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Biochemical and Biophysical Research Communications 329 (2005) 58–63
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Cigarette smoke extract induces endothelial cell injury via JNK pathway

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Received 12 December 2004
Available online 2 February 2005

Abstract

Cigarette smoking is the most crucial factor responsible for chronic obstructive pulmonary disease (COPD). The precise mechanisms of the development of the disease have, however, not been fully understood. Recently, impairment of pulmonary endothelial cells has been increasingly recognized as a critical pathophysiological process in COPD. To verify this hypothesis, we examined how cigarette smoke extract (CSE) damages human umbilical vein endothelial cells (HUVECs). CSE activated c-Jun N-terminal kinase (JNK), and treatment of HUVECs with SP600125, a specific inhibitor of the JNK pathway, significantly suppressed endothelial cell damage by CSE. In contrast, inhibition of the extracellular-regulated kinase or the p38 pathway did not affect the cytotoxicity of CSE. Furthermore, anti-oxidants superoxide dismutase and catalase reduced CSE-induced JNK phosphorylation and endothelial cell injury. These results indicate that CSE damages vascular endothelial cells through the JNK pathway activated, at least partially, by oxidative stress.

Keywords: COPD; Cigarette smoke extract; Endothelial cell; c-Jun N-terminal kinase; Oxidative stress; Superoxide dismutase; Catalase

Cigarette smoking is known to be associated with various kinds of diseases, including atherosclerotic cardiovascular disease, cancer, and chronic obstructive pulmonary disease (COPD) [1–3]. It is predicted that COPD, which was in sixth place in the global ranking of the causes of death in 2000, will move to third place by 2020. No currently available treatments have, however, been shown to suppress the progression of this disease. Therefore, new agents for the treatment of COPD are greatly and urgently needed.

Emphysema is the major pathological change in the lungs of patients with COPD. An imbalance between proteinase and antiproteinase has been proposed in the pathogenesis of emphysema [4]. The oxidative stress hypothesis has also evolved to explain the association between smoking and emphysema [5]. Moreover, impairment of pulmonary endothelial cells has been increasingly recognized as a crucial pathophysiological process in emphysema and COPD [6]. A VEGF receptor inhibitor induces endothelial cell apoptosis in rat lungs leading to emphysematous changes [7], suggesting the involvement of endothelial cells in the pathogenesis of emphysema and COPD. Thus, it is reasonable to hypothesize that cigarette smoke induces endothelial cell injury, death-causing emphysema, and COPD. Consequently, further studies on the precise mechanism of cigarette smoke injury to endothelial cells may lead to new insights into the prophylactic and therapeutic strategies for COPD.

Here, we investigated the cytotoxic effect of cigarette smoke on endothelial cells and examined the roles of

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doi:10.1016/j.bbrc.2005.01.095

JNK, p38, and ERK in CSE-induced cell damage. Our data demonstrated that CSE activated the JNK pathway in endothelial cells and that a JNK inhibitor attenuated CSE-induced endothelial cell damage, suggesting that CSE induced endothelial cell injury through the JNK pathway. In addition, the results disclosed that oxidative stress generated by CSE was involved in the CSE-induced JNK activation that causes vascular endothelial cell injury.

Materials and methods

Reagents. SP600125 (a specific JNK inhibitor), SB203580 (a specific p38 inhibitor), and PD98059 (a specific inhibitor of mitogen-activated protein (MAP) kinase kinase (MEK)) were purchased from CALBIOCHEM (Darmstadt, Germany). Superoxide dismutase (SOD) was purchased from MP Biomedicals (Eschwege, Germany); Catalase was from Wako Pure Chemical Industries (Osaka, Japan); and the LDH Cytotoxicity Detection kit was from TaKaRa Bio Laboratories (Tokyo, Japan).

Cell culture. Human umbilical vein endothelial cells (HUVECs), purchased from the Japan Health Sciences Foundation (Osaka, Japan), were cultured in MCDB131 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 ng/ml basic fibroblast growth factor (bFGF) (PEPROTECH EC, London, UK), 1460 mg/L L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. HUVECs between passages 4 and 6 were used in the experiments.

Cigarette smoke extract. Cigarette smoke extract (CSE) was prepared as previously described with some modification [8]. Briefly, the smoke from five cigarettes (''Seven Stars,'' Japan Tobacco, tar 14 mg, nicotine 1.2 mg) was bubbled through 10 ml of pre-warmed (37 °C) phosphate-buffered saline (PBS) at a constant negative pressure (5 L/min) [9]. PBS containing CSE was sterilized by filtration through a 0.22 μm syringe filter; the resulting solution, considered 100% CSE, was stored at −20 °C until use.

