Inhibitory Effect of Chebulic Acid on Alveolar Epithelial to Mesenchymal Transition in Response to Urban Particulate Matter Using Co-treatment and Post-treatment Exposure

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Urban particulate matter (UPM) is atmospheric particulate samples obtained from industrialized urban areas. It is known that pulmonary fibrosis can result directly or indirectly from particulate matter. In this study, the protective effect of chebulic acid (CA) against UPM-induced epithelial–mesenchymal transition (EMT) in the pulmonary alveolar epithelial (PAE) cells were investigated. Our findings revealed that PAE cells were changed from the epithelial phenotype to mesenchymal one after exposure to UPM. Furthermore, co-treatment and post-treatment of CA inhibited EMT progression. Especially the key epithelial marker, E-cadherin, was down-regulated by UPM and recovered by CA. Also, gelatin zymogram showed that the activity of matrix metalloproteinase (MMP)-2 and MMP-9 decreased by co-treatment and post-treatment of CA. Further investigation revealed that CA attenuated UPM-stimulated PAE cells invasion ability. These data showed that UPM promoted PAE cells invasion, reactive oxygen species-mediated extracellular matrix degradation and CA reduced the potential health risks associated with UPM.

Key words urban particulate matter; epithelial–mesenchymal transition; chebulic acid; lung fibrosis

INTRODUCTION

Global air pollution causes various types of problem to human health and the relationship between particulate matter (PM) increment and public health hazards has attracted an increasing attention. This worldwide problem is especially relevant to the release of urban particulate matter (UPM), which has the aerodynamic diameter less than 2.5 \( \mu \)m and originates from incomplete coal combustion and diesel vehicle exhaust.\(^3\) PM pollution was identified Group I carcinogen based on adequate evidence of carcinogenicity in humans and laboratory animals by the International Agency for Research on Cancer.\(^2\) PM consists of organic chemicals including polycyclic aromatic hydrocarbons (PAHs), which promote the generation of intracellular reactive oxygen species (ROS).\(^3\)

Accumulated evidence indicates that oxidative stress cause cellular damage,\(^9\) and PM-induced mitochondria dysfunction leads to generation of ROS.\(^9\) Several toxicological and epidemiological studies have suggested that PM triggers negative effects on respiratory system, including the lung,\(^6\) immune system,\(^7\) and cardiovascular system.\(^8\) PM exposure exacerbates pulmonary inflammation. Epithelial mesenchymal transition (EMT), which promotes the phenotype transition of epithelial cells to mesenchymal cells, is activated during the process of fibrosis and cancer.\(^9\) EMT can be used as a biological marker of tumors formation and carcinogenesis of lung cancer.\(^10\) Therefore, knowledge of how PM-mediated EMT process in the pulmonary system is crucial for PM-relevant cancer studies. The steps of EMT involve extracellular matrix degradation by matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases and a clear markers of EMT.\(^11\) Many studies in pulmonary fibrosis models have shown that MMPs promote pulmonary fibrotic responses to injury.\(^12\)

Also, previous studies have demonstrated that the correlation between PM2.5 and invasion of lung cancer cells.\(^13\) Although the relation between extended exposure to PM and risk of lung cancer is established, the defense mechanism of PM-induced pulmonary fibrosis is still poorly understood. Antioxidant-rich diets or chemicals might be potentially useful in ameliorating the effects of PM by counteracting the associated oxidative stress.\(^14\) Therefore, we focused on the role of chebulic acid (CA) in UPM-induced pulmonary EMT, which promotes metastasis by protein-degrading MMPs.

Chebulic acid, which was isolated from Terminalia chebula Retz.\(^15\) This plant possesses the biological activities, including anti-asthmatic, anti-cardiovascular, antioxidant, anti-diabetic, and wound healing properties.\(^16\) Recently, our group demonstrated that CA eliminates the free radicals and protects integrity of the tight junctions of pulmonary epithelial cells barrier against UPM exposure.\(^17\) In this study, we analyzed the EMT markers, including fibronectin, \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA), and E-cadherin on human pulmonary alveolar epithelial (PAE) cell. In addition, we investigated the mechanism of UPM-induced EMT to understand the intracellular signaling. Also, we examined protective and therapeutic effects of CA against UPM exposure.

