gap junctions play many roles in vascular biology, including control of vascular tone, permeability, angiogenesis, and remodeling. Changes of gap junctions have been correlated to the development of vascular diseases, such as hypertension, atherosclerosis, or restenosis [1]. It has been reported that endothelial cells mainly express Cx43, Cx40, and Cx37, of which Cx43 is predominant in cultured endothelial cells [2]. Apart from forming gap junction channels for exchange of ions and small molecules between adjacent cells, Cx43 also participates in the modification of cell-cycle and transcription regulation [3]. Cx43 deficiency causes outflow obstruction associated with infundibular pouches, indicating Cx43 plays a pivotal role in coronary vasculogenesis and vascular remodeling [4]. Previous studies demonstrated that Cx43 was down-regulated in endothelial cells exposed to risk factors for atherosclerosis, such as ageing [5], nicotine [6,7], diabetes [8,9], and hypertension [10]. Down-regulation of Cx43 by Cx43-specific small interference RNA (siRNA) inhibits gap-junctional communication, and activated endothelial cells to pathological status [2]. Vascular endothelial cells-specific knockout of Cx43 causes hypotension and bradycardia [11]. These findings suggest that reduced expression of Cx43 gap junctions is a potential indicator of endothelial dysfunction.

(−)-Epigallocatechin gallate (EGCG), the major constituent found in green tea polyphenols, is responsible for the majority of the potential health benefits attributed to green tea consumption. Beneficial effects of EGCG therapy have been reported in a number of human and animal studies, including the prevention of LDL oxidation, reduction of platelet aggregation, lipid regulation, and inhibition of proliferation and migration of smooth muscle cells [12,13]. Any of these factors might be promising in reducing cardiovascular diseases. Despite a wealth data about the chemo-preventive properties of EGCG for cancer and cardiovascular system, its effect on Cx43 gap junction in endothelial cells, which is associated with the promotion and progression of endothelial dysfunction, has not been studied.

In the present study, we aimed to investigate the effect of EGCG on Cx43 gap junction in endothelial cells and the implication of MAPKs signaling in this process.

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## 2. Materials and methods

### 2.1. Cell culture and chemicals

Human umbilical vein endothelial cells (HUVECs) obtained from ScienCell (Carlsbad, CA, USA) were maintained according to the manufacturer's instructions in RMPI 1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in a humidified incubator maintained at 37 °C and supplied with 5% $CO_2$ and 95% air. 3–8 passages were used in all experiments.

EGCG, Lucifer Yellow CH, Rhodamine–dextran, 2′,6′-diamidino-2-phenylindole (DAPI), 18 α-glychrrhetinic acid (18-AGA), LY294002, $N^G$-nitro-arginine methyl ester (L-NAME) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO, USA). PD98059, SB203580 and SP600125 were purchased from Calbiochem (La Jolla, CA, USA).

### 2.2. Determination of GJIC by scrape loading/dye transfer technique

The scrape loading/dye transfer (SL/DT) technique was performed as previously described [14] with minor modifications. Cells ($2 \times 10^5$) were plated onto 35 mm Petri dishes 2 days before the experiment. Before scrape loading the confluent cell layer was washed twice with PBS and was then covered with a preheated (37 °C) solution of 0.05% Lucifer Yellow CH and 0.05% Rhodamine–dextran dissolved in PBS without $Ca^{2+}$ and $Mg^{2+}$. For each dish of cells, the cell monolayer was cut 5–6 times with a surgical scalpel. After 3.5 min following scraping the dye was removed, dish rinsed three times with PBS and cells fixed with 4% paraformaldehyde. The distance traveled by the dye in a direction perpendicular to the scrape was observed with an inverted fluorescent microscope (Olympus, Tokyo, Japan).

### 2.3. Quantitative RT-PCR

Total RNA was isolated from cells using the TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA (1–2 μg) was converted into cDNA using murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). Specific primers matching the published sequences were used to identify and amplify Cx43 (216 bp, sense primer: 5′-AAT TCA GAC AAG GCC CAC AG-3′; anti-sense primer: 5′-CAT GGC TTG ATT CCC TGA CT-3′). Glyceraldehyde-3-dehydrogenase (GAPDH, 136 bp, sense primer: 5′-GGG TGT GAA CCA TGA GAA GT-3′; anti-sense primer: 5′-GAC TGT GGT CAT GAG TCC T-3′) was amplified as a reference. For the quantitative measurement of mRNA, real-time PCR was performed on the ABI 7500 cycler (Applied Biosystems, CA, USA) using SYBR green PCR mix (Takara, Shiga, Japan). Fold change of relative mRNA expression of Cx43 was calculated using the 2-ΔΔCt method as described before [15].

### 2.4. Small interference RNA transfection

siRNA duplexes were synthesized by GenePharma (Shanghai, China). Twenty-one base sequences of the human Cx43 gene targeting the trans-membrane domain of Cx43 were chosen as described previously [2]. Cx43 sense siRNA sequence is 5′-GGU GUG GCU GUC AGT ACU UdTT-3′; Cx43 anti-sense siRNA sequence is 5′-AAG UAC UGA CAG CCA CAC CdTT-3′. A non-silencing siRNA (sense: 5′-UUC UCC GAA CGU GUC ACG UdTT-3′; anti-sense: 5′-ACG UGA CAC GUU CGG AGA AdTT-3′) was used as negative control. Cells in six-well plates were transfected with siRNAs using Hiperfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, 100 pmol siRNA against Cx43 or control siRNA was diluted in 500 μl culture medium without serum, and mixed with 12 μl Hiperfect transfection reagent by vortexing for 10 s. Cells were incubated with the transfection complexes for 3 h under normal growth conditions, before 1 ml fresh culture medium containing serum was added. Verification of siRNA efficacy was achieved by real-time polymerase chain reaction and Western blot.

### 2.5. Immunocytochemistry

Cells were seeded on round glass coverslips coated with collagen in 24-well plates, then fixed with 4% paraformaldehyde. After fixation, cells were permeabilized with 0.1% Triton-X-100 in PBS, and non-specific binding sites were saturated with 10% goat serum for 30 min. Cells were further incubated with the connexin43 antibody in PBS-1% BSA, and then with goat secondary antibodies coupled to Alexa Fluor® 568 (Molecular Probes). Nuclei were stained with 0.5 μg/ml DAPI for 5 min. Coverslips were observed with an inverted fluorescent microscope.

### 2.6. Western blot and antibody

Cells were scraped in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA) supplemented with 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 1 mM $Na_3VO_4$, and 10 mM NaF. Proteins (20–40 μg) were separated by 10% SDS–polyacrylamide gels and electro-transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were incubated overnight with primary antibody in the appropriate dilution, before incubation for 1 h with a secondary antibody conjugated to horseradish peroxidase (1:10,000). After reaction with enhanced chemiluminescence reagent (Amersham, Haemek, Isreal), the images were captured on the image reader LAS-4000 system (Fujifilm, Tokyo, Japan).

Anti-Akt, anti-phospho-Akt, anti-eNOS, anti-phospho-eNOS, anti-ERK1/2, anti-phospho-ERK1/2, anti-JNK 1/2, anti-phospho-JNK 1/2, anti-p38 MAPK and anti-phospho-p38 MAPK for Western blotting were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-Cx43 was from Sigma. Anti-GAPDH polyclonal antibody was purchased from Santa Cruz (CA, USA).

### 2.7. Statistical analysis

All experiments were performed at least three times. Data were expressed as means ± SEM and analyzed by unpaired Student's *t*-test for comparisons between two groups or one-way ANOVA for multiple comparisons. *P* values <0.05 were considered statistically significant.

## 3. Results

### 3.1. EGCG treatment ameliorated the GJIC-inhibitory effect of serum deprivation

To investigate the effect of EGCG on GJIC in endothelial cells, the GJIC activity of HUVECs was assessed using SL/DT assay. Cells were loaded with Lucifer Yellow CH after serum deprivation with or without EGCG treatment. We also used a high molecular weight dye, Rhodamine–dextran, which is gap junction-impermeant, to exclude the non-gap junction-mediated dye loading [16]. Cells were loaded with a 0.05% Rhodamine–dextran solution for 3.5 min. Only primary loaded cells of the scrape line were observed to contain Rhodamine–dextran, indicating that non-gap junction-mediated dye transfer in present study was insignificant (data not shown). After serum-deprivation 48 h, significant inhibition of GJIC activity was observed as compared to control. A 24-h treat-

ment with EGCG markedly ameliorated the GJIC-inhibitory effect of serum deprivation in a concentration-dependent manner (Fig. 1).

### 3.2. Effect of EGCG on Cx43 expression and localization

Cx43 protein level of HUVECs was significantly down-regulated by serum deprivation, whereas Cx43 mRNA level determined by real-time RT-PCR did not vary significantly compared with cells grown in media containing 10% FBS (Fig. 2A and B). Western blot analysis revealed that the down-regulating effect of serum deprivation on Cx43 protein in HUVECs was attenuated by EGCG treatment in a time and dose-dependent manner (Fig. 2B and C). We also examined the effect of EGCG on Cx43 without serum deprivation. Both Cx43 mRNA and protein of HUVECs remained unchanged under EGCG treatment 24 h (data not shown).

The abundance of Cx43 does not necessarily correlate with the amount of intracellular communication. Intercellular communication is either associated with changes in gap junction activity or with the levels of Cx43 at the plasma membrane available to form gap junctions [17]. HUVECs were immunostained with antibodies directed against Cx43 and imaged by immunofluorescence microscopy (Fig. 2D). In control cells, Cx43 was localized both to the plasma membrane, to cell–cell contacts, and intracellularly, and gap junctions appeared as a fine and continuous line bordering the cells. In cells under serum deprivation, significant decreases were observed in the abundance of Cx43 detected at the sites of contacts between adjacent cells. EGCG treatment significantly increased the membrane-associated Cx43 amount as compared to cells under serum deprivation alone.

### 3.3. Recovery of GJIC activity by EGCG is mediated by Cx43

We also examined the effect of siRNA mediated knock-down of Cx43 on EGCG-induced GJIC recovery in HUVECs. After transfection with siRNA, Cx43 expression was decreased approximately 70% as compared to non-silencing RNA controls which was confirmed by real-time PCR and western blotting (Fig. 3A and B). The recovery of HUVECs GJIC activity by EGCG treatment was completely abrogated by Cx43 silencing (Fig 3C), indicating that the effect of EGCG on HUVECs GJIC was specifically via Cx43 rather than other connexins of gap junction, such as Cx37 and Cx40.

### 3.4. EGCG treatment activates MAP kinases and PI3K/Akt/eNOS pathway in HUVECs

We examined the activation of several MAPKs (ERK1/2, p38 and JNK) to identify the protective action of EGCG on the Cx43 gap junction and GJIC activity. As shown in Fig. 4A, EGCG could activate both ERK1 and ERK2. The phospho-ERK1/2 level reached a maximum 60 min after 40 μM EGCG treatment. EGCG also induced P38 (Fig. 4B) and JNK (Fig. 4C) activation, which were comparable with the time course observed for ERK1/2.

We also investigated the effects of EGCG on Akt and eNOS phophorylation. As illustrated in Fig. 4D and E. EGCG treatment (40 μM) of HUVECs stimulated Akt phosphorylation in a time dependent manner. The phospho-Akt-Ser$^{473}$ level reached a maximum 30 min after EGCG treatment, whereas no significant change in total Akt expression was observed over the course of the experiments. EGCG induced eNOS phosphorylation was similar to Akt phosphorylation while peaked at 60 min after EGCG treatment.

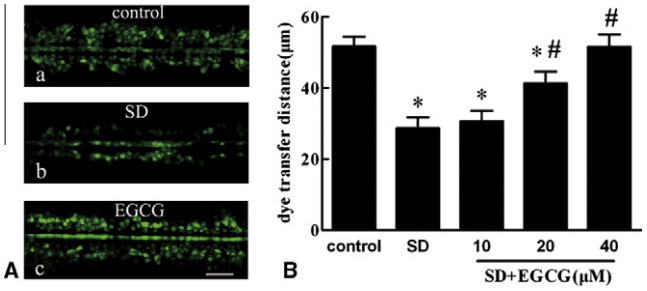


**Fig. 1.** Effect of EGCG on intercellular communication between HUVECs as assessed by SL/DT technique. Cells were loaded for 3.5 min with a 0.05% (w/v) Lucifer Yellow CH solution. (A) Confluent cells were treated control (a), serum-deprivation-only 48 h (b), serum-deprivation 48 h plus EGCG treatment (40 μM, 24 h) (c). (B) Quantification of dye transfer distance. Data are represented as mean ± SEM of three independent experiments. Control, cells without any treatment; SD, serum-deprivation-only 48 h; EGCG, serum-deprivation 48 h plus EGCG treatment (40 μM, 24 h). $P < 0.05$ compared to *control and #SD. Bar, 50 μm.

### 3.5. EGCG improved Cx43 gap junction was ERK MAP kinase dependent

We investigated the roles of MAP kinases and PI3K/Akt on improving HUVECs GJIC inhibition when treated with EGCG. Confluent HUVECs were pretreated for 30 min with 100 μM eNOS inhibitor L-NAME or 10 μM LY294002, a PI3K inhibitor, or 20 μM PD98059, a selective inhibitor of MEK1/2, the upstream activator of ERK1/2, or 10 μM SB203580, a specific inhibitor of p38 MAPK, or 10 μM SP600125, a specific inhibitor of JNK. As shown in Fig. 4H, the pharmacological inhibition of MEK1/2 (PD98059) completely abolished the protective effect of EGCG on GJIC in HUVECs. In contrast, other two MAPK inhibitors (SB203580, SP600125) and PI3K inhibitor LY294002 or eNOS inhibitor L-NAME did not attenuate the effect of EGCG on GJIC. PD98059 also completely abolished the effect of EGCG on Cx43 protein expression (Fig. 4G), which was comparable with the result observed for GJIC. These results demonstrated that the ERK1/2 MAPK signal transduction pathway may be involved in the effect of EGCG on Cx43 gap junction.

## 4. Discussion

It is demonstrated that Cx43 plays multiple roles in ischemia-induced arrhythmia and ischemia preconditioning cardioprotection [18–20]. However, little information is actually available about the effect of ischemia environment on endothelial Cx43, which plays multiple roles in vascular biology. Here, we used serum deprivation to mimic the harsh ischemic microenvironment in vitro as previous studies reported [21,22]. Preliminary studies show that serum starvation down-regulated Cx43 expression in cultured cells [23,24]. Serum starvation caused reversible lysosomal degradation of Cx43, which can be reversed by lysosomal inhibitor $NH4^+$ (1 mM) or proteinase inhibitor leupeptin (1 mg/ml) in osteoblast cells [23]. Data reported in this study showed that the total amount of Cx43 protein in HUVECs decreased approximately 50% by serum deprivation, whereas the levels of Cx43 mRNA did not vary significantly, indicating serum starvation reduce Cx43 expression through a post-transcriptional regulation, most likely through augmented degradation of Cx43. In good accordance with the results of Western blot, immunocytochemistry exhibited similar decreases of membranous Cx43 at the sites of contacts between adjacent cells. Most importantly, our dye transfer experiments indicated that reduced Cx43 expression was accompanied with decrease of GJIC, indicating that the reduced Cx43 expression seems to be of functional relevance.

Although promising experimental and clinical data demonstrate that EGCG have beneficial effects against cancer and cardiovascular disease [13], its effect on connexin43 and gap-junction intercellular communication remains rather controversial. Takahashi et al. [25] demonstrated that the levels of GJIC and Cx43 expression were markedly decreased in Mardin-Darby canine

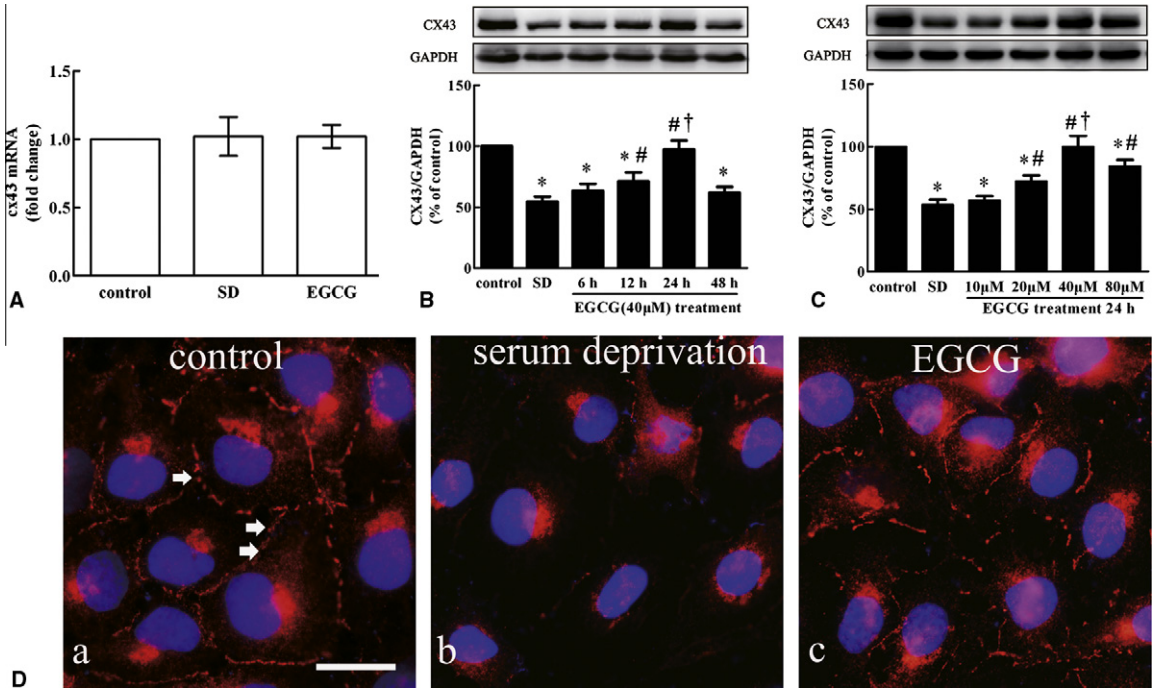


**Fig. 2.** Effects of EGCG on Cx43 gene and protein expressions. (A) Effects of EGCG on Cx43 gene expressions. (B) Time-dependent effects of EGCG on Cx43 protein expressions. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to SD and $^{\dagger}P < 0.05$ compared to 6, 12 and 48 h. (C) Dose-dependent effects of EGCG on Cx43 protein expressions. $^{*}P < 0.05$ compared to control, $^{\#}P < 0.05$ compared to SD and $^{\dagger}P < 0.05$ compared to 10, 20 and 80 μM. Data are mean ± SEM. (D) Epifluorescence micrographs of HUVECs, stained with anti-Cx43 antibody in combination with DAPI. (a) In control group, Cx43 gap junctions appear as a fine and continuous line (arrows) bordering the cells. Immunoreactivity is also present in the cytoplasm. (b) Significant decreases were observed in the abundance of Cx43 detected at the sites of contacts between adjacent cells under serum deprivation compared to control group. (c) Incubation of cells with EGCG (40 μM, 24 h) significantly increased the membrane-associated Cx43 compared to serum-deprivation-only group. Bar, 20 μm.

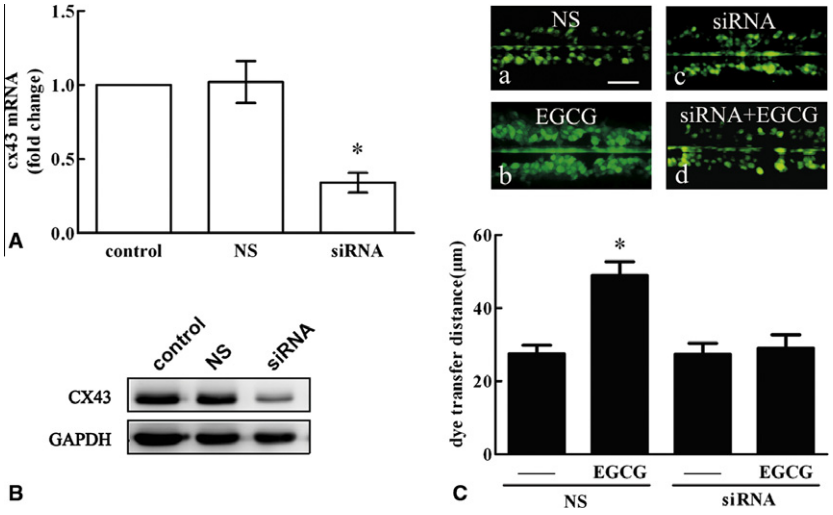


**Fig. 3.** Connexin43-specific siRNA abrogated the effect of EGCG on GJIC under serum deprivation. Uptake of Cx43 siRNA reduces connexin43 expression, as confirmed using quantitative RT-PCR (A), and western blotting (B). (C) scrape loading/dye transfer experiments were performed in cells under serum deprivation treated with non-silencing control (a), non-silencing control plus EGCG treatment (b), Cx43 siRNA alone (c), and Cx43 siRNA co-treatment with EGCG (d). $^{*}p < 0.05$ compared with EGCG-untreated for each group. NS, non-silencing control. $^{*}p < 0.05$ compared to control. Bar, 50 μm.

kidney cells exposed to dimethylnitrosamine. A 12 h pretreatment with EGCG greatly ameliorated the GJIC-inhibitory effects of dimethylnitrosamine. However, in normal rat liver epithelial cells [26], EGCG (100–400 μM) inhibited GJIC activity in a dose-dependent and reversible manner. In our study, reduced Cx43 protein and GJIC in HUVECs induced by serum starvation were significantly attenuated by treatment with EGCG in a concentration-dependent manner. It was noteworthy that Cx43 mRNA was not affected by EGCG treatment, suggesting Cx43 expression may be regulated by EGCG through decreasing Cx43 degradation rather than enhancing Cx43 synthesis. The different concentrations of EGCG and cell types used in different experiments may partly contribute to the different effects of EGCG between current and previous study, which need further investigations to explore the detailed mechanisms.

The effect of EGCG on GJIC activity might also be mediated by other junction protein components such as Cx40, Cx37, and Cx32, which was also expressed in various cultured human endothelial

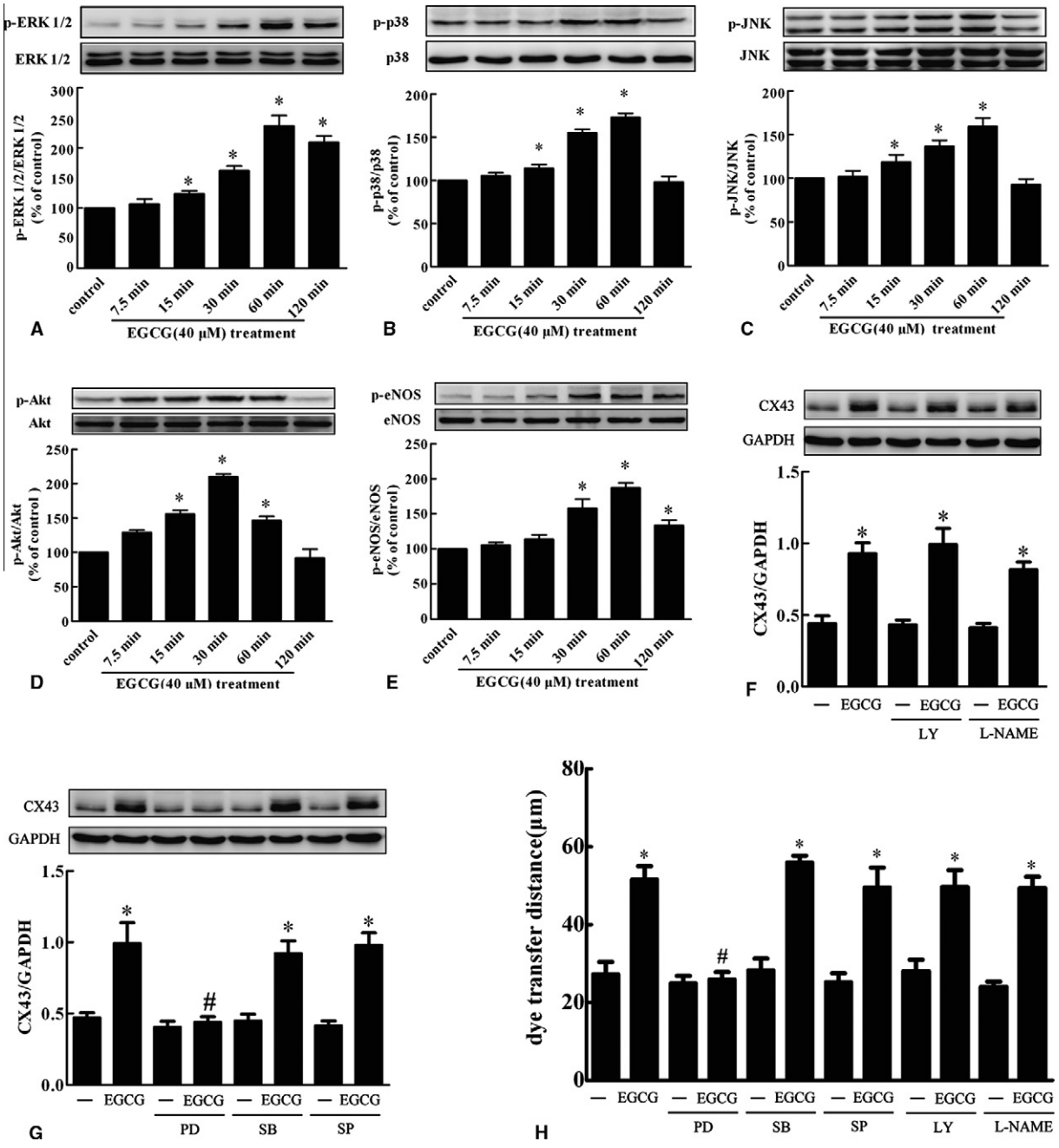


**Fig. 4.** ERK MAPK plays a role in EGCG-mediated effects on Cx43 gap junction within HUVECs. (A–E) Effects of EGCG on phosphorylated-ERK (p-ERK), Phosphorylated-p38 (p-p38), phosphorylated-JNK (p-JNK) MAPK pathway and phosphorylated-AKT (p-AKT), phosphorylated-eNOS PI3K/Akt/eNOS pathway in HUVECs. $^{*}p < 0.05$ compared to control, Data are represented as mean ± SEM. (F–H) After cells were treated with inhibitors (PD98059, SB203580, SP600125, LY294002 or L-NAME) alone or in the present of EGCG (40 μM, 24 h) under serum deprivation, western blot analysis and SL/DT assay were performed to examine Cx43 protein expression and GJIC activity separately. (F–G) Representative example and quantification of the Western blot analysis of Cx43 protein expression. (H) Quantification of dye transfer distance. Each bar represents mean ± SEM. of three independent experiments. Data are expressed as a ratio of Cx43 to GAPDH. Each bar represents mean ± SEM. ($n = 3$). $^{*}P < 0.05$ compared to EGCG-untreated for each group, $^{\#}P < 0.05$ compared to serum-deprivation 48 h plus EGCG treatment (40 μM, 24 h).

cells and participated in endothelial gap-junction intercellular communication [27]. In the present study, Cx43 protein reduction and impairment of GJIC were rectified by EGCG treatment in a dose-dependent manner. Cx43-silencing by specific siRNA drastically abolished the effect of EGCG on GJIC activity. Our findings indicated that increased GJIC activity in HUVECs exposed to EGCG treatment is at least partly due to the up-regulation of Cx43.

It is well established that MAPKs play critical roles in regulating Cx43 expression. Cho et al. [28] reported that mushroom Phellinus linteus extract prevented inhibition of GJIC by hydrogen peroxide via the ERK1/2 and p38 MAP kinases. Activation of P38 MAP kinase by anisomycin leaded to loss of Cx43 in WB-F344 cells, possibly owing to accelerated degradation, and these losses might be responsible for the reduction in numbers of gap junctions and in function of intercellular communication [29]. Tacheau et al. [30] recently reported that TNF-alpha repressed connexin43 expression and GJIC activity in HaCat keratinocytes via activation of JNK signaling. In the present study, we showed that EGCG activated the time-dependent phosphorylation of MAPK family proteins (ERK, p38, JNK) in HUVECs. Inhibition of ERK by co-treatment of PD98059 was able to completely abolish the ameliorative effects of EGCG on Cx43 protein and GJIC activity in HUVECs. However,

SB203580 (a inhibitor of p38 MAPK) and sp600125 (a inhibitor of JNK MAPK) failed to block the effects of EGCG on impaired Cx43 gap junction. Taken together, the data presented here suggested that ERK MAPK was responsible for the protective effects of EGCG on Cx43 gap junction.

Besides MAPKs, PI3K/Akt [10] and eNOS [31] may also participate in the regulation of Cx43 expression. It has been reported that treatment with EGCG induced a sustained activation of Akt and eNOS $Ser^{1179}$ phosphorylation in endothelial cells [32], which was similar as Kim et al. [33] demonstrated recently. In accordance with preliminary studies, the results of our study discovered that treatment of EGCG time-dependently activated both Akt and eNOS. Inhibition of Akt and eNOS by pretreatment with LY294002 and L-NAME respectively, failed to abrogate the ameliorative effects of EGCG on Cx43 gap junction, suggesting that the activation of Akt and eNOS by EGCG treatment does not correlate with the regulation of Cx43 gap junction and intercellular communication in HUVECs.

## 5. Conclusion

We provide the first experimental evidence that the green tea polyphenol EGCG protected Cx43 gap junction from serum starvation induced impairment via ERK MAPK pathway.

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