LDH assay. HUVECs were seeded onto a 96-well collagen-coated plate at a density of 1×10^4 cells per well and allowed to attach overnight. The medium was then changed to the MCDB131 medium containing 2% FCS; the cells were again cultured overnight for quiescence and exposed to CSE. In some experiments, the cells were preincubated with various reagents for 30 min before exposure to CSE. The LDH cytotoxicity detection assay was conducted according to the manufacturer's protocol. In brief, 100 μl supernatant of the cultured HUVECs was mixed with 100 μl solution of the assay kit. The reaction mixture was incubated for 30 min at room temperature in the dark. The absorbance of the sample was then measured at 490 nm with a microplate reader at a reference wavelength of 630 nm.

Western blotting. The treated or untreated cells exposed to CSE were washed with PBS and then lysed with RIPA buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride, 0.04 TIU/μl aprotinin, 1 mM sodium orthovanadate, and 50 mM sodium fluoride. Protein concentrations were measured with the Detergent-Compatible (DC) protein assay (Bio-Rad, Hercules, CA). Samples were separated by gel electrophoresis on 4/20% SDS–polyacrylamide gradient gels (SDS–PAGE). Separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were then incubated with rabbit primary antibodies against phospho-JNK, phospho-p38, and phospho-ERK (all from Cell Signaling, Beverly, MA) at a dilution of 1:200 for 1 h at room temperature, before incubation for another 1 h at room temperature with donkey anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) at a dilution of 1:1000. The membranes were visualized by a chemiluminescence reagent (Perkin-Elmer Life Science, Boston, MA) according to the manufacturer's instructions, and the image was obtained by exposure to X-ray films. To determine the total amounts of JNK, p38, and ERK, antibodies against total JNK-1, total p38, and the total ERK1 and ERK2 mixture (Santa Cruz Biotechnology, Santa Cruz, CA) were used at dilutions of 1:2000, 1:200, and 1:1000, respectively.

Statistics. All values are expressed as means ± SD. The statistical significance of the differences was evaluated by ANOVA and then by Bonferroni *t* test. *P* values < 0.05 were considered statistically significant.

Results

CSE damage to endothelial cells in a time- and dose-dependent manner

To characterize the toxic effects of CSE on human vascular endothelial cells, HUVECs were first treated with 10% CSE for varying periods. Cytotoxicity determined by LDH assay revealed that CSE specifically induced LDH release from HUVECs in a time-dependent manner (Fig. 1A). To determine whether the effect was dose-dependent, HUVECs were incubated with varying concentrations of CSE (Fig. 1B). CSE at a concentration of 3% had only a background effect on LDH release from HUVECs; however, significant cytotoxicity was observed in a dose-dependent manner at CSE concentrations between 5% and 10%. Subsequent experiments were carried out at a CSE concentration of 10%, and the amount of LDH release was determined at 18 h after CSE exposure.

JNK pathway activation by CSE

Since MAP kinases, including ERK, p38, and JNK, are activated in response to a variety of extracellular stimuli [10–12], we investigated whether CSE stimulated the phosphorylation and activation of the MAPK pathway, by Western blot analysis using anti-phospho-ERK, anti-phospho-p38, and anti-phospho-JNK. The amount of phosphorylated ERK increased as early as 60 min after adding CSE and remained elevated for up to 240 min (Fig. 2A). JNK was also phosphorylated progressively; phospho-JNK signaling was up-regulated at 120 min after adding CSE, persisted, and increased up to 240 min (Fig. 2C). In contrast, p38 was transiently phosphorylated within 5 min of CSE stimulation, reached a maximum at 30 min, and then reverted to nearly basal levels (Fig. 2B).