MATERIALS AND METHODS

**UPM Preparation** UPM was gained from the National Institutes of Standards and Technology (NIST, Gaithersburg, MD, U.S.A.). NIST Standard Reference Material (SRM 1648a) was gathered in St. Louis, MO in the 1970s and has been analyzed for health effects of urban particulate matter in biological systems.\(^18\) UPM were suspended in cell culture medium at a concentration of 10 \( \mu \)g/mL and treated for 24h. We used two methods for treatment of UPM. Co-treatment means UPM and CA is suspended in cell culture medium at the same time.

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Post-treatment means after suction of UPM-contained medium and then CA is added.

**Cell Culture** The human pulmonary alveolar epithelial cell (NCI-H441) was obtained from ATCC (Manassas, VA, U.S.A.), and cultured in RPMI 1640 medium including 2.5 g/L dextrose, 2.383 g/L N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 0.11 g/L sodium pyruvate, 10% fetal bovine serum (FBS) (v/v), 2.2 g/L sodium bicarbonate, 100 units/mL of penicillin, and streptomycin. Cells were plated at 2.0 × 10^5 cells/mL in RPMI 1640 medium. The cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

**Cell Viability Assay** The viability of cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. H441 cells of 2 × 10^5 cells/mL were seeded in 96 well plates for 24 h. After treatment of UPM for 24 h, MTT solution was added to each well and further incubated for 3 h. Medium were removed, and dimethyl sulfoxide (DMSO) were added to each well to dissolve intracellular generated insoluble formazan crystals. The optical density was measured by multiplate reader (EL-808, BioTek, Winooski, VT, U.S.A.) at 540 nm.

**Detection of Intracellular ROS** For the measurement of intracellular ROS, H441 cells were seeded in 96-well plates. First co-treatment method, the cells were loaded with 100 μM of 2',7'-dichloro-dihydro-fluorescein diacetate (DCFH-DA) for 30 min followed by co-treatment of UPM and CA for 3 h. After then the cells were washed with phosphate-buffered saline (PBS) for removing debris. The fluorescence intensity was measured using a multi-detection microplate reader (HIDEX, Turku, Finland) at excitation 485 nm/emission 535 nm. Second post-treatment method, the cells were loaded with 100 μM of DCFH-DA for 30 min followed by pre-treatment of UPM for 3 h and post-treatment of CA for 24 h. After then the same process of co-treatment was performed.

**mRNA Expression by Real Time PCR** Total RNA was isolated using RNAiso PLUS (TaKaRa Korea Biomedical Co., Seoul, Korea). cDNA was synthesized from total RNA using the LeGene Premium Express first-strand cDNA Synthesis System (Legene Biosciences, San Diego, CA, U.S.A.) according to the manufacturer’s instructions. Quantitative real time PCR (qPCR) were conducted with PreMIX SYBR green (Enzymics, Seoul, Korea) kit using 1 μL of cDNA in a total reaction volume of 20 μL using an iQ5 Thermal Cycler (Bio-Rad, Hercules, CA, U.S.A.). The results were normalized to housekeeping gene of β-actin and expressed as 2^ΔΔCT values (Supplementary Table 1). The forward and reverse primers for specific oligonucleotides were as follows: fibronectin (FN), 5'-CTG GCC GAA AAT ACA TTG TAA A-3' and 5'-CCA CAG TCG GGT CAG GAG-3'; α-SMA, 5'-CTC TTG GCC AC CTT CCA G-3' and 5'-TAA CGA GTC ACA GCT TTT GC-3'; E-cadherin, 5'-ATT TTT TCC TCG ACA CCC GAT-3' and 5'-ATT TTT TCC TCG ACA CCC GAT-3'; transforming growth factor-β (TGF-β), 5'-CAAA ACTT CTTG GCGATA CC-3' and 5'-GCT AAG GGCA AAG GCC CTC AAT-3'; β-actin 5'-AGC GAG CAT CCC CCA AAG TT-3' and 5'-GGG CAC GAA GGC TCA TCA TT-3'.

**Protein Expression by Western Blot** Cells were lysed in RIPA buffer at 4°C, and the supernatant was used as total cell lysate. An equal amount of protein (30 μg) was separated by 10% gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica MA, U.S.A.) for 12 h at 50 mA using tank transfer device (Bio-rad). Membranes were blocked with 5% non-fat dry skim milk in TBST solution at room temperature, and then incubated with primary antibodies at 4°C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Antibodies against the following proteins were used fibroactin (1:1000), α-SMA (1:1000), E-cadherin (1:2000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000) (all from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). Immunoreactive bands were developed using the supersignal west pico plus (Thermo, Schaumburg, IL, U.S.A.) and quantified using the Image J software (National Institutes of Health, Maryland, MD, U.S.A.).

**Immunofluorescence Staining** NCI-H441 cells were fixed with 3.7% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 15 min. The cells were blocked with 1% bovine serum albumin (BSA) followed by incubation with anti-e-cadherin primary antibody (1:100, Cell Signaling, MA, U.S.A.) at 4°C overnight. Then, anti-mouse Alexa 488 green fluorescent secondary antibody was added to plates for 2 h, and nuclei were marked with 500 ng/mL of 4',6'-diamidino-2-phenylindole (DAPI). The cells were observed under a confocal laser-scanning microscope 700 (Carl-Zeiss, Oberkochen, BA, Germany).

**Gelatin Zymography Assay** Activity of MMP-2, MMP-9 was assessed in the culture supernatants by gelatin zymography. H441 cells were exposed to 10 μg/mL of UPM for 24 h with CA using co-treatment and post-treatment exposure. At the end of incubation, 1 mL of culture supernatant was mixed with 2X sample buffer (Koma Biotech, Seoul, Korea) and resolved on a zymogram-PAG 10% gel (Koma Biotech) under non-reducing conditions. Gel was washed with rehydration buffer (2.5% Triton X-100) at room temperature before incubation in the developing buffer (Koma Biotech) at 37°C for 12 h. Subsequently, gel was stained for 30 min in 1.5% (w/v) coomassie brilliant blue R-250 and then washed with destaining solution (40% methanol and 10% acetic acid). White bands were observed on a blue background after de-staining, which represents MMP-2, -9’s gelatinolytic activity. Band intensities were measured using the Image J software (National Institutes of Health, MD, U.S.A.).

**Cell Invasion Assay** NCI-H441 cells were seeded to 24 well transwells chambers (Corning, Tewksbury, MA, U.S.A.), which were pre-coated with matrigel solution in inserts. RPMI medium containing 10% FBS was added to the basolateral sides. The matrigel coated chambers were incubated at 37°C under 5% CO₂ for 24 h. After incubation, the non-invading cells were eliminated from the apical layer of the membrane by cotton swab scrubbing. The invaded cells were fixed with 100% methanol for 10 min and stained with 0.1% crystal violet staining solution for 30 min. Inserts were rinsed twice in the distilled water to remove excess stain and allowed to air dry. Subsequently, the cells were quantified using a microscope (CKX41, Olympus, Tokyo, Japan).

**Statistical Analysis** All results are expressed as means ± standard deviation (S.D.) (n = 3). Different letters indicate significant differences at p < 0.05 by Tukey’s multiple range tests. All statistical analyses were performed using SAS version 9.4 (SAS institute, Cary, NC, U.S.A.).
RESULTS

Effect of CA and UPM on NCI-H441 Cell Viability  To determine NCI-H441 cell viability of CA and UPM, MTT assay was performed. As shown in Fig. 1A, there were no cytotoxicity after treatment of CA (0.63–5 µM) and N-acetyl cysteine (NAC) (125–1000 µM) for 24h. Treatment with UPM up to 100 µg/mL for 24h decreased cell viability by 53% compared to the control group (Fig. 1B). Approximately, 10 µg/mL of UPM concentration gave 80% of cell viability.

Effect of CA on UPM-Induced Intracellular ROS Generation of NCI-H441 Cells Intracellular ROS generation was measured by DCF fluorescence intensity in NCI-H441 cells. As shown in Fig. 2A, intracellular ROS generation significantly decreased compared to the controls after co-treatment of UPM (10 µg/mL) and samples (0.5–4 µM CA and 125–1000 µM NAC) for 3h. Similar tendency was observed in the post-treatment method in which the 10 µg/mL of UPM was treated for 3h and then the samples (0.5–4 µM CA and 125–1000 µM NAC) were treated for 24h (Fig. 2B). Especially, post-treatment of CA attenuated dose-dependently the UPM-induced ROS generation. In addition, the treatment of 0.25–0.5 µM CA only did not significantly affect intracellular ROS production compared with untreated control, although ROS generation was slightly decreased with 1 µM CA treatment (Supplementary Fig. 1). This result indicates that CA and NAC had UPM mediated intracellular ROS scavenging activity. In particular, the difference in the molar concentration of CA (1–4 µM) compared with NAC (125–1000 µM) treated in cells confirms that CA is more effective in reducing intercel-

Fig. 1. Effect of Chebulic Acid (CA) and Urban Particulate Matter (UPM) on NCI-H441 Cell Viability
(A) NCI-H441 cells were incubated with CA (0.63, 1.25, 2.5, and 5 µM) and NAC (125, 250, 500, and 1000 µM) for 24h. (B) Exposure to UPM up to 100 µg/mL for 24h. MTT assay was carried out to determine the UPM concentration showed that 80% or more cell viability. Data represent the mean ± S.D. of 3 experiments with triplicate samples and different letters mean significant differences at the p < 0.05 by Tukey’s studentized range test.

Fig. 2. Effect of CA on UPM-Induced Intracellular Reactive Oxygen Species (ROS) Production of NCI-H441 Cells
Intracellular ROS scavenging activities of CA against UPM was detected by 2',7'-dichlorofluorescein (DCFA) fluorescence. DA, diacetate was hydrolyzed by the esterase in the cells. (A) Cells were co-treated with 10 µg/mL of UPM and samples (CA, NAC) for 3h. (B) Cells were incubated with samples (CA, NAC) for 24h after treatment of UPM for 3h. Data represent the mean ± S.D. of 3 experiments with triplicate samples and different letters mean significant differences at the p < 0.05 by Tukey’s studentized range test.
Effect of CA on mRNA Expression of EMT Markers and TGF-β in UPM-Treated PAE Cells

The mRNA expression of fibronectin, α-SMA, E-cadherin and TGF-β was measured to establish whether the 10 µg/mL of UPM for 24 h induces pulmonary EMT alterations NCI-H441 cells. The Fig. 3A results demonstrated that UPM induced the mRNA expression of mesenchymal markers (fibronectin and α-SMA), while it suppressed that of the epithelial marker (E-cadherin). Additionally, UPM exposure upregulated the mRNA level of TGF-β, which are indicator of the tumor progression. To investigate whether CA reverses UPM-induced EMT in cells, cells were co-treated with CA (0.25, 0.5, and 1 µM) and UPM for 24 h (Fig. 3A). The mRNA expression of EMT markers and TGF-β in the PAE cells were recovered by CA. The mRNA expression of fibronectin, α-SMA, E-cadherin and TGF-β was

Fig. 3. Effect of CA on mRNA Expression Levels of Epithelial to Mesenchymal Transition (EMT) Markers and TGF-β as Changed by UPM Exposure

(A) NCI-H441 cells were co-treated with 10 µg/mL of UPM and CA (0.25, 0.5, and 1 µM) for 24 h. (B) NCI-H441 cells were treated with UPM for 24 h and post-treated with CA for 24 h. These levels are normalized by expression levels of β-actin. Data represent the mean ± S.D. of 3 experiments with triplicate samples and different letters mean significant differences at the p < 0.05 by Tukey’s studentized range test.
also measured to determine post-treatment effects of CA for 24 h on UPM-induced EMT process. The Fig. 3B showed that CA treatment attenuated the UPM induction of fibronectin, α-SMA, and TGF-β and increased E-cadherin mRNA expression. These results indicate that CA reversed UPM-induced alterations in pulmonary EMT in PAE cells.

Effect of CA on Protein Expression of EMT Markers in UPM-Treated PAE Cells  To investigate the influence of CA (0.25–1 µM) on 10 µg/mL of UPM-induced pulmonary EMT, the changes in protein expression of EMT markers was examined. As shown in Figs. 4A (co-treatment) and B (post-treatment), Western blot analysis revealed that 24 h treat-
ment of CA suppressed UPM-induced mesenchymal markers (fibronectin and α-SMA) and increased epithelial marker (E-cadherin). The protein level of α-SMA did not significantly increase during UPM exposure via both treatment method, but was significantly reduced by CA. The protein level of E-cadherin, epithelial marker, was recovered by CA, but was not statistically significant compared with the control. Additionally, the level of E-cadherin protein was increased by 1 µM CA treatment (Supplementary Fig. 2). On the other hand, fibronectin expression in the cells significantly increased with UPM treatment for 24 h was significantly reduced by both co- and post-treatments.

Effect of CA on Adherens Junction in PAE Cells Treated with UPM We performed immunofluorescence staining of NCI-H441 cells to detect E-cadherin. To determine whether CA (0.5 and 1 µM) attenuates the loss of adherens junction, the cells were co-treated and post-treated with CA for 24 h in the presence of UPM (10 µg/mL). As shown in Figs. 5A and B, the expression of E-cadherin was significantly lower in only UPM-treated group and recovered by CA to a level comparable with that of the untreated control. These results indicated that CA has protective properties on alveolar epithelial cells adherens junction.

Expression of Matrix Metalloproteinase (MMP)-2 and MMP-9 Activities To investigate proteolytic activity in the medium of NCI-H441 cells, zymography assay was performed. In the case of co-treatment, the proteolytic activity of MMP-9, which have molecular weights of 88kDa, was increased in 10 µg/mL of UPM for 24 h treated group, but there was no changes of MMP-2 expression level. Also, there was no change in the NAC group, but the CA (0.25–1 µM) for 24 h decreased MMP-2 activities significantly, especially in lower concentration of CA (0.25 and 0.5 µM). In the case of post-treatment, the induction of MMP-2 and MMP-9 was significantly decreased by lower concentration of CA, and no differences were found in the NAC group. This tendency was similar to that of the EMT markers protein expression levels in Western blot analysis.

Effect of CA on UPM-Induced Cell Invasion Ability We quantitated the invaded cells through extracellular proteins in response to 10 µg/mL of UPM for 24 h by carrying out using matrigel chamber. Following exposure of UPM, the number of NCI-H441 cells that invaded from the apical chamber to the basolateral chamber compared with the untreated control cells was increased (Figs. 7A, B). Furthermore, co-treatment and post-treatment of 0.5 µM of CA significantly at-

Fig. 5. Effect of CA on Adherens Junction Protein Expression Levels after Exposure to UPM
Immunofluorescence staining of the effect of UPM (10 µg/mL) and CA (0.5 and 1 µM) on NCI-H441 cells. (A) NCI-H441 cells were co-treated with UPM and CA for 24 h. (B) NCI-H441 cells were treated with UPM for 24 h and post-treated with CA for 24 h. Cells were preprocessed and stained with an anti-E-cadherin (1:100) antibody and DAPI (500 ng/mL). (Color figure can be accessed in the online version.)
tenuated the invaded cell number. These results suggested that CA for 24h treatment inhibits UPM-promoted cell invasion.

DISCUSSION

Air pollution due to particulate matter becomes one of the most serious problems faced by world in the view of its adverse impacts on human health.19) Also, Environmental Performance Index reported that Korea air quality score was 173th/180 countries, and actually got the lowest score in the world.20) Fine particulate matter less than 2.5 micron in aerodynamic diameter (PM2.5), one of the most relevant and prevalent atmospheric environmental pollutants in the world, is increasingly associated with lung disease.21) Larger particulate matter is mostly filtered in the bronchial, while PM2.5 facilitates particles to reach the gas exchange system and enter the circulatory system to stimulate hazardous injury.22)

In this study, we used urban particulate matter (SRM 1648a) as a quality control material and evaluation of atmospheric particulate matter. The mean particle diameter of SRM 1648a is 5.85 µm and consist of polycyclic aromatic hydrocarbons (PAHs), nitro-substituted PAHs (nitro-PAHs), polychlorinated biphenyl (PCB) congeners, and chlorinated pesticides.

Many studies have shown that particulate matter affects lung health though oxidative stress-involved signaling pathway.23) A recent research demonstrated that abnormal increase of ROS generation by PM2.5 induces changes in cell cycle progression, apoptosis,24) and autophagy,25) in human lung epithelial cells. The major chemical components of PM2.5 include alkanes, fatty acids, alkanols, and PAHs, and many studies have reported associations between PAHs and EMT.26,27) PM2.5 increases numbers of the mesenchymal cell and EMT markers through TGF-β activation, which is associated with EMT induction and maintenance.28,29) Furthermore, evidence revealed that MMPs can stimulate EMT processes associated with activation of tumor cells during cell invasion and metastasis.30)

The PM2.5 is characterized by remote transportation distance, longer retaining period and no filtering resistance.31) Therefore, the studies of potential antagonists against PM2.5-induced pulmonary damage are very urgent and important. It has been reported that the treatment of taurine recovered mitochondrial function, affording airway epithelia protection from PM-induced damages.14) It remains unclear to what extent the effect of antioxidant chemicals on pulmonary responsiveness. Naturally occurring components from Terminalia chebula, possess antioxidant activities and useful as a treatment for asthma.32) Our previous published studies have shown that pre-treatment of CA isolated from T. chebula reduces UPM-induced alveolar epithelium inflammation and junctional molecules-related barrier integrity.17) Therefore, in the current study, we extended our study to investigate whether CA attenuates the damage of alveolar EMT by UPM. Also, we delivered CA to PAE cells in the presence of UPM with co-treatment and post-treatment representing protective and therapeutic effect, respectively against pulmonary alveolar cell injury. The co-treatment method was started with UPM and chebulic acid at the same time. Also, the post-treatment method was treated with only chebulic acid after removing of UPM. The present study confirmed that 10 µg/mL of UPM can

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**Fig. 6.** Expression of Matrix Metalloproteinase (MMP)-2 and MMP-9 Activities

Gelatin zymogram reveals the activity of MMP-2, -9 in the conditioned medium. (A) NCI-H441 cells were co-treated with 10 µg/mL of UPM and CA (0.25, 0.5, and 1 µM) for 24h. (B) NCI-H441 cells were treated with UPM for 24h and post-treated with CA for 24h. Data represent the mean ± S.D. of 3 experiments with triplicate samples and different letters mean significant differences at the $p<0.05$ by Tukey’s studentized range test.
induce EMT through the intracellular ROS generation, and the expression of EMT markers were restored by co-treatment and post-treatment with CA (0.25–1 µM) and NAC (1000 µM). NAC is well known for a precursor of l-cysteine and scavenger of free radicals as it interacts with ROS such as OH· and H2O2.33) Our data indicate that treatment of 10 µg/mL UPM promoted ROS generation and upregulated the EMT related mRNA expression levels. Especially, reduced mRNA expression of E-cadherin and upregulated expression of mesenchymal markers were recovered by co-treatment and post-treatment with CA 0.25–1 µM. The expression of E-cadherin is negatively associated with tumor invasion, and the disappearance of epithelial cell polarity is accompanied by an increase in the expression of mesenchymal cell markers fibronectin and α-SMA.9,34) Previous published study demonstrated that the potent fibrogenic cytokine TGF-β could induce EMT process in the human alveolar epithelial cells.35) In this study, we observed mRNA expression level of TGF-β could be reduced by treatment of CA.

Fig. 7. Effect of CA on UPM-Induced Cell Invasion Ability

UPM-induced cell invasion ability was determined using a microscope. (A) NCI-H441 cells were co-treated with 10 µg/mL of UPM and CA (0.5 µM) for 24h. (B) The cells were treated with UPM for 24h and post-treated with CA for 24h. The left of the image is the representative images of the invaded cells, and the right graph is the calculation of the average number of invaded cells in each treated groups. Data represent the mean ± S.D. of 3 experiments with triplicate samples and different letters mean significant differences at the p < 0.05 by Tukey’s studentized range test.

Also, the EMT process is activated by the disruption of cell-cell adherens junctions and extracellular matrix (ECM) components adhesions.36) Through degradation of ECM components, MMPs can destroy the alveolar epithelium, as well as disrupt reorganization during the repair process.37) We examined to the proteolytic activity of the gelatinases (MMP-2 and -9), which are most effective in the cleavage of type IV and V collagen, in alveolar epithelial cells. We found that CA (0.25–1 µM) effectively inhibits pulmonary epithelial MMPs secretion and activity, compared with 2000-fold higher molar concentration of positive control, 1000 µM of NAC. As shown in Figs. 6A, B, treatment with CA for 24h suppressed MMPs expression, but not a dose-dependent manner. But we observed that UPM-induced MMPs activity was effectively suppressed by CA treatment, indicating that it might exert anti-carcinogen effect on the human alveolar cells. The WHO has set daily exposure limits for PM2.5 and PM10 of 25 and 50 mg/m3, respectively.38) Namely, the UPM concentration of 10 µg/mL used in our experiment is not high, compared with the daily exposure limits for particulate matter. When the EMT process having the loss of adherent phenotype is progressed, cell invasion occurs along with the secretion of MMPs.39) Because the high mortality of lung cancer is closely associated with its early metastasis,26) we investigated whether...
0.5 μM of CA attenuates the human alveolar cells invasion. Our results provided further evidence that protective effect of CA on UPM-induced the invasion which is typical characteristics of tumor development.

In this study, we showed that co-treatment and post-treatment of CA had not only scavenging activity of the intracellular ROS, but also inhibitory activity of EMT process *via* TGF-β signaling. Excessive intracellular ROS derived from UPM may play a key role of EMT, enhance downregulation of the epithelial makers, and upregulate of the mesenchymal markers. Therefore, we can expect that CA is a promising candidate to modulate EMT process and a novel therapeutic compound for the UPM-derived pulmonary fibrosis.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

**REFERENCES**


