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Involvement of early growth response gene 1 in the modulation of microsomal prostaglandin E synthase 1 by epigallocatechin gallate in A549 human pulmonary epithelial cells

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Abbreviations:
EGCG, (−)-epigallocatechin gallate
PGES, prostaglandin E synthase
COX, cyclooxygenase
EGR-1, early growth response gene 1
cPGES, cytosolic PGES
mPGES, microsomal PGES
NSCLC, non-small cell lung cancer
MAP kinase, mitogen activated protein kinase
ERK, extracellular signal-regulated protein kinase
PDGF, platlet derived growth factor
EGF, epithermal growth factor
NF-κB, nuclear factor κB

ABSTRACT

The prostaglandin E_2 (PGE_2) can play critical roles in the pulmonary inflammation or carcinogenesis. It is the first investigation of the effect of a green tea polyphenol, (−)-epigallocatechin gallate (EGCG), on the PGE_2-producing microsomal prostaglandin E synthase 1 (mPGES-1) expression in the lung alveolar type II pneumocytes, A549 cells as an epithelial model. EGCG enhanced cyclooxygenase (COX)-2 and mPGES-1 gene expression as well as PGE_2. Among several tea catechins, EGCG was most effective in inducing mPGES-1 expression. Moreover, even in the cytokine-stimulated cells, mPGES-1 protein was super-induced by EGCG treatment. As signaling mediators in mPGES-1 induction by EGCG, active ERK1/2 MAP kinases and early growth response gene 1 (EGR-1) were increased after exposure to EGCG. Moreover, EGCG stimulated the nuclear translocation of the EGR-1 protein in A549 cells through ERK signaling pathway. Recent studies demonstrate that EGR-1 is a key transcription factor in mPGES-1 gene expression. When blocking the gene expression of EGR-1 with EGR-1 siRNA or ERK inhibitor, EGCG-induced mPGES-1 was suppressed in both cases. mPGES-1 promoter with deleted or point-mutated EGR-1 binding sites showed significantly less response to the EGCG stimulation, which also implicated the importance of EGR-1 binding in promoting mPGES-1 gene expression. Taken all, EGCG was strong inducer of EGR-1 expression and mediated EGR-1 nuclear translocation via ERK signaling pathway in A549 pulmonary epithelial cells. Induced EGR-1 then stimulated the induction of mPGES-1 gene expression and this effect mechanistically can be linked to the pharmacological or toxicological actions after human exposure to green tea catechins.

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AP-1, activator protein-1
MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium
TBST, Tris-buffered saline plus Tween 0.05%
RT-PCR, reverse transcription-polymerase chain reaction
PGH_2, prostaglandin H_2
PGE_2, prostaglandin E_2
IRAK, interleukin-1β receptor-associated kinase
CREBP, cyclic AMP response element binding protein

1. Introduction

Prostaglandins are arachidonic acid metabolites involved in both the normal and pathologic responses. Prostaglandins are formed from a common unstable endoperoxide intermediate, prostaglandin H_2 (PGH_2) via enzymatic oxygenation of arachidonic acid that is catalyzed by cyclooxygenase (COX) [1]. Among the various downstream metabolites of COX-derived PGH_2, prostaglandin E_2 (PGE_2) is commonly regarded as a pro-inflammatory or a resolving anti-inflammatory mediator in the inflammatory process. Some investigations suggested that PGE_2 could be beneficial in the lung and some tissues like brain because of PGE_2's protective function in these regions despite many convincing data addressing PGE_2 as a pathologically aggravating mediator in the general inflammation and carcinogenesis [2,3].

The biosynthesis of prostaglandin E_2 (PGE_2) from arachidonic acid requires two enzymatic activities. First, COX converts arachidonic acid into prostaglandin H_2 (PGH_2). The second enzyme prostaglandin E synthase (PGES) then converts PGH_2 into PGE_2 [1,4]. Two isoforms of cyclooxygenase, COX-1 and COX-2, are well characterized and at least three PGES isoforms have been identified, including microsomal PGES-1 (mPGES-1), mPGES-2, and cytosolic PGES (cPGES). cPGES is constitutively and ubiquitously expressed and is preferentially coupled with COX-1 to promote immediate secretion of PGE_2. mPGES-2 is ubiquitously expressed, but its role remains unclear. In contrast, mPGES-1 is markedly up-regulated by inflammatory or mitogenic stimuli and is functionally coupled with COX-2 for the delayed PGE_2 synthesis. Recent reports demonstrated that early growth response gene product (EGR-1) as well as a NF-κB and AP-1 are key transcription factors in regulating the inducible expression of mPGES-1 [5,6].

Green tea is rich in bioactive flavonoids and indeed its consumption has been associated with health benefits, including decreased risk of cardiovascular diseases, diabetes, obesity, and cancer [7,8]. These effects have been largely attributed to (−) epigallocatechin-3-gallate (EGCG), the most abundant polyphenol from green tea leaves. EGCG has been extensively investigated as a phenolic antioxidant with anti-inflammatory or anti-cancer potential [9,10]. In the animal models, EGCG suppresses fibrotic lung diseases as well as airway inflammation in chemical-mediated asthma. Moreover, oral administration of green tea extract enhances resolution in a pulmonary inflammation, significantly reducing chronic fibrosis [11]. Previous report describes some adverse events such as upset stomach, nausea, heartburn, and abdominal pains [12]. Moreover, some epidemiological reports demonstrated that EGCG was associated with the green tea-induced respiratory dysfunctions found in the tea-packers. Inhalated EGCG aggravates the IgE-mediated responses by elevating histamine release [13–15]. Because of these toxicological actions of green tea in human exposure, application of green tea catechins in terms of the respiratory disease needs to be carefully considered with diverse research approaches.

Whereas EGCG has been known to have inhibitory action on the COX-2 enzymatic activity and expression in some tissues [16,17], recent studies demonstrated that EGCG can also induce COX-2 expression in the monocytes and astrocyte cells, which can imply some controversial roles of EGCG in the inflammatory processes in the different tissues because enhanced COX metabolites can exacerbate the disease processes [18,19].

The present study was performed to address the effect of EGCG on the PGE_2 production and a COX-2-downstream biosynthetic enzyme, mPGES-1 in the human pulmonary epithelial cells. We also investigated the signaling pathway including EGR-1 and ERK1/2 which mediated mPGES-1 gene expression. The study will provide a promising molecular evidence for the pulmonary PGE_2 production by EGCG.