JNK involvement in CSE-induced endothelial cell injury

CSE up-regulated the phosphorylation of ERK, p38, and JNK, suggesting that MAP Kinase plays a part in the mechanisms of CSE-induced endothelial cell damage. Consequently, we examined the possible involvement of MAP Kinase in endothelial cell injury induced by CSE. Cells were pretreated with specific MAP Kinase

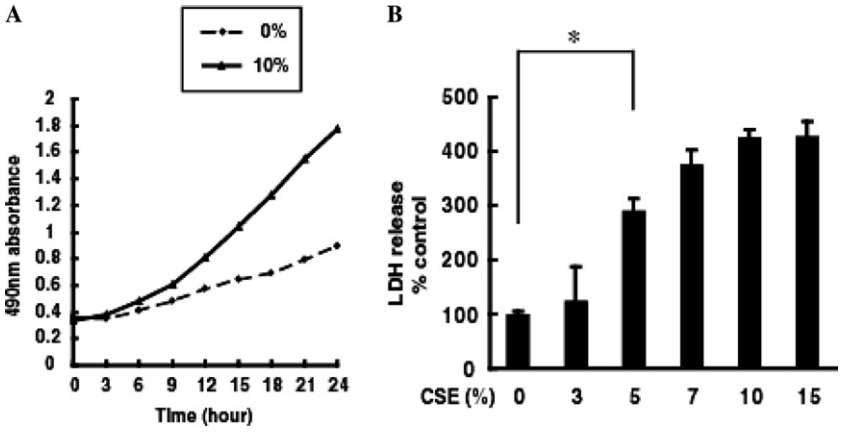

Fig. 1. LDH release from HUVECs treated with CSE. (A) Time course measurement of LDH release from HUVECs. Cells were incubated with (solid line) or without (dashed line) 10% CSE for several hours. The figure is representative of three separate experiments with similar results. (B) LDH release from HUVECs treated with various concentrations of CSE. Cells were incubated with the indicated concentrations of CSE for 18 h. Results are expressed as a percentage of the control value, means ± SD ($n = 8$). The figure is representative of three separate experiments with similar results. $^{*}P < 0.05$.

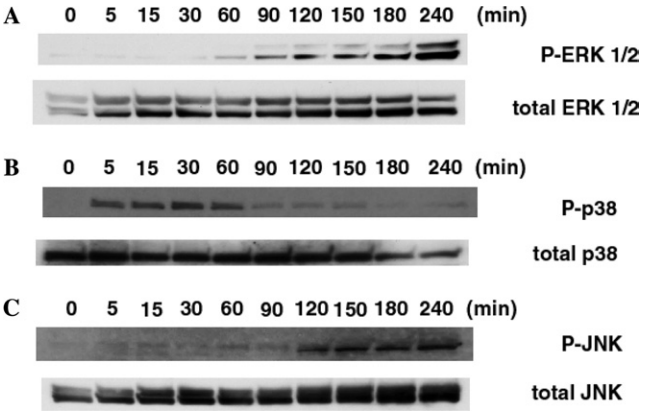

Fig. 2. Western blot analyses of the phosphorylation of JNK, p38, and ERK 1/2 after CSE treatment. HUVECs were treated with 10% CSE for the indicated durations. Phosphorylation of ERK 1/2 (A), p38 (B), and JNK (C) was detected with antibodies specific for phosphorylated MAP Kinases (upper panels). As loading controls, membranes were blotted with antibodies against total ERK, p38, and JNK (lower panels). The results are representative of three separate experiments with similar results.

inhibitors before exposure to CSE. SP600125 markedly reduced LDH release induced by CSE in a dose-dependent manner (Fig. 3A). In contrast, neither SB203580 nor PD98059 affected LDH release from CSE-treated HUVECs (Figs. 3B and C). Taken together with the data of Western blotting, these results suggest that the activity of JNK is requisite for CSE-induced endothelial cell injury, whereas that of either ERK or p38 is not.

Oxidative stress involvement in endothelial cell injury induced by CSE

Oxidative stress, responsible for many of the cytotoxic effects of CSE [13,14], also induces JNK activation [15]. We, therefore, examined oxidants with regard to their correlation with JNK signaling and, furthermore, with injury to endothelial cells. Western blot analysis demonstrated that pretreatment with SOD or catalase partially suppressed JNK phosphorylation induced by CSE (Fig. 4A). Similarly, the amounts of LDH release from

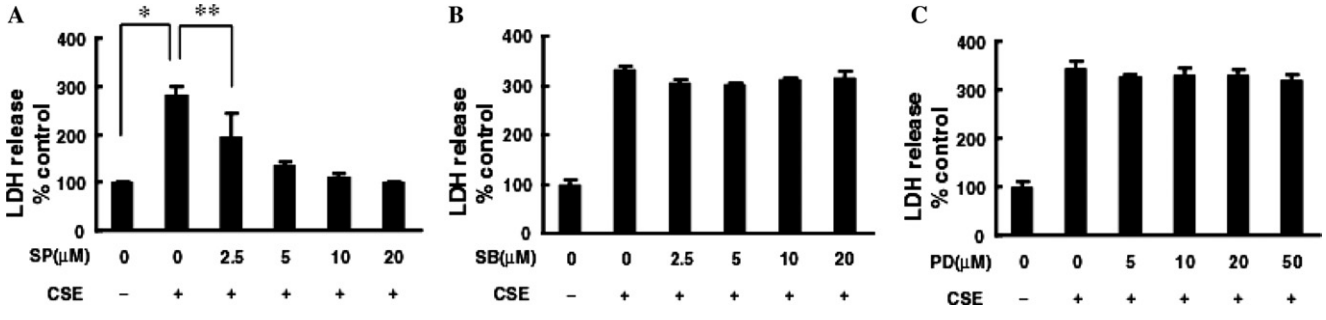

Fig. 3. Effects of MAP Kinase inhibitors of CSE-induced endothelial cell injury. LDH release from HUVECs with or without (A) SP600125 (B) SB203580 or (C) PD98059. Results are expressed as a percentage of control value, means ± SD ($n = 4$). The figure is representative of three separate experiments with similar results. $^{*,**}P < 0.05$.

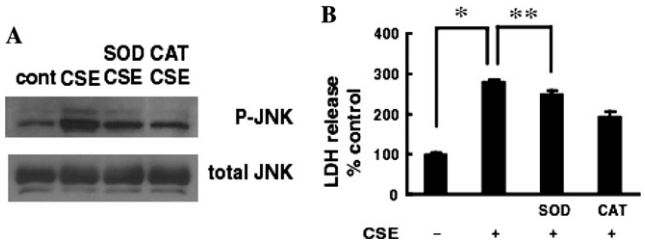

Fig. 4. Effects of antioxidants on CSE-induced endothelial cell injury. (A) The amount of JNK phosphorylation induced by CSE in HUVECs treated with or without 50 U/ml SOD or 1000 U/ml catalase (CAT) was determined by Western blotting. The figure is representative of three separate experiments with similar results. (B) The amount of LDH released from HUVECs treated with or without SOD or CAT is expressed as a percentage of the control value, means ± SD ($n = 4$). The figure is representative of three separate experiments with similar results. *·**$P < 0.05$.

endothelial cells preincubated with the antioxidants were significantly, but partially, attenuated compared with those from untreated cells (Fig. 4B), suggesting that oxidative stress, at least partially, activated CSE-induced JNK signaling resulting in vascular endothelial cell injury.

Discussion

The current study demonstrated that CSE induced endothelial cell injury in a dose- and time-dependent manner. Western blot analysis showed that CSE phosphorylated the three MAP kinases. A JNK inhibitor attenuated CSE-induced LDH release from endothelial cells, whereas neither the p38 inhibitor nor the ERK inhibitor affected LDH release from CSE-treated HUVECs. Pretreatment with antioxidants partially suppressed JNK phosphorylation and endothelial cell injury by CSE. Taken together, these results indicate that CSE induces vascular endothelial cell damage through the JNK pathway activated, at least partially, by oxidative stress.

The MAP kinase pathway is involved in a variety of biological events, including cell proliferation, differentiation, and cell death [16]. JNK is activated by various types of stresses such as ultraviolet irradiation, heat shock, proinflammatory cytokines, and osmotic stress [17], and is, therefore, also called stress-activated protein kinase (SAPK). Accumulating evidence supports the concept that JNK activation contributes to the induction of cell death in various kinds of cells [18–22]. On the other hand, several studies have reported that under various conditions JNK can protect cells from damage [23,24]. From these observations, it can be speculated that the JNK pathway is bidirectional depending on cell types, stimuli, and environmental circumstances. Nonetheless, our results clearly demonstrated that JNK activation was a prerequisite to CSE-induced endothelial cell injury.

This study showed that CSE-induced cell damage is dependent on the JNK pathway; however, the molecules involved downstream of this pathway remain unknown. c-Jun, an important transcription factor activated through the JNK pathway, is crucial in UV-induced cell death [25]. Cycloheximide, a protein synthesis inhibitor, did not, however, affect CSE cytotoxicity in our study (personal observation), suggesting that CSE-induced cell death through the JNK pathway might not be mediated by the activation of c-Jun or by its downstream transcription of various genes. This observation is consistent with a previous report [18]. Moreover, accumulating evidence has proposed a variety of mechanisms for JNK-mediated cytotoxicity: JNK induces cell damage by the activation of death receptors 4 and 5 [26]; inactivