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Simvastatin inhibits induction of matrix metalloproteinase-9 in rat alveolar macrophages exposed to cigarette smoke extract

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Abbreviations: AM, alveolar macrophage; CSE, cigarette smoke extract; FPP, farnesyl pyrophosphate; FTI, farnesyl transferase inhibitor; GGPP, geranylgeranyl pyrophosphate; GGTI, GGPP transferase inhibitor; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MMP, matrix metalloproteinase

Abstract

Matrix metalloproteinase-9 (MMP-9) may play an important role in emphysematous change in chronic obstructive pulmonary disease (COPD), one of the leading causes of mortality and morbidity worldwide. We previously reported that simvastatin, an inhibitor of HMG-CoA reductase, attenuates emphysematous change and MMP-9 induction in the lungs of rats exposed to cigarette smoke. However, it remained uncertain how cigarette smoke induced MMP-9 and how simvastatin inhibited cigarette smoke-induced MMP-9 expression in alveolar macrophages (AMs), a major source of MMP-9 in the lungs of COPD patients. Presently, we examined the related signaling for MMP-9 induction and the inhibitory mechanism of simvastatin on MMP-9 induction in AMs exposed to cigarette smoke extract (CSE). In isolated rat AMs, CSE induced MMP-9 expression and phosphorylation of ERK and Akt. A chemical inhibitor of MEK1/2 or PI3K reduced phosphorylation of ERK or Akt, respectively, and also inhibited CSE-mediated MMP-9 induction. Simvastatin reduced CSE-mediated MMP-9 induction, and simvastatin-mediated inhibition was reversed by farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP). Similar to simvastatin, inhibition of FPP transferase or GGPP transferase suppressed CSE-mediated MMP-9 induction. Simvastatin attenuated CSE-mediated activation of RAS and phosphorylation of ERK, Akt, p65, IκB, and nuclear AP-1 or NF-κB activity. Taken together, these results suggest that simvastatin may inhibit CSE-mediated MMP-9 induction, primarily by blocking prenylation of RAS in the signaling pathways, in which Raf-MEK-ERK, PI3K/Akt, AP-1, and IκB-NF-κB are involved.

Keywords: macrophages, alveolar; matrix metalloproteinases-9; pulmonary disease, chronic obstructive; pulmonary emphysema; simvastatin; smoking

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive and irreversible airflow limitation, which is usually associated with an abnormal inflammatory response to noxious particles or gases, mainly cigarette smoke (Rabe *et al*., 2007). According to the World Health Organization, (COPD) will emerge as the third leading cause of death and rank fifth in 2020 in burden of disease worldwide by 2020 (Rabe *et al*., 2007). COPD manifests with two pathological features, emphysema and chronic bronchitis. Emphysema is caused by destruction of walls of the distal airways and alveolar sacs, leading to enlargement of airspaces.

Cigarette smoke harbors a multitude of chemical compounds and causes direct oxidative lung damage and indirect damage through the activation of various lung cells including alveolar macro-

phages (AMs) (Barnes *et al*., 2003). Activated AMs are suggested to amplify the inflammatory response through the liberation of various chemokines/cytokines required for recruitment and activation of other inflammatory cells such as neutrophils. The number of AMs is elevated in sputum, bronchoalveolar lavage fluid, airways, and lung parenchyma of smokers and patients with COPD (Barnes, 2004). The numbers of AMs in the alveolar walls correlates to the severity of COPD (Di Stefano *et al*., 1998). Moreover, AMs are localized to the sites of alveolar wall destruction in patients with emphysema (Finkelstein *et al*., 1995; Meshi *et al*., 2002). AMs activated by cigarette smoke extract (CSE) release inflammatory mediators such as TNF-α (Churg *et al*., 2003) and IL-8 (Culpitt *et al*., 2003) to provoke inflammation in the lungs of the individual smoker.

In addition, AMs also secrete several elastolytic enzymes including matrix metalloproteinase (MMP)-2, MMP-9, and MMP-12; cathepsins K, L, and S; and neutrophil elastase taken up from neutrophils (Punturieri *et al*., 2000; Russell *et al*., 2002). Among the proteases liberated from AMs, MMP-9 has been suggested to play an important role in development of emphysema in COPD. In patients with emphysema, expression of MMP-9 is elevated in AMs (Betsuyaku *et al*., 1999), and AMs from normal smokers express more MMP-9 than those from normal subjects (Lim *et al*., 2000). Moreover, AMs from patients with COPD display elevated basal- and CSE-induced MMP-9 activities (Russell *et al*., 2002).

Previously (Lee *et al*., 2005), we demonstrated that simvastatin, an inhibitor of HMG-CoA reductase, can successfully attenuate emphysematous changes and reduce the increase in MMP-9 activities in the lung tissues of rats exposed to cigarette smoke for 16 weeks. Beyond a lipid lowering effect by inhibiting HMG-CoA, statins have other pleiotropic effects that include improving or restoring endothelial function, inhibiting the proliferation and migration of smooth muscle cells, decreasing vascular inflammation, and enhancing stability of atherosclerotic plaques (Liao and Laufs, 2005). Furthermore, statins suppress MMP-9 expression in macrophages in atheromatous lesions (Aickawa *et al*., 2001), vascular smooth muscle cells (Turner *et al*., 2005), and cancer cells (Denoyelle *et al*., 2001).

While simvastatin alleviated emphysematous changes possibly through inhibiting MMP-9 induction in the lung tissues of rats exposed to cigarette smoke in our previous study, it remains unknown whether simvastatin inhibits CSE-mediated MMP-9 induction in AMs which are believed to orchestrate inflammatory and destructive responses to cigarette smoking (Barnes, 2004). Furthermore, it is unclear how cigarette smoke mediates MMP-9 induction in AMs. Presently, we sought to deter-

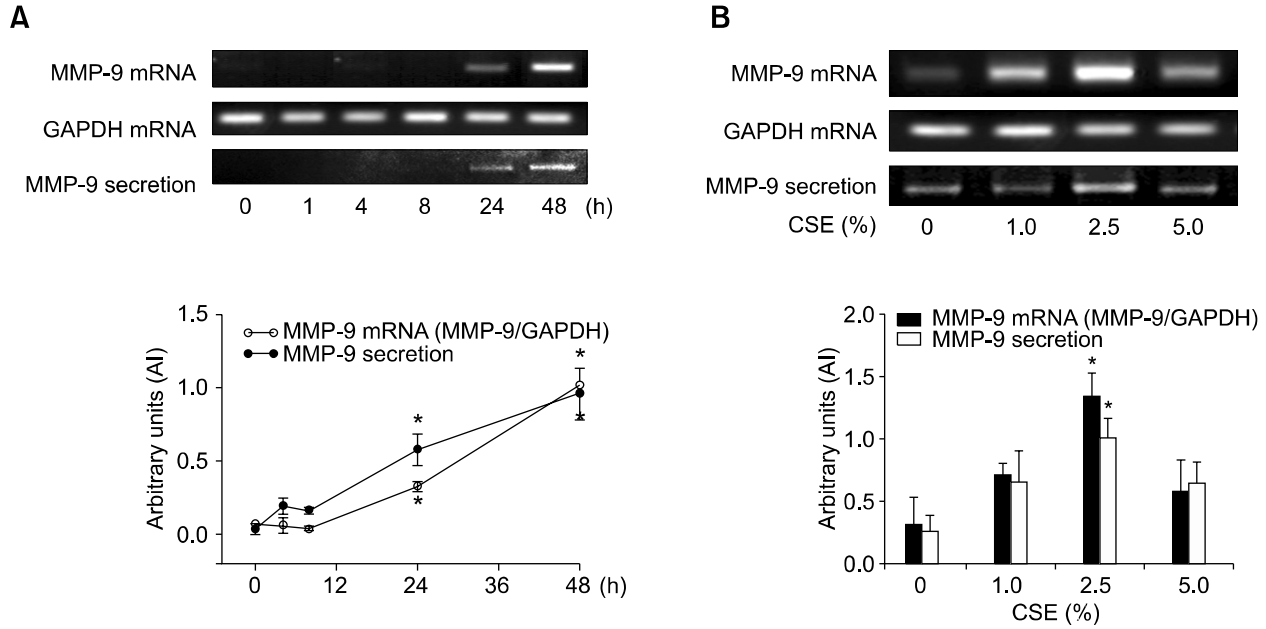

Figure 1. Induction of MMP-9 in AMs by CSE in time-dependent (A) and concentration-dependent (B) manners. (A) After exposing AMs to 2.5% CSE, conditioned media and AMs were harvested at indicated time points for gelatin zymography and RT-PCR of MMP-9 mRNA, respectively. (B) Forty-eight hours after exposure to different concentrations of CSE, gelatin zymography and RT-PCR of MMP-9 were performed. Expression of MMP-9 mRNA was normalized with expression of GAPDH mRNA. * or ** indicates statistical difference ($P < 0.05$) in comparison with levels at 0 h or 0% CSE, respectively.

mine the related signaling for MMP-9 induction and the inhibitory mechanism of simvastatin on MMP-9 induction in rat AMs exposed to CSE.

Results

CSE increases MMP-9 secretion and MMP-9 mRNA expression in rat AMs

MMP-9 induction by CSE exposure was investigated in isolated AMs. After exposure of AMs to 2.5% CSE, the MMP-9 activities in the conditioned medium and the expression of MMP-9 mRNA were determined at various times by gelatin zymography and RT-PCR, respectively (Figure 1A). Significant increase in the expression of MMP-9 mRNA was observed 24 h and 48 h after CSE treatment, with maximum induction at 48 h. In accordance with the changed expression of MMP-9 mRNA, gelatinolytic activities around 92 kDa increased in a similar manner. However, the 82 kDa active form of MMP-9 was not evident in gelatin zymograms. This absence of active MMP-9 implies the deficiency of extracellular proteolytic cleavage of secreted pro-MMP-9 under the experimental conditions.

Next, varying concentrations of CSE were added to AMs, and gelatin zymography and RT-PCR detection of MMP-9 were done 48 h later (Figure 1B). CSE induced both MMP-9 activity and mRNA expression in a concentration-dependent manner, whereas 5% CSE suppressed MMP-9 induction. Since higher concentrations of CSE might cause cytotoxicity (Aoshiba *et al*., 2001) or nonspecific suppression of cellular function, we subsequently used 2.5% CSE to maximally induce MMP-9.

MEK-ERK and PI3K-Akt are involved in CSE-mediated induction of MMP-9

MAPK and PI3K/Akt are known to be involved in MMP-9 induction in different types of cells stimulated by different stimuli (Gum *et al*., 1997; Lai *et al*., 2003; Lu and Wahl, 2005; Cheng *et al*., 2006; Han *et al*., 2006). Therefore, to identify the signaling pathways related to CSE-mediated MMP-9 induction, AMs were pretreated with inhibitors of each signaling molecule 1 h before CSE exposure, with MMP-9 secretion and MMP-9 mRNA expression being determined 48 h later. Both 10 μM PD98050 and 1 μM U0126, which are both MEK1/2

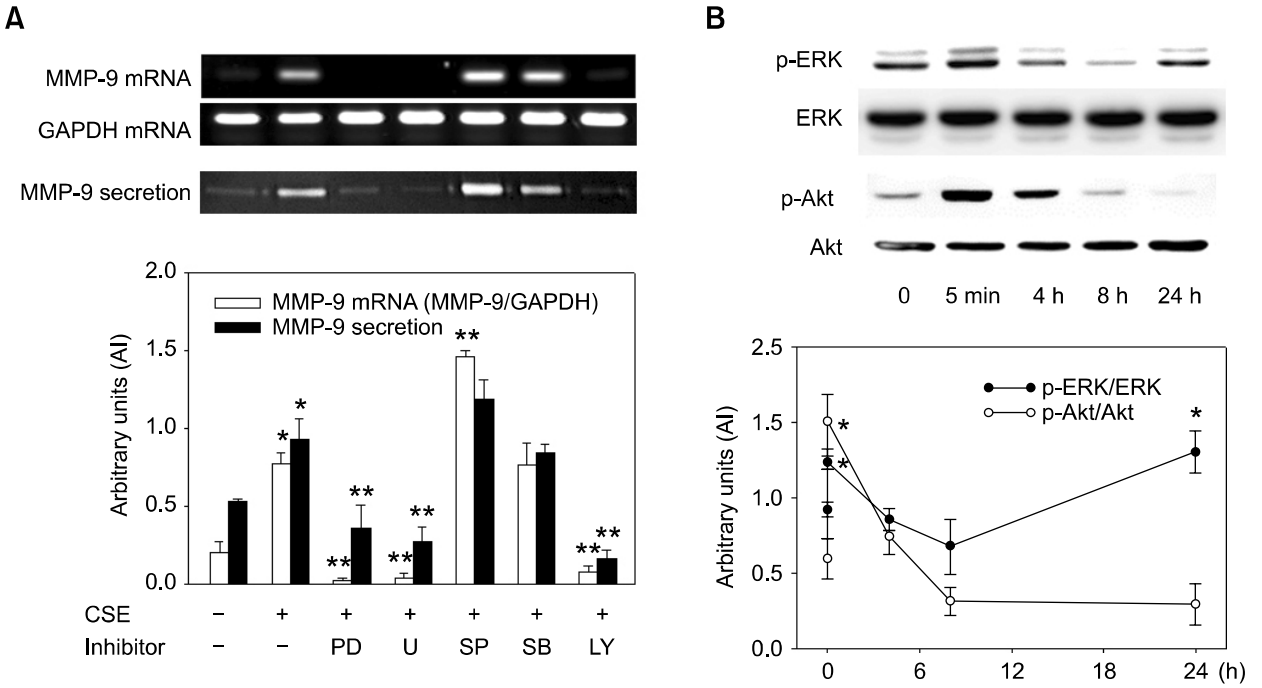

Figure 2. Involvement of MEK-ERK or PI3K-Akt in CSE-mediated MMP-9 induction in AMs. (A) Inhibition of CSE-mediated MMP-9 induction by chemical inhibitors. Chemical inhibitors were given 1 h prior to CSE administration to suppress MEK [10 μM PD98059 (PD), or 1 μM U0126 (U)], p38 MAPK (10 μM SB203580), JNK [10 μM SP600125 (SP)], or PI3K (10 μM LY294002). Forty-eight hours after exposure to 2.5% CSE, gelatin zymography and RT-PCR of MMP-9 were performed. * or ** indicates statistical difference ($P < 0.05$) in control vs. CSE-exposed group, or CSE-exposed vs. inhibitor-treated groups, respectively. (B) Phosphorylation of ERK and Akt by CSE in AMs. After CSE exposure, AMs were harvested at the indicated times, and phosphorylation of ERK and Akt was determined by Western blot. Levels of phosphorylated ERK or Akt were normalized with total ERK or Akt, respectively. * indicates statistical difference ($P < 0.05$) in comparison with the levels at 0 h.

inhibitors, effectively abrogated CSE-induced increase in MMP-9 secretion and MMP-9 mRNA expression (Figure 2A). Similarly, 10 μM of the PI3K inhibitor LY294002 decreased MMP-9 induction. However, neither SB203580 (a p38 MAPK inhibitor) or SP600125 (a JNK inhibitor) displayed inhibitory effect on CSE-mediated MMP-9 induction.

As ERK1/2 are downstream to MEK1/2, and Akt downstream to PI3K is involved in MMP-9 expression (Chung *et al*., 2004; Lu and Wahl, 2005; Cheng *et al*., 2006), phosphorylation status of ERK or Akt was checked by Western blot to confirm the involvement of MEK-ERK or PI3K-Akt in CSE-mediated MMP-9 induction. Phosphorylation of ERK1/2 was increased 5 min after CSE exposure, and gradually returned to basal level (Figure 2A). Phosphorylation of ERK1/2 significantly increased again at 24 h. Similarly, phosphorylation of Akt was increased by CSE at 5 min. However, dissimilar to ERK, there was no late phosphorylation of Akt at 24 h. Similar to the effects on MMP-9 induction, the MEK and PI3K inhibitors successfully inhibited phosphorylation of ERK1/2 and Akt, respectively (Figure 3). However, MEK inhibitors did not affect phosphorylation of Akt and the PI3K inhibitor did not decrease phosphorylation of ERK1/2. These observations suggest the involvement of MEK-ERK1/2 and PI3K-Akt in CSE-mediated MMP-9 induction in AMs in a process that does not involve pathway cross-talk.

Simvastatin inhibits MMP-9 induction in rat AMs exposed to CSE

As we observed attenuation of MMP-9 induction in a rat model of cigarette smoke-induced emphysema, we determined whether simvastatin might inhibit CSE-mediated MMP-9 induction in AMs. Simvastatin successfully reduced both MMP-9 secretion and MMP-9 mRNA expression in a concentration-dependent manner, with maximum inhibition at 10 μM simvastatin (Figure 4).

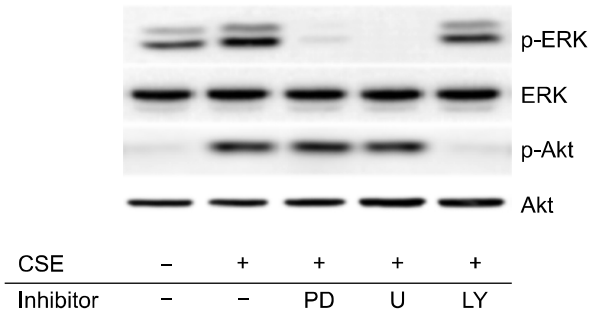

Figure 3. Absence of cross talk between MEK-ERK and PI3K-Akt pathways. MEK inhibitors [(10 μM PD98059 (PD) or 1 μM U0126 (U)] or a PI3K inhibitor (10 μM LY294002) were given 1 h prior to CSE exposure. Five min after CSE exposure, AMs were harvested and phosphorylation of ERK and Akt was determined by Western blot.

Simvastatin inhibits MMP-9 induction through protein prenylation

In addition to their inhibitory action on cholesterol synthesis, the pleiotropic effects of statins are partly explained by inhibition of synthesis of mevaolonate, a precursor of isoprenoids including FPP and GGPP, which are required for prenylation of small GTPase including RAS and Rho proteins (Jain and Ridker, 2005; Liao and Laufs, 2005). Moreover, RAS (Moon *et al*., 2004; Lee *et al*., 2006) and Rho proteins (Turner *et al*., 2005) are related to MMP-9 expression. Accordingly, we investigated whether simvastatin could suppress CSE-mediated MMP-9 induction via the inhibition of protein prenylation. Supplementation of FPP or GGPP before CSE exposure restored CSE-mediated MMP-9 induction (MMP-9 secretion and MMP-9 mRNA expression) in AMs treated with simvastatin (Figure 5). Conversely, 10 μM of either FTI-277 (a farnesyl transferase inhibitor) or GGTI-2133 (a geranylgeranyl transferase inhibitor) suppressed CSE-mediated MMP-9 induction similar to simvastatin. In accordance with the inhibitory action on MMP-9 induction, simvastatin, FTI, or GGTI redu-

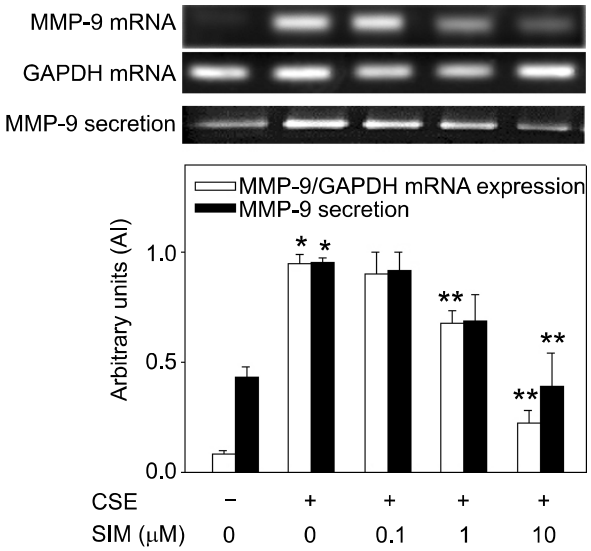

Figure 4. Inhibition of CSE-mediated MMP-9 induction by simvastatin in AMs. AMs were pretreated with vehicle (DMSO) or simvastatin (SIM) at the indicated concentrations 1 h prior to addition of 2.5% CSE. After 48 h, conditioned media and AMs were examined by gelatin zymography and MMP-9 RT-PCR, respectively. * or ** indicates statistical difference ($P < 0.05$) in comparison with the control or CSE-exposed group, respectively.

ced phosphorylation of ERK and Akt in AMs exposed to CSE similar to simvastatin (Figure 6).

Next, we determined the activation status of RAS, since lovastatin inhibits MMP-9 expression via the inhibited prenylation of RAS in vHRAS-transformed NIH 3T3 cells (Wang *et al*., 2000), and, as found presently, simvastatin, FTI or GGTI reduced CSE-mediated MMP-9 induction. CSE activated RAS in AMs, whereas simvastatin suppressed CSE-mediated RAS activation (Figure 7A). However, Rho activation was not detected, and neither cell-permeable C3 exoenzyme (a Rho inhibitor) nor Y-7632 (a ROCK inhibitor) inhibited MMP-9 induction (data not shown), which suggests no involvement of Rho-ROCK pathway in CSE-mediated MMP-9 induction.

Since the RAS-Raf-MEK-ERK pathway is activated by various stimuli, the involvement of Raf in CSE-mediated MMP-9 induction was investigated by the application of the specific Raf inhibitor GW 5074. GW 5074 successfully inhibited CSE-mediated MMP-9 induction (Figure 7B). These data suggest that CSE induces MMP-9 expression through the activation of RAS, and that simvastatin inhibits MMP-9 induction possibly by inhibiting prenylation of RAS, which subsequently transmits the signal to Raf.

Effect of simvastatin on downstream signaling molecules involved in MMP-9 expression

As AP-1 and NF-κB binding sites reside in the MMP-9 promoter region (Kim *et al*., 2007), and IKK-IκBα-p65 and AP-1 are involved in MMP-9 expression (Eberhardt *et al*., 2000, 2002; Takada *et al*., 2005), we checked the inhibition of these downstream signaling by simvastatin. The IKK inhibitors BMS-335541 and IKK inhibitor VII suppressed CSE-mediated MMP-9 induction (Figure 8A), consistent with the involvement of IKK in MMP-9 induction. Simvastatin also inhibited phosphorylation of IκBα and p65, which were increased by CSE exposure (Figure 8B). In addition, simvastatin inhibited the binding activities of NFκB and AP-1 in a gel shift assay (Figure 8C).

Discussion

In this study, we have demonstrated that CSE induces MMP-9 with activation of RAS, ERK, and

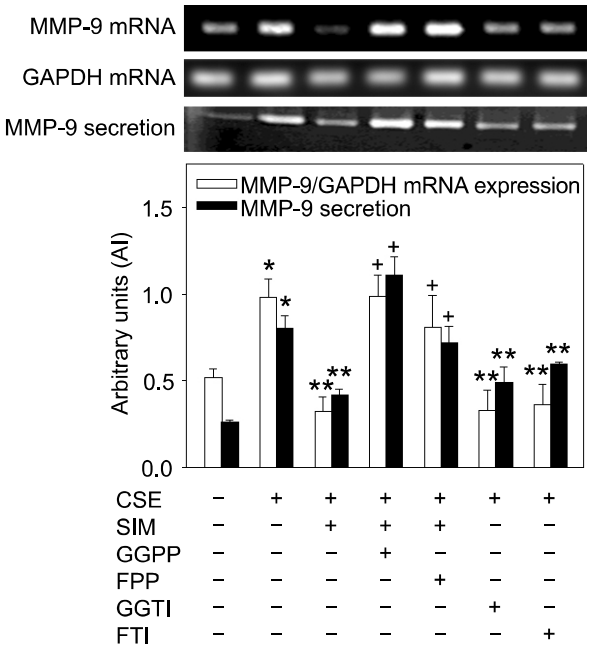

Figure 5. Simvastatin reduces MMP-9 induction through inhibition of isoprenoid synthesis. Isoprenoids (100 μM GGPP or 100 μM FPP) were added to AMs 1 h prior to administration of 10 μM simvastatin. CSE was added to AMs at a final concentration of 2.5%. A GGPP transferase inhibitor (GGTI-2133) or a FPP transferase inhibitor (FTI-277) was added at a final concentration of 10 μM 4 h before CSE exposure. After 48 h, MMP-9 secretion and MMP-9 mRNA expression were determined. *, +, or ** denotes statistical difference ($P < 0.05$) in comparison with the control, simvastatin-treated, or CSE-exposed group, respectively.

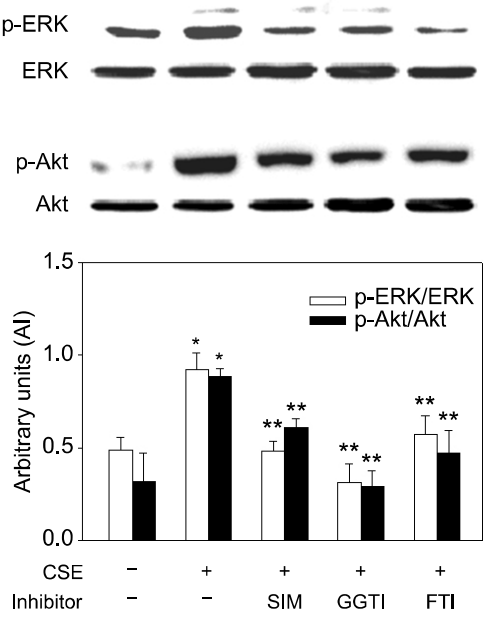

Figure 6. Protein prenylation mediates activation of ERK and Akt by CSE in alveolar macrophages. Simvastatin was added 1 h before CSE exposure, and GGTI-2133 or FTI-277 was added 4 h before CSE exposure. Five min after CSE exposure, phosphorylation of ERK and Akt were determined by Western blot. * or ** indicates statistical difference ($P < 0.05$) in comparison with the control or CSE-exposed group, respectively.

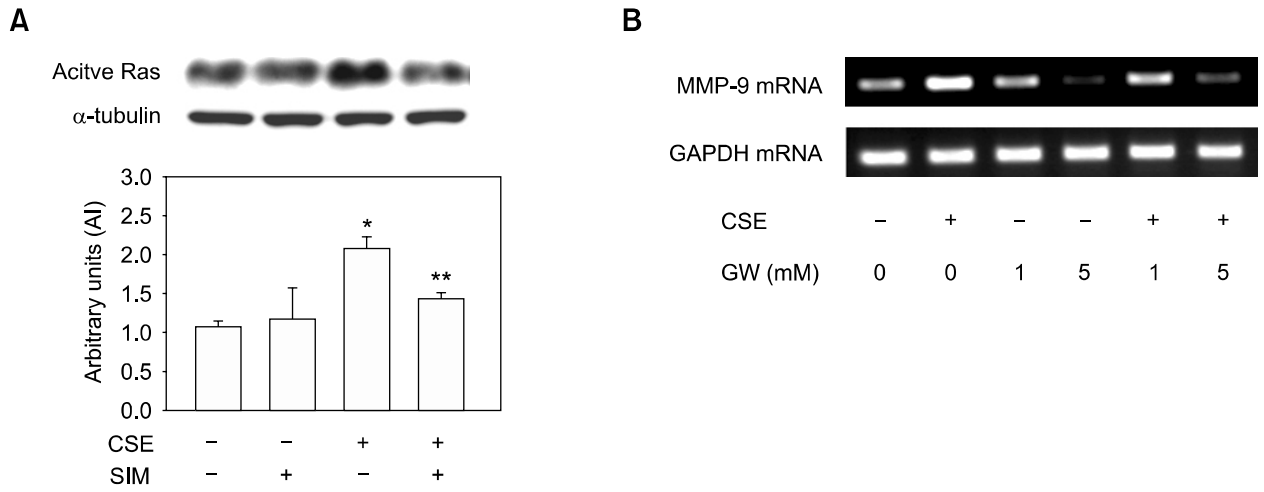

Figure 7. Involvement of RAS-Raf in CSE-mediated MMP-9 induction in AMs. (A) Inhibition of CSE-induced RAS activation by simvastatin. Three million rat AMs were seeded in a 35 mm-diameter dish. Simvastatin at 10 μM was added 1 h before addition of 2.5% CSE. Five min later, AMs were harvested and a RAS activation assay was performed. * or ** indicates statistical difference ($P < 0.05$) in comparison with control or CSE-exposed group, respectively. (B) Involvement of Raf in CSE-mediated MMP-9 induction. The selective Raf inhibitor GW 5074 (10 μM) was added 1 h prior to CSE exposure. After 48 h, AMs were taken for MMP-9 RT-PCR.

Akt together with increased binding activities of AP-1 and NF-κB in AMs. The data obtained from chemical inhibitor experiments suggests the involvement of Raf-MEK, PI3K, or IKK in the activation of these signaling molecules. Simvastatin successfully inhibited CSE-mediated MMP-9 induction by blocking isoprenoid synthesis, which is required for prenylation and activation of small GTPases, mainly RAS in this study. Simvastatin also reduced activation of ERK, Akt, AP-1, and NF-κB. These data support the suggestion that simvastatin may reduce CSE-mediated MMP-9 induction by inhibiting the activation of RAS, subsequently Raf-MEK-ERK or PI3K-Akt, and, finally, AP-1 and NF-κB.

Three RAS genes are translated into four RAS proteins: HRAS, NRAS, KRAS4A, and KRAS4B (KRAS4A and KRAS 4B are splice variants of a single gene). RAS proteins contain a CAAX motif (C, cysteine; A any aliphatic amino acids; X, any amino acid), which serves as a substrate for a series of post-translational modifications (Konstantinopoulos *et al*., 2007). These modifications include the covalent attachment of FPP or GGPP to the cysteine residue of the CAAX motif by prenylation. HRAS is only farnesylated, whereas NRAS, KRAS4A, and KRAS4B can be farnesylated and geranylgeranylated. In this study, the inhibitory action of simvastatin was reversed by either FPP or GGPP, and FTI or GGTI mimicked the inhibitory action of simvastatin. Even though we did not identify the form of RAS, it seems unlikely that HRAS is the only RAS protein capable of inducing MMP-9 expression, because HRAS is only farnesylated, and either GGTI or FTI inhibited CSE-mediated MMP-9 induction in this experiment. Also, cross-prenylation may not be operative in RAS activation in CSE-mediated MMP-9 induction. Whereas FTIs prevents HRAS farnesylation and reverse HRAS-induced transformation (Kohl *et al*., 1993), KRAS and NRAS can be geranylgeranylated (cross-prenylation) in FTI-treated cells, resulting in persistent membrane association of KRAS and NRAS, and persistent downstream activation of MAPK/ERK (Whyte *et al*., 1997; Law *et al*., 2000). However, presently, single treatment of GGTI or FTI inhibited MMP-9 induction. So, the contribution of each form of RAS in CSE-mediated MMP-9 induction remains unclear.

RAS proteins activate Raf/MEK/ERK and PI3K/Akt pathways. In human primary monocytes, LPS stimulation results in MMP-9 induction through a PI3K-Akt-IKK-NFκB pathway, in which p65 is activated (Lu and Wahl, 2005), while ERK mediates TNF-α-induced MMP-9 expression in human vascular smooth muscle cells via activation of NF-κB and AP-1 (Moon *et al*., 2004). In contrast, both MEK-ERK and PI3K-Akt pathways are suggested to activate both AP-1 and NF-κB in HBV X protein-transfected cells (Chung *et al*., 2004). Interestingly, ERK activity is increased not only in AMs of the mice chronically exposed to cigarette smoke but also in AMs of emphysema patients (Mercer *et al*., 2004). However, it is not clear whether ERK activation may be caused directly by exposure to cigarette smoke or indirectly by pro-inflammatory chemokines/cytokines from activated AMs (Yang *et*

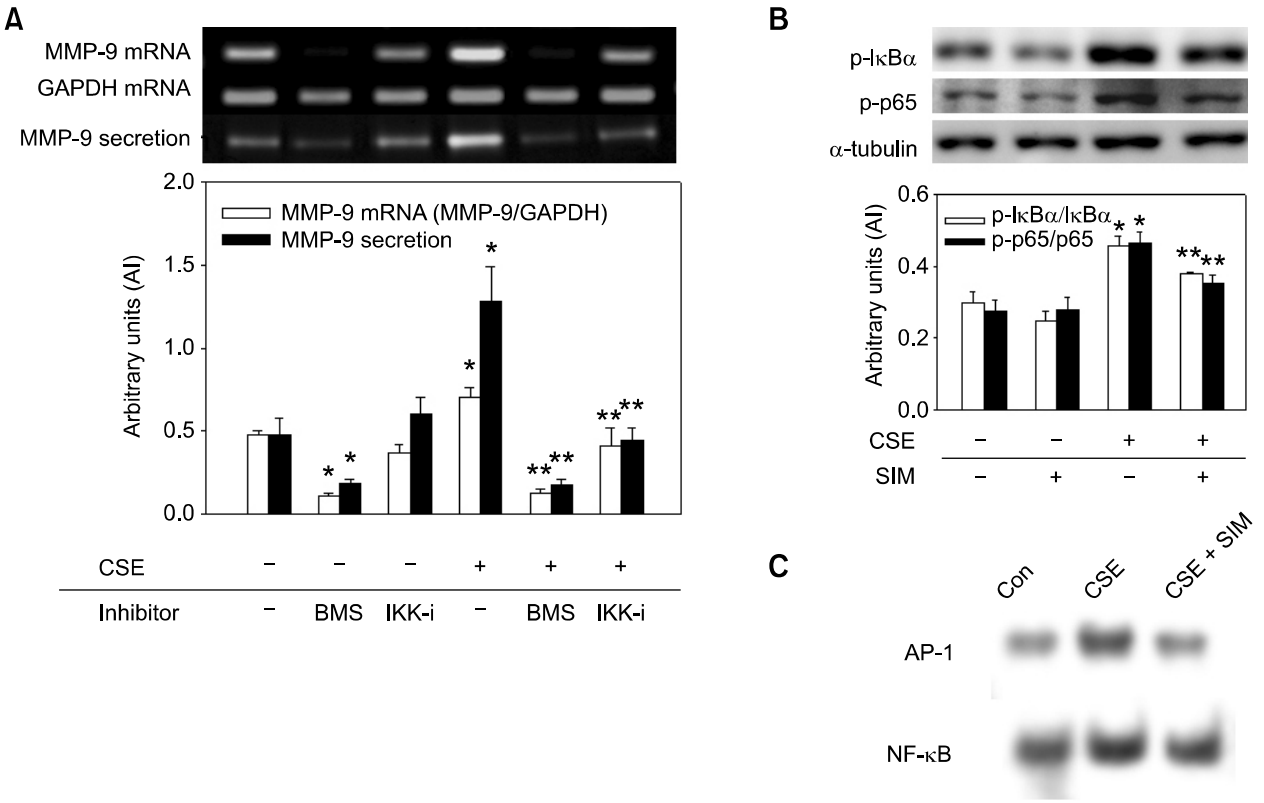

Figure 8. Effect of simvastatin on downstream signaling molecules involved in MMP-9 expression. (A) Involvement of IKK in CSE-mediated MMP-9 induction. IKK inhibitors (10 μM BMS-345541 (BMS) or 1 μM IKK VII inhibitor (IKK-i)) were added to rat AMs 1 h prior to exposure to 2.5% CSE. After 48 h, MMP-9 mRNA expression was determined. * or ** indicates statistical difference in comparison with control group, or CSE-treated group, respectively. (B) Inhibition of CSE-mediated phosphorylation of IκBα and p65 by simvastatin. Simvastatin at 10 μM was added 1 h prior to addition of 2.5% CSE. 24 h later, AMs were harvested for Western blot. * or ** indicates statistical difference in comparison with CSE-treated group, or CSE-exposed group. (C) Effect of simvastatin on binding activity of AP-1 or NF-κB in AMs exposed to CSE. Simvastatin at 10 μM was added 1 h prior to the addition of 2.5% CSE. 24 h later, AMs were harvested for a gel shift assay.

al., 2006) or other types of cells (Moodie *et al*., 2004). Our present observation concerning late ERK activation raises the possibility that CSE may activate AMs to release some autocrine factors, which may induce MMP-9.

In this study, we demonstrated inhibitory effect of simvastatin on MMP-9 induction using an *in vitro* model. Hence, the present *in vitro* data may raise questions in the context of physiological relevancy. However, several reports imply correlation between *in vitro* and *in vivo* data. Russell *et al*. (2002) reported that CSE treatment for 24 h induced higher production of MMP-9 in AMs isolated from COPD patients than from normal volunteers or healthy smokers. In MonoMac6, a human monocyte-macrophage cell line, CSE treatment for 1 h caused increased IKKα expression, which was also observed in AMs *in vivo* in mouse lungs after 3 days or 8 weeks of cigarette smoking (Yang *et al*., 2008). Moreover, cultured alveolar epithelial cells exposed to CSE for 2 h showed increased expression in receptor for advanced glycation end-products (RAGE), similar to alveolar/airway epithelial cells in lungs of mice exposed to cigarette smoke for 6 months (Reynolds *et al*., 2008). However, care must be taken in interpreting or extrapolating *in vitro* data because of *in vivo* interaction of AMs with other types of lung cells or circulatory blood cells. Therefore, *in vivo* experiments are needed to confirm whether the beneficial effects of simvastatin may be mediated by inhibition of MMP-9 induction in AMs.

Here, we demonstrate that simvastatin can inhibit CSE-mediated MMP-9 induction. However, the contribution of MMP-9 in emphysema in COPD has been questioned, even with indirect evidence including higher levels of MMP-9 in COPD patients (Ohinishi *et al*., 1998; Russel *et al*., 2002). Recently, converse to this notion, experimentally induced adult onset emphysema was reported in MMP-9 transgenic mice (Foronjy *et al*., 2008). After 12 months of age, MMP-9 transgenic mice developed

significant air space enlargement together with decreased levels of elastin in their alveolar walls. In contrast, ablation of the MMP-9 gene did not protect mice from the development of emphysema induced by repeated intratracheal administration of lipopolysaccharide (Brass *et al*., 2008). Thus, it remains debatable whether MMP-9 plays a central role in the development of emphysema in COPD. However, the contribution of MMP-9 to lung injury in the exacerbation phase of COPD cannot be excluded, because MMP-9 elevated in the exacerbation phase may aggravate inflammation through several mechanisms including proteolytic activation of pro-inflammatory cytokines such as TNF-α (Gearing *et al*., 1998) and IL-8 (Van den Steen *et al*., 2000).

In our previous study, we showed that simvastatin attenuates emphysematous change and pulmonary hypertension, both of which are observed in terminal COPD patients (Lee *et al*., 2005). Similar to our previous findings, Takahashi *et al*. (2008) recently reported that simvastatin reverses elastase-induced emphysema with alveolar epithelial proliferation, increases VEGF synthesis, and elevates eNOS expression. However, whether simvastatin can regenerate damaged lungs in cigarette smoking-induced emphysema remains to be demonstrated. This question may be originated from RAS potentially mutated by chronic exposure to cigarette smoke. In the epithelium of smokers' lung, somatic mutations have been identified in RAS, p53, EGFR, and PTEN (Anderson and Bozinovski, 2003). Until now, these mutations have been intensively investigated in the context of carcinogenesis. However, evidence is lacking that the somatic mutations play a role in persistent activation in the context of inflammation and tissue remodeling process including MMP-9 induction in COPD. In addition, it is unknown whether mutated RAS remains as a target of simvastatin in inflammatory cells including AMs. These uncertainties need to be resolved before any clinical trial of simvastatin in human COPD can be undertaken.

Methods

Reagents

Simvastatin, PD98059, U0126, LY294002, SP600125, SB203580, geranylgeranyl transferase inhibitor (GGTI), farnesyl transferase inhibitor (FTI), BMS- 345541, and IκB kinase (IKK) inhibitor VII were purchased from Calbiochem (San Diego, CA). GW 5074 was purchased from Tocris (Elliville, MI). Dulbecco's DPBS, MEM, and penicillin/streptomycin were obtained from Gibco (Calsbad, CA). Geranylgeranyl pyrophosphate (GGPP), farnesyl pyrophosphate (FPP), BSA, protease inhibitor cocktail, and gelatin were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to p-ERK, p-Akt, Akt, and p-IκB were purchased from Cell Signaling Technology (Beverly, MA), and antibody to ERK1/2 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence detection reagents were purchased from Amersham (Piscataway, NJ).

Isolation and culture of rat AMs

Animal experimentation protocols were approved by the Institutional Animal Care and Use Committee of Sungkyunkwan University School of Medicine. Male Sprague-Dawley rats weighing 250-350 g (Orient, Sungnam, Korea) were used for isolation of AMs. After rats were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg) and xylazine (5 mg/kg), the trachea were cannulated and AMs were obtained by three bronchoalveolar lavages (BALs) using 10 ml of Ca^{2+}- and Mg^{2+}-free DPBS each time. Cells recovered from the pooled BAL fluid were suspended in MEM containing 0.02% BSA, 25 mM HEPES, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were seeded in either 24-well culture plates (Nalge Nunc, Napersville, IL) or 35 mm-diameter culture dishes (Nalge Nunc). After incubation for 2 h at 37^{o}C in a 5% CO_2 humidified atmosphere to adhere, non-adherent cells were removed by washing one time with complete medium. AMs were incubated overnight to make them quiescent. Cell viability as determined by trypan blue exclusion was $>$ 95% and the purity of AMs as determined by nonspecific esterase staining (Sigma-Aldrich) was $>$ 90%. AMs from the same rat were used for the same series of experiments to reduce inter-individual variation.

CSE preparation

CSE was prepared as previously described (Gibbs *et al*., 1999) with slight modification. Briefly, mainstream smoke from two cigarettes (Eighty Eight Lights, KT&G, Korea) yielding 8.5 mg of tar and 0.9 mg of nicotine were simultaneously bubbled in 10 ml of MEM containing 25 mM HEPES. After determining the absorbance at 340 nm, the CSE solution was filtered through a 0.22 μm syringe filter (Millipore, Billerica, MA). The filtered CSE solution was applied to AMs within 30 min.

Gelatin zymography

Semi-quantitative measurement of MMP-9 activities in conditioned media was carried out using zymography (Gibbs *et al*., 1999). After centrifugation of conditioned media at 400 *g* for 5 min, MMP-9 in the conditioned media was separated by 8% SDS-PAGE gels containing 2 mg/ml porcine skin gelatin. After electrophoresis, the gels were washed three times in a washing buffer (20 mM Tris/HCl, pH 7.8 and 2.5% Triton X-100) for 15 min at room temperature. The gels were subsequently washed twice for 15 min in a developing buffer (20 mM Tris/HCl, pH 7.8, 1% Triton X-100, 10 mM $CaCl_2$, and 5 μM $ZnCl_2$), incubated for 18 h at 37^{o}C, stained with 1% Coomassie Blue R-250 in a destaining solution containing 40% methanol, 10% acetic

acid and 50% water for 20 min, and washed by immersion in the destaining solution for 1 h. Gel images were acquired using the LAS 3000 (Fuji, Japan), and analyzed with the Image Gauge software (Fuji).

RT-PCR

RT-PCR was performed to analyze mRNA expression of MMP-9 and GAPDH. Total RNAs were isolated from attached AMs using a kit (iNtRON, Sungnam, Korea) according to the manufacturer's instruction. cDNA was synthesized using 0.2 μg total RNAs in 10 μl of Superscript II First Strand System reaction mixture (Invitrogen). PCR primers used for amplifying MMP-9 and GAPDH were as follows: MMP-9, 5'-CAAACCCTGCGTATTTCC-3' and 5'-AGAGTACTGCTTGCCCAGGA-3'; GAPDH gene, 5'-CTCATGACCACAGTCCATGC-3' and 5'-TTCATCGGGATGACCTT-3'). PCR was performed with 2 μl of cDNA and 0.2 mM of each primer in the Gene Amp PCR system 9700 (Perkin Elmer, Norwalk, CT) using touchdown PCR consisting of 2 cycles of PCR at 94°C for 1 min, 64°C for 1 min and 72°C for 1 min, followed by 25 cycles (MMP-9) or 20 cycles (GAPDH) of PCR at 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. The PCR products (MMP-9, 223 bp; GAPDH, 155 bp) in 20 μl were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Band intensities were quantified by the Gel-Doc system (Kodak, Rochester, NY).

Western blot analysis

One million AMs were lysed in 100 μl of cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM p-nitrophenyl phosphate, and a protease inhibitor cocktail). The lysates were centrifuged at 16,000 *g* for 20 min at 4°C. Proteins in the supernatant were separated by 12% SDS-PAGE and transferred to a PVDF membrane (Schleicher & Schuell, Riviera Beach, FL). Each membrane was blocked with 5% skim milk in T-TBS (Tween 20-Tris buffered saline; 8 g/l NaCl, 0.2 g/l KCl, 3 g/l Tris-HCl, pH 7.4, and 0.1% Tween 20) for 1 h at room temperature. Then, each membrane was incubated overnight at 4°C with a primary antibody at a titer of 1:1,000. After washing with T-TBS, the membrane was incubated with HRP-conjugated secondary antibodies at a titer of 1:5,000 for 1 h at room temperature. Proteins were visualized using enhanced chemiluminescence reagents and LAS-3000.

Determination of RAS activity

RAS activity was assessed using a kit (Upstate Biotechnology, Billerica, MA). Briefly, AMs in a 60 mm-diameter dish were washed twice with ice-cold PBS and lysed in 200 μl of MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% IGEPAL CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol) containing a protease inhibitor cocktail and 1 mM Na_3VO_4. After centrifugation at 14,000 *g* for 10 min, protein concentrations were determined using a kit (Bio-Rad, Hercules, CA). The cell extract containing 300 μg protein was incubated at 4°C for 45 min with 5 μl of agarose slurry containing a fusion protein consisting of glutathione S-transferase and human RAS-binding domain (residues 1-149) of RAF1. After the samples were washed three times with MLB, activated RAS (RAS-GTP) in the reaction mixture was detected by Western blot with anti-RAS monoclonal antibody.

Gel shift assay

Nuclear activity of AP-1 or NF-κB was determined with a gel shift assay using a kit according to manufacturer's instructions (Promega, Madison, WI). In brief, 5'-radiolabeled AP-1 or NF-κB probe was incubated with 10 μg of nuclear proteins at 20°C for 20 min. DNA-protein complex was separated in a 4% nondenaturing polyacrylamide gel and images were taken with a FLA 3000 phosphoimager (Fuji).

Statistical analysis

Data are presented as mean ± SEM of 3 to 6 independent experiments. Statistical significance ($P < 0.05$) was determined by ANOVA followed by the multiple comparison *t*-test.

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