2. Materials and methods

2.1. Cell culture and reagents

Human pulmonary epithelial cell line A549 cells and human colonic epithelial cell HT-29 and HCA-7 were purchased from American Type Culture Collection (Rockville, MD). A549 and HCA-7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) and HT-29 cells were cultured in RPMI 1640 (Invitrogen), respectively, in a 5% CO_2 humidified incubator at 37 °C. Each culture media was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Hyclone, South Logan, UT), 50 unit/ml penicillin (Sigma, St. Louis, MO), and 50 μg/ml streptomycin (Sigma). Cell

number and viability were assessed by Trypan Blue (Sigma) dye exclusion using a hemacytometer. All chemicals were purchased from Sigma.

2.2. Cellular viability assay

Colorimetric analysis of cell growth was performed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) according to the manufacturers' protocol (Promega, Madison, WI). Cells (5×10^4/well) were cultured in 96-well plate for each time and the MTS (50 μl) was treated onto cells for 2 h. The absorbance at 490 nm of the culture media was measured.

2.3. Prostaglandin E_2 (PGE_2) assay

PGE_2 was measured using an EIA kit (Cayman Chemical Co., Ann Arbor, MI). Cell culture supernatants were collected 24 h after EGCG treatment. The culture supernatant was centrifuged to remove cellular debris and the assays were conducted according to the instructions of the supplier.

2.4. Construction of plasmids

The luciferase constructs containing the mPGES-1 (−650/−21, −179/−21 and −100/−21) in pGL3 basic vectors were generated after PCR of each promoter region with *Pfu*Turbo DNA polymerase (Stratagene, La Jolla, CA). The fragment was cloned into the TA vector (Invitrogen), sequenced, and further cloned into the pGL-Basic3 vector. Mutant plasmids (MT1, MT2, and MT1/2) were created by QuikChange® II XL Site-Directed Mutagenesis Kits (Stratagene). CMV-driven small interference RNA (siRNA) expression vector was constructed by inserting the hairpin siRNA template into *pSilencer 4.1-CMV-neo* vector (Ambion Inc., Austin, TX). The empty vector and siEGR-1 insert-containing vector were named as pSilence and pSiEGR1, respectively. Insert Egr-1 siRNA (Dharmacon, Lafayette, CO) was targeting the sequence, AAGTTACTACCTC TTATCCAT.

2.5. Western immunoblot analysis

Levels of protein expression were compared using Western immunoblot using polyclonal anti-mPGES-1 antibody (Oxford Biomedical, Oxford, MI), monoclonal anti-human COX-2 antibody (Cayman Chemical Co.), goat polyclonal anti-human Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-human ERK1/2 antibody (Cell Signaling Technology, Beverly, MA), and polyclonal anti-human EGR-1 antibody (Santa Cruz Biotechnology). Cell lysate was prepared in RIPA buffer containing 1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 1 mM sodium orthovanadate, and Protease Inhibitor Cocktail (Sigma). After brief sonication of samples, lysate protein was quantified using BCA protein assay kit (Pierce, Rockford, IL) and 50 μg of protein was separated by NuPAGE Novex Bis–Tris gel electrophoresis (Invitrogen). Protein was transferred onto a Nitrocellulose membrane (Invitrogen) and the blots were blocked for 1 h with 5% skim milk in Tris-buffered saline plus Tween 0.05% (TBST) and probed with each antibody for 2 h at room temperature or overnight at 4 °C. After washing three times with TBST, blots were incubated with horseradish-conjugated secondary antibody for 1 h and washed with TBST three times. Protein was detected by ECL detection system (Amersham) according to the manufacturer's instruction.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted with RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA (100 ng) from each sample was transcribed to cDNA by BD Sprint PowerScript (Clontech, Mountain View, CA). The amplification was performed with Takara HS ExTaq DNA Polymerase (Takara Bio Inc., Shiga, Japan) in Mycycler Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) using the following parameters: denaturation at 94 °C for 2 min and 25 cycles of reactions of denaturation at 98 °C for 10 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 45 s. An aliquot of each PCR product was subjected to 1.2% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide. The 5′ forward and 3′ reverse-complement PCR primers for amplification of each gene were as follows: human COX-2 (5′-TATACTAGAGCCCTTCCTCCTGTGCC-3′ and 5′-ACATCGCA-TACTCTGTTGTGTTCCC-3′), human mPGES-1 (5′-CACAGCC-TGGTGATGAG C-3′ and 5′-CCGCTTCCCAGAGGATCT-3′), and human GAPDH (5′-TCAACGGATTTGGTCGTATT-3′ and 5′-CTGTGGTCATGAGTCCTTCC-3′).

2.7. Transient and stable transfection

Cells were transfected with mixture of plasmids using FuGENE6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. For transfection of the luciferase reporter gene, a mixture of 2.8 μg firefly luciferase reporter and 0.2 μg renilla luciferase, pRL-null vector (Promega) per 9 μl of FuGENE6 reagent was applied for a 6 mm tissue culture dish. At 24 h after transfection, cells were exposed to chemicals for the next 24 h and lysed for dual-luciferase reporter assay system (Promega). All transfection efficiency was maintained at around 50–60%, which was confirmed with pMX-enhanced GFP vector. To create pSilence and pSiEGR1-expressing stable cell lines, A549 cells were transfected using FuGENE6 reagent. After 48 h, the cells were subjected to selection for stable integrants by exposure to 1000 μg/ml G418 (Invitrogen) in complete medium containing 10% fetal bovine serum. Selection was continued until monolayer colonies formed. The transfectants were then maintained in medium supplemented with 10% fetal bovine serum and 500 μg/ml G418.

2.8. Luciferase assay

Cells were washed with cold PBS, lysed with passive lysis buffer (Promega) and then centrifuged at 12,000 × *g* for 4 min. The supernatant was collected isolated and stored at −80 °C until assessment of luciferase activity. Luciferase activity was measured with a dual-mode luminometer (Model TD-20/20, Turner Designs Co., Sunnyvale, CA) after briefly mixing the supernatant (20 μl) with 100 μl *firefly* luciferase assay substrate

solution, followed with 100 μl stopping *renilla* luciferase assay solution (Promega). The *firefly* luciferase activity was normalized against *renilla* luciferase activity using the following formula: *firefly* luciferase activity/*renilla* luciferase activity.

2.9. Confocal microscopy

A549 cells incubated on the glass bottom culture dish (MatTek Corp., Ashland, MA) were starved for at least 18 h in serum-free medium prior to chemical exposure. After treatment with EGCG or vehicle DMSO, cells were fixed with 4% formaldehyde diluted in phosphate-buffered saline (USB Corp., Cleveland, OH). Fixed cells were permeabilized with 0.1% NP-40 in phosphate-buffered saline for 10 min. After 1 h blocking with 3% BSA in PBS, cell were incubated with the rabbit polyclonal anti-EGR-1 (Santa Cruz Biotechnology, 1:500) antibody at room temperature for 1 h and repeatedly washed using phosphate-buffered saline. Incubation of the Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, 1:1000) was done for 1 h at room temperature followed by repeated washes using phosphate-buffered saline. After the subsequent staining with 100 ng/ml DAPI in PBS for 30 min, confocal images were obtained on Zeiss LSM510 NLO laser scanning microscope using single line (488 nm) or multitrack sequential excitation (488 and 633 nm). Images were acquired and processed with Zeiss LSM Image Browser software.

2.10. Statistics

Data were analyzed using SigmaStat for windows (Jandel Scientific, San Rafael, CA). For comparison of two groups of data, Student's *t*-test was performed.

3. Results

3.1. EGCG enhanced PGE_2 production and expression of the involved enzymes in A549 human pulmonary epithelial cells

The previous studies demonstrate that COX-2 and its subsequent terminal product PGE_2 are up-regulated by EGCG in astrocytes and murine monocyte cells whereas some cells showed the opposite responses [18,19]. A549 pulmonary epithelial cells were treated with each dose of EGCG for 24 h and then supernatant PGE_2 was detected using ELISA kit. PGE_2 production was significantly enhanced by EGCG treatment in a concentration dependent manner (Fig. 1A). Since PGE_2 increase can be derived from the increased cell number by EGCG, cellular viability was measured using MTS reagent (Section 2). However, there was no observable increase in the cellular number. Thus, enhancement in PGE_2 by EGCG was due to an increased cellular secretion. Significant reduced cellular number compared with the vehicle control group was observed at the treatment with 100 μM EGCG (Fig. 1B). Therefore, all of the following experiments were thus performed by applying 0–50 μM EGCG.

To investigate the responsible mechanisms of EGCG-mediated PGE_2 production, expressions of COX-2 and mPGES-1 were measured since expression levels of other involved enzymes such as COX-1, cPGES, and mPGES-2 were not affected

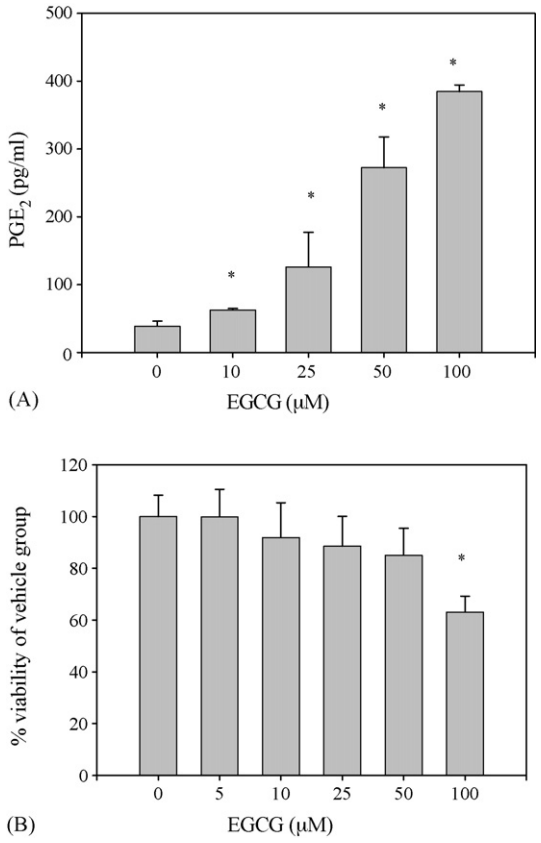

Fig. 1 – Effects of EGCG on prostaglandin E_2 production and cellular viability. (A) A549 cells were cultured with each dose of EGCG (0–100 μM) for 24 h. Culture media was harvested and directly analyzed by PGE_2 ELISA kit. All asterisks indicate significant difference ($p < 0.05$) from the vehicle control. (B) A549 cells were incubated with each dose of EGCG for 24 h and MTS reagent was applied at 2 h before the end of the EGCG incubation to measure the cellular viability. Asterisk indicates significant difference ($p < 0.05$) from the vehicle control group. All results are representative of three experiments.

(data not shown). However, treatment with EGCG elevated mPGES-1 expression both in time- and dose-dependent manners (Fig. 2A and B). COX-2 induction was only prominent at 50 μM EGCG and it got to peak earlier than mPGES-1 in A549 cells. The production of mPGES-1 and COX-2 was also assessed in the human colonic epithelial cells, HT-29 and HCA-7. Unlike responses in the A549 pulmonary epithelial cells, EGCG had slight inducible effects on mPGES-1 and COX-2 expression in the colon epithelial cells (Fig. 2C and D). Additionally, mPGES-1 and COX-2 mRNA were also increased by EGCG treatment in A549 cells, which was measured by RT-PCR method (Fig. 2E).

We assessed effects of EGCG on mPGES-1 production in interleukin 1β-activated cells as well as the normal unstimulated cells. Co-treatment with EGCG plus interleukin-1β enhanced mPGES-1 production when compared with the production in presence of only interleukin-1β (Fig. 3A). mPGES-1 promoter activity also showed super-induced pattern by EGCG in the cytokine-activated A549 cells (Fig. 3B).

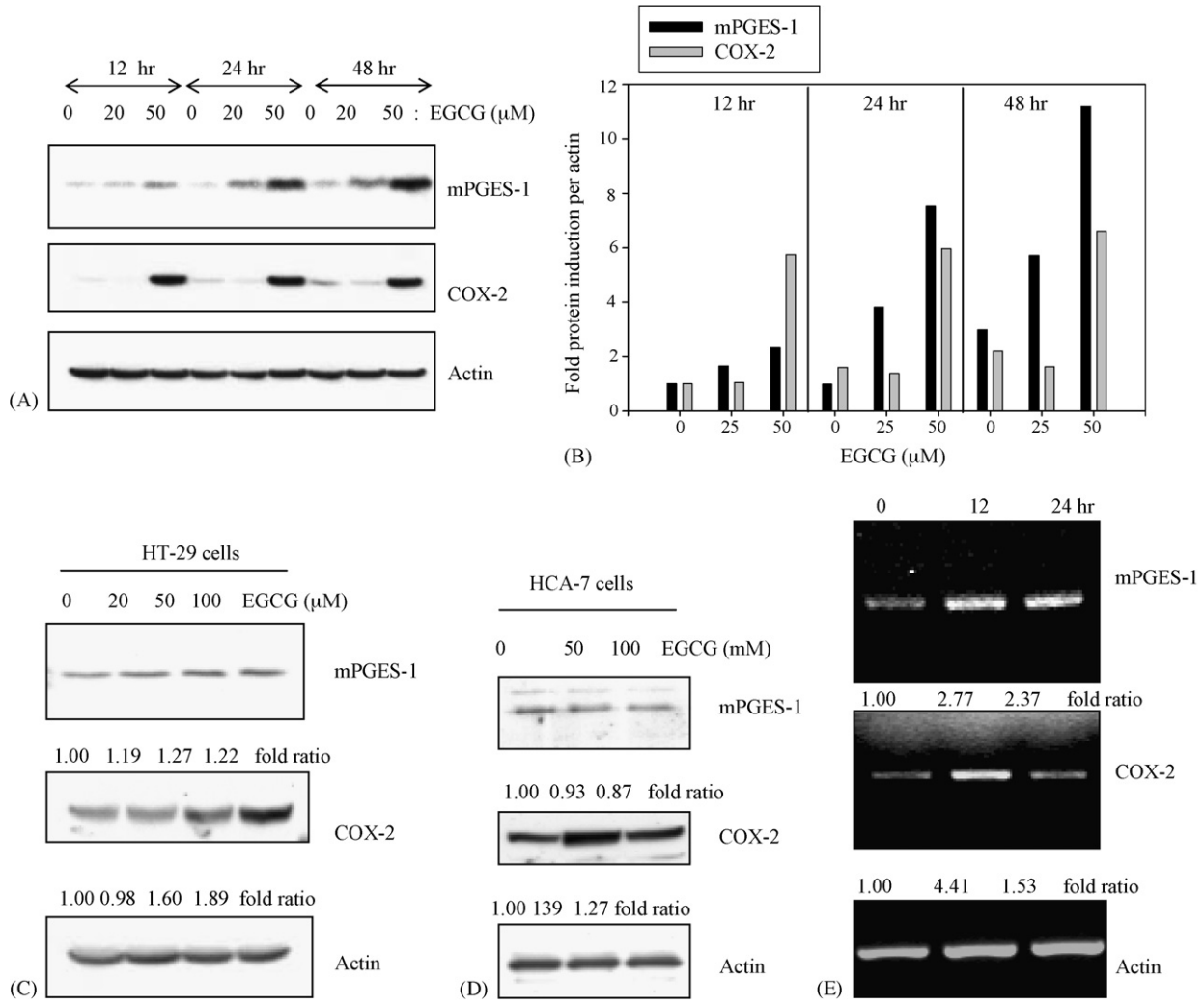

Fig. 2 – EGCG-mediated induction of mPGES-1/COX-2 expression. (A) Type II pulmonary epithelial A549 cells were cultured with each dose of EGCG (0, 20 and 50 μM) for 12, 24 and 48 h. (B) Relative density of bands of the Western blot were quantified using Multi-Gauge Version 3.0 (Fuji film Ltd. Japan). (C and D) HT-29 and HCA-7 colonic epithelial cells were each cultured with each dose of EGCG (0–100 μM) for 24 h. Total cell lysates were subjected to Western blot analysis. (E) Type II pulmonary epithelial A549 cells were cultured with each dose of EGCG (25 μM) for 12 and 24 h. Total RNA was analyzed using RT-PCR methods (Section 2). All results are representative of three experiments.

Taken all, the production of mPGES-1 protein or mRNA was enhanced in EGCG-treated human lung epithelial cells. In addition, the induction of mPGES-1 was more prominent in A549 lung epithelial cells than the colonic epithelial cells. The following studies will be then focused on the gene regulation of mPGES-1 by EGCG in A549 pulmonary cells.

3.2. *EGR-1 and ERK signaling pathways are involved in induction of mPGES-1 by EGCG*

Since EGR-1 is a critical transcription factor in regulating cytokine- or mitogen-mediated induction of mPGES-1, we assessed whether EGCG could affect EGR-1 which then might mediate mPGES-1 induction in A549 cells. EGCG treatment enhanced EGR-1 expression at the early exposure time even from 30 min and reached maximum level at 1–2 h after EGCG exposure (Fig. 4B). EGR-1 was also dose-dependently increased by EGCG (Fig. 4A). Expression of EGR-1 has been known to be mainly regulated by early responsive MAP kinases such as ERK1/2 signals [20,21]. We analyzed ERK1/2 signals and found the active phosphorylated ERK1/2 was maximal from 30 min to 1 h after EGCG exposure, relatively earlier than EGR-1 induction (Fig. 4B).

From the assumption that ERK1/2 signals could modulate mPGES-1 expression via EGR-1 signals, the ERK signaling pathway was blocked to assess the effects on the gene induction. Pre-treatment with a specific ERK inhibitor (U0126) suppressed not only the basal level of mPGES-1 but also EGCG-induced mPGES-1 in A549 cells (Fig. 4C). EGR-1 was directly interfered by introducing EGR-1 siRNA-expression vector into A549 cells. siRNA-mediated blocking of EGR-1 expression decreased EGCG-induced mPGES-1, suggesting the positive association of EGR-1 with mPGES-1 gene induction by EGCG (Fig. 4D).

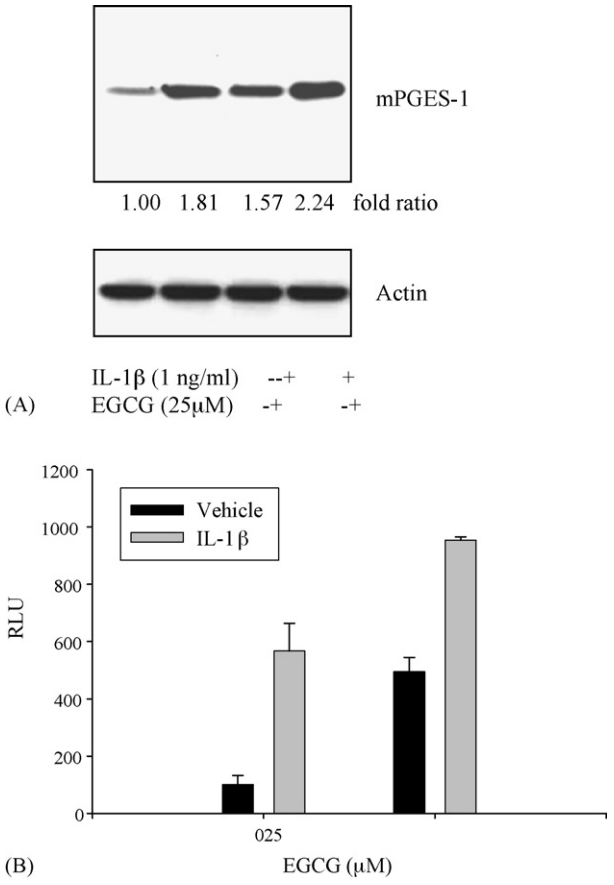

Fig. 3 – EGCG-induced of mPGES-1 expression in Interleukin 1β-activated cells. (A) Type II pulmonary epithelial A549 cells were cultured with each combination of EGCG and interleukin-1β for 24 h. Total cell lysates were subjected to Western blot analysis. (B) A549 cells were co-transfected with mPGES-1 promoter (−650 to −21) luciferase plasmid plus pRL-null vector for 24 h and then treated with each treatment combination for another 24 h. Results are representative of three experiments.

3.3. *EGCG stimulated the nuclear translocation of EGR-1, which was mediated by ERK1/2 signaling pathway*

Several evidences have been suggested about the positive modulation of EGR-1 by ERK signaling pathways [20,22]. In terms of the relationship between ERK1/2 and EGR-1, ERK inhibition by its specific inhibitor U0126 retarded EGCG-induced EGR-1 production (Fig. 5A). In the un-stimulated cellular state, EGR-1 was mainly located in the cytoplasm (Fig. 5B). EGR-1 can be induced and move into the nuclei when activated by external stimuli [20]. Two-hour treatment with EGCG strongly enhanced levels of total EGR-1 as shown in Western blot analysis and also promoted nuclear sequestration of EGR-1 protein in A549 cells (Fig. 5B). When ERK signal was inhibited, both of the expression and nuclear translocation of EGR-1 were suppressed. Taken all, EGCG-activated ERK MAP kinase mediated EGR-1 production and its nuclear translocation in the pulmonary epithelial cells.

3.4. *EGR-1 binding mediated EGCG-induced mPGES-1 promoter activity*

Stimulation of mPGES-1 promoter activity by TNF-α is mediated by tandem GC box-binding EGR-1 which play a significant role in regulating the transcription of both human and mouse mPGES-1 genes [6,23]. Promoter region of human mPGES-1 gene includes two EGR-1 binding GC boxes located around −101 to −119 bp. We created deleted or mutated 5′-UTR in GC boxes and measured promoter activity using luciferase assay in A549 cells. When the upstream sequence (−650 to −101) was truncated, the EGCG-mediated promoter activation was significantly suppressed (Fig. 6B). Additionally, another upstream sequence (−650 to −180) was also proved important in mPGES-1 transcriptional activation by EGCG, implying the cooperation of other transcription factors in regulation of mPGES-1 together with EGR-1 (Fig. 6B).

We constructed three mutant mPGES-1 promoter at GC boxes. Cells were transfected with each mutant promoter reporter plasmid to monitor EGCG-activated mPGES-1 transcriptional activity (Fig. 6C). Among these mutants, the double mutant promoter was the least responsive to EGCG. To address the direct effect of EGR-1 in the mPGES-1 transcriptional activation, EGR-1 knockout cell line was assessed for the transcriptional activation by EGCG. EGR-1-silenced A549 cells showed significantly reduced response to EGCG, implying the importance of EGR-1 in EGCG-stimulated mPGES-1 promoter activity (Fig. 6D). Taken together, EGCG-induced mPGES-1 transcriptional activity was mediated by EGR-1 binding in the lung alveolar A549 cells.

3.5. *EGCG was peculiar in induction of mPGES-1 compared with other catechins*

We tested other catechins for their capability to induce mPGES-1 protein. Each catechin of EGCG, (−)epigallocatechin (EGC), (−)epicatechin (EC), or (−)catechin gallate (CG) was applied to the A549 cells and mPGES-1 levels were detected. Among these catechins, EGCG was only significant inducer of mPGES-1 in the cells, which was also confirmed at levels of mPGES-1 promoter activity (Fig. 7A and B). Therefore, EGCG was one of most promising candidate compound that modulated mPGES-1 expression in the lung alveolar epithelial cells.

4. Discussion

In this study, mPGES-1 and PGE_2 production by EGCG were shown to be up-regulated in the alveolar epithelial A549 cell. Induction of mPGES-1 was mediated by ERK and EGR-1 signaling pathways. Enhanced PGE_2 production is associated with diverse pathophysiological events in the airway inflammation and carcinogenesis.

Definite role of mPGES-1 in the pulmonary cancer have been little described previously. However, some reports indicate that overproduction of mPGES-1 or COX-2 is found in non-small cell lung cancer (NSCLC) patients and in the established NSCLC cell lines with activating oncogene mutation [24,25]. Several lines of evidences suggest the up-regulation of mPGES-1 could be also

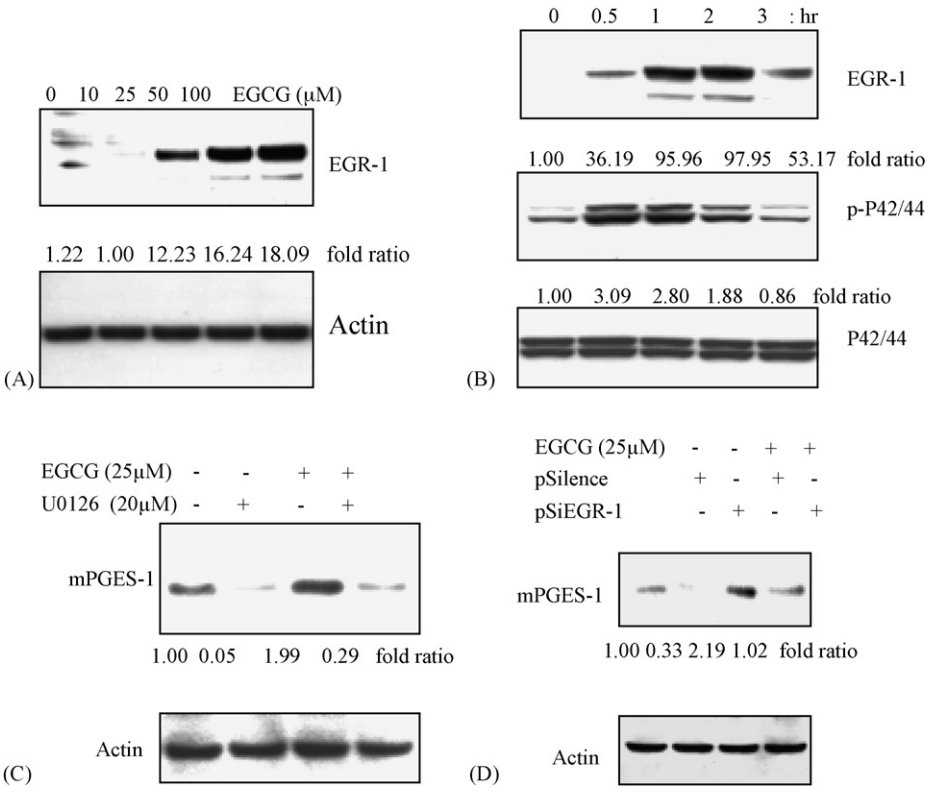

Fig. 4 – Involvement of EGR-1 and ERK signaling pathway in induction of mPGES-1 by EGCG. (A) A549 cells were treated with each dose of EGCG (0–100 μM) for 2 h. (B) A549 cells were treated with 25 μM EGCG for each time (0–3 h). (C) A549 cells were treated with each chemical combination for 24 h. (D) Stably transfected cell lines (pSilence- or pSiEGR-transfected A549 cells) were treated with 25 μM EGCG for another 24 h. Total cell lysates were subjected to Western blot analysis. Results are representative of three experiments.

important in other experimental tumorigenesis models [6,26]. In particular, mPGES-1 is over-expressed in human colorectal adenomas and in culture and nude mice with tumor xenograft, suggesting a potential role in colon tumorigenesis. Considering the roles of mPGES-1 in the tumorigenesis, EGCG could be no longer safe since increase in mPGES-1 expression by the catechin can be linked to the lung tumor aggravation. However, some evidences suggest intriguing questions on the role of mPGES-1 induction in the lung tumorigenesis [27]. The targeted overproduction of mPGES-1 and PGE_2 production in the alveolar airway epithelial pneumocytes did not promote lung tumorigenesis. Moreover, carcinogen-treatment had no differential effect on the lung tumor progression between mPGES-1 transgenic mice and the wild type mice. By contrast with mPGES-1, COX-2 is directly associated with lung tumor progression and its inhibition is sufficient to suppress the lung cancer in the many experimental models. Therefore, it can be postulated that the simple modulation of PGE_2 or mPGES-1 is not enough for the lung tumorigenesis but shifts in organized metabolic profile of COX-2 products like overproduced PGD_2 may play more critical roles in promoting the lung tumorigenesis. Considering all together, results from up-regulation of mPGES-1 by EGCG should be further assessed to detect the actual negative aspect of EGCG application.

Green tea intake is generally considered as safe, but the bioavailability of green tea polyphenols is broadly measured in the tissues, depending on exposure routes or formulations. When human ingests green tea, plasma EGCG levels are detected as 0.2–2% of the ingested amount. Peak plasma EGCG levels of 0.4–4 μM can be achieved after the administration of the formulations at doses equivalent to the EGCG contents in 5–20 cups of green tea [28,29]. However, local EGCG exposure levels at primary contact sites such as gastrointestinal tract or airway after repeated drinking or inhalation are much higher. Therefore, experimental treatment levels in this study (10–50 μM) are supposed as efficient to simulate the real chemical behavior in the pulmonary and intestinal epithelial tissues. Previous report describes some adverse events such as upset stomach, nausea, heartburn, and abdominal pains [12]. Moreover, clinical investigations indicate the association of EGCG component with the green tea-induced asthma in the tea factory workers [13–15]. Linked to the asthmatic inflammation, the elevated prostaglandin E_2 could be a causing factor of such adverse effects of green tea catechins by directly promoting the allergic IgE-mediated asthmatic responses [30,31]. However, there is little direct evidence of the association between PGE_2 levels and green tea-induced asthma and thus additional investigations will uncover such relationship.

Since PGE_2 is generally considered as pro-inflammatory mediators, the up-regulation of mPGES-1 production can mediated the detrimental actions of green tea. In our study,

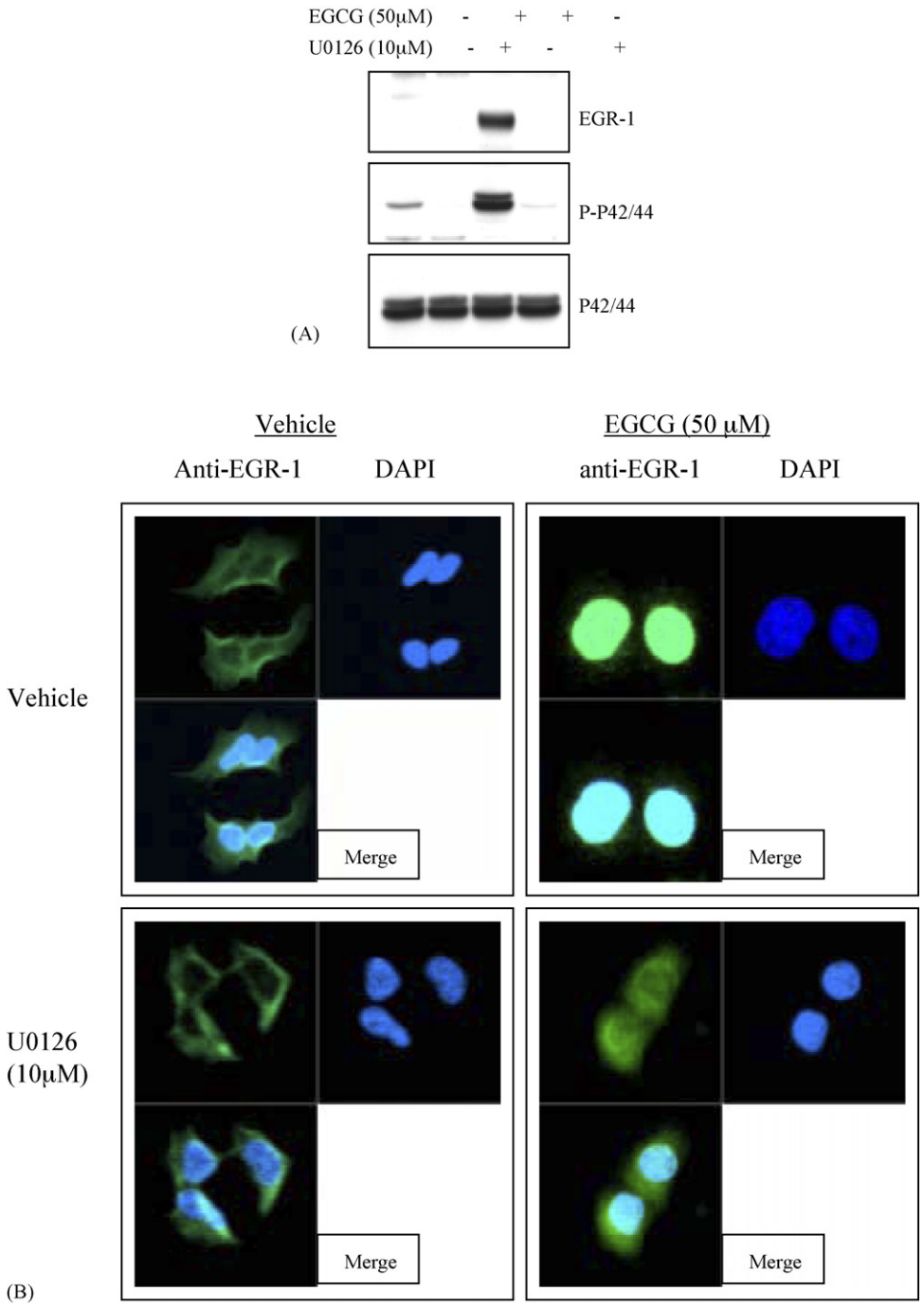

Fig. 5 – EGCG-mediated nuclear translocation of EGR-1. A549 cells were pre-treated with U0126 for 1 h and then incubated with EGCG for another 2 h. Total cell lysates were subjected to Western blot analysis (A). After cellular fixation and staining after the same treatment like Fig. 4A, cells were visualized under fluorescence microscope (B). Results are representative of three experiments.

EGCG enhanced mPGES-1 expression not only in the unstimulated cells but also in the cytokine-activated cells. Therefore, it can be speculated that EGCG might exacerbate the actively inflamed airway condition by eliciting the pro-inflammatory PGE_2 secretion. However, several opposite reviews suggest pulmonary PGE_2 plays beneficial roles in the airway inflammation although PGE_2 is generally considered as a potent pro-inflammatory metabolite in most other parts of body [32,33]. Lung represents a privileged site for the action of PGE_2. Normal pulmonary tissues maintain higher levels of PGE_2 than the plasma, which contributes to the normal physiological functions as well as the protective activity in the lung inflammation. Human studies have shown that direct inhalation of PGE_2 or its receptor agonist can inhibit both the early and late phase pulmonary responses to inhaled allergen [32,34]. Airway epithelial cells and airway smooth

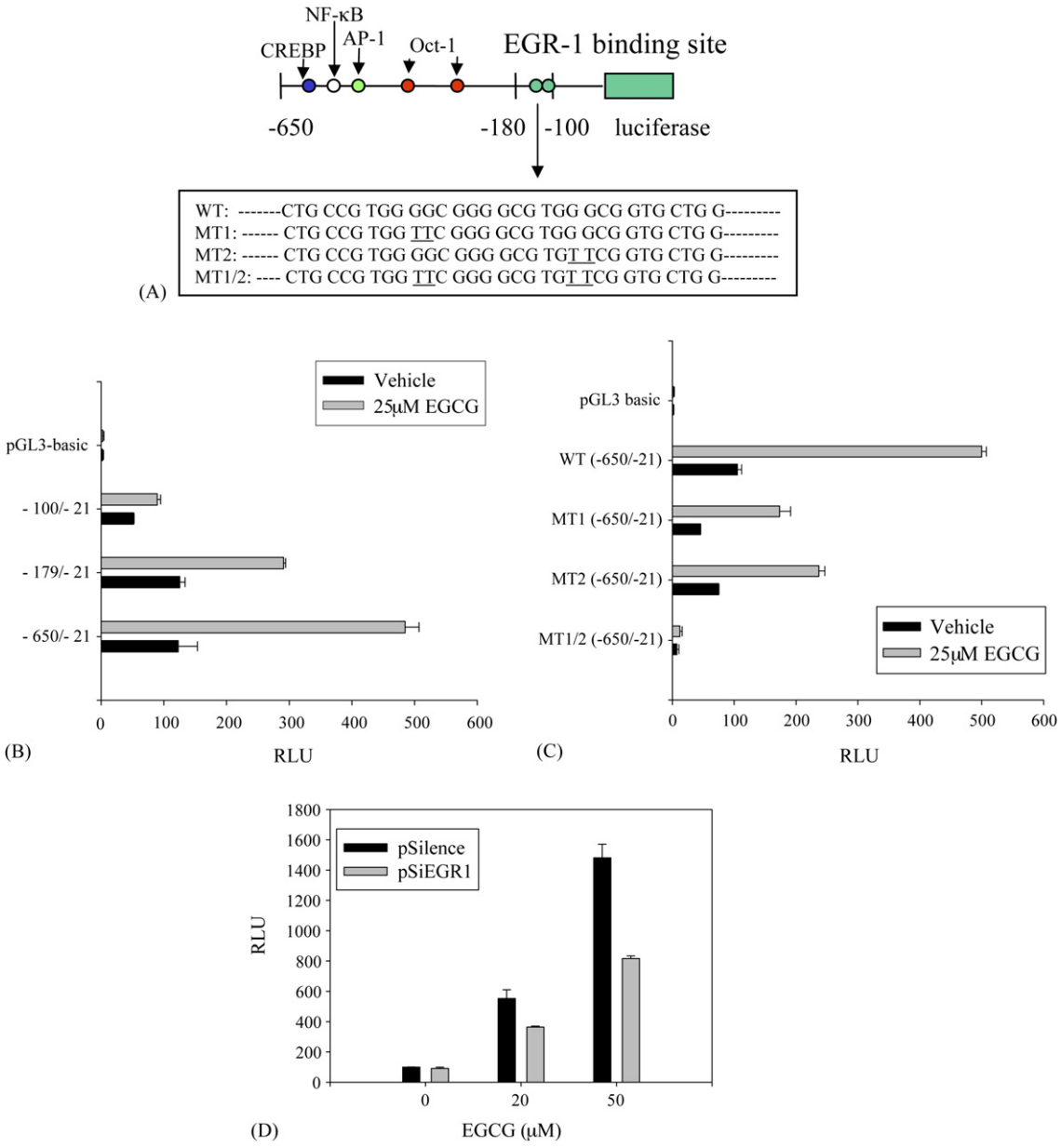

Fig. 6 – Involvement of EGR-1 binding GC box in EGCG-mediated mPGES-1 transcriptional activation. (A) Location of two EGR-1 binding GC boxes in the 5′ untranslated region in mPGES-1 gene. (B and C) A549 cells were co-transfected with mPGES-1 promoter luciferase plasmid plus pRL-null vector for 24 h and then treated with vehicle or EGCG for another 24 h. (D) Stably transfected cell lines (pSilence and pSiEGR1 transfectants) were co-transfected with mPGES-1 promoter luciferase plasmid plus pRL-null vector for 24 h and then treated with vehicle or EGCG for another 24 h. Results are representative of three experiments.

muscle cells are a rich source of the bronchoprotective prostanoids, particularly PGE_2, which suppresses the fibroblast proliferation and collagen synthesis in response to inflammatory stimulations [35]. Experimentally, PGE_2 loss from injuries in alveolar epithelium is a major cause of development of the pulmonary fibrosis [36]. Considering both anti-inflammatory and pro-inflammatory properties of PGE_2 in the lung from many controversial opinions, more careful assessment of EGCG in the pulmonary inflammation should be run by using diverse experimental models in terms of the interplay with other inflammatory mediators as well.

Several reports demonstrate that EGCG have anti-inflammatory effects on the cytokine- or mitogen-activated phagocytes and respiratory epithelial cells [37,38]. EGCG strongly suppresses the pro-inflammatory mediators such as nitric oxide or interleukin-8 in the cytokine-inflammed cells. EGCG markedly inhibits interleukin-1β receptor-associated kinase (IRAK) degradation and the downstream signaling events such as I-κBα degradation and NF-κB activation, and finally reduced interleukin-8 production. NF-κB signaling pathway also plays very important roles in inducing mPGES-1 [5]. Despite of these suppressive effects of EGCG on the NF-κB signals, mPGES-1

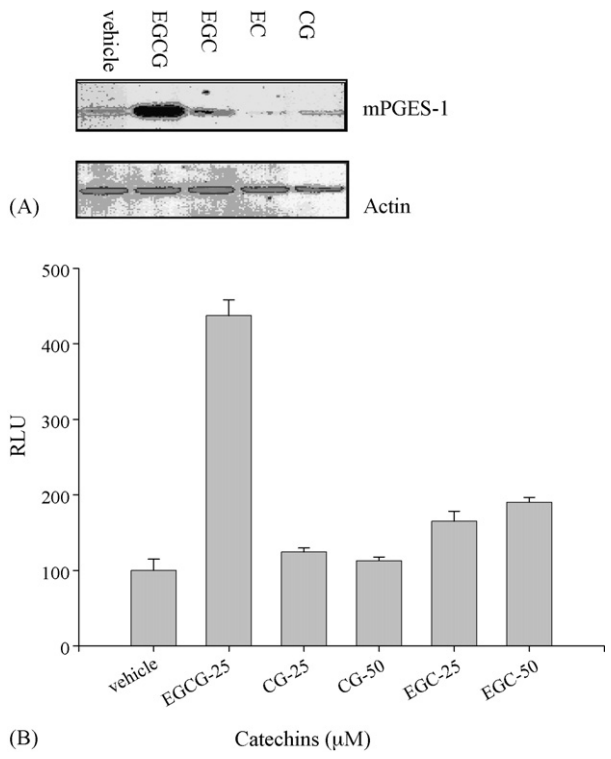

Fig. 7 – Effect of catechins on mPGES-1 expression. (A) Type II pulmonary epithelial A549 cells were cultured with 25 μM catechin (EGCG, (−)epigallocatechin (EGC), (−)epicatechin (EC), or (−)catechin gallate (CG)) for 24 h. Total cell lysates were subjected to Western blot analysis. (B) A549 cells were co-transfected with mPGES-1 promoter (−650 to −21) luciferase plasmid plus pRL-null vector for 24 h and then treated with each catechin treatment for another 24 h. Results are representative of three experiments.

was up-regulated by EGCG in the same cells. It thus can be supposed that there are some hierarchy in the transcription factors such as EGR-1 and NF-κB in inducing mPGES-1. In our study, when the upstream sequence (−650 to −180) over EGR-1 was deleted from mPGES-1 reporter, activation of promoter activity by EGCG was also significantly suppressed. Moreover, blocking of EGR-1 expression did not suppress mPGES-1 expression completely. It thus suggests that other transcription factors as well as EGR-1 cooperate in the promoter activation. The relative contribution and interplay of transcription factors to EGCG-mediated mPGES-1 induction should be compared in the further study.

Putative promoter region of human mPGES-1 contains the binding sites for cyclic AMP response element binding protein (CREBP), AP-1, NF-κB, Oct-1, and EGR-1 (Fig. 6A). EGR-1 has been known as the key transcription factor in the regulation of mPGES-1 gene expression [6]. EGR-1 was up-regulated by EGCG in the lung epithelial cells, which contributed to mPGES-1 production. Additionally, ERK signaling pathway was also involved in EGCG-mediated EGR-1 induction and mPGES-1 production. ERK1/2 MAP kinases have been known to mediate EGR-1 production in the other systems as well [20,22]. There are some contrasting reports of the effect of EGCG on EGR-1 in the vascular smooth muscle cells when cells are activated by PDGF. EGCG suppresses the PDGF-induced EGR-1 production [39,40]. Since EGCG is a selective inhibitor of the tyrosine phosphorylation of PDGF-Rβ, its downstream signaling ERK1/2 phosphorylation and EGR-1 induction were suppressed. By contrast, ERK1/2 phosphorylation and EGR-1 induction by EGF or serum are not reduced or even slightly enhanced by EGCG. Comparing with up-regulation of EGR-1 by EGCG in our result, it can be speculated that EGR-1 and ERK responses by EGCG might be differentially regulated, depending on the tissue environment and external stimuli.

Taken together, EGCG was a strong inducer of EGR-1 expression and mediated EGR-1 nuclear translocation via ERK signaling pathway in A549 pulmonary epithelial cells. Induced EGR-1 then mediated the induction of mPGES-1 gene expression. These whole events can account for EGCG behavior in human body, which need to be further investigated in terms of the global interplays among multiple signaling transcriptional factors and diverse inflammatory events.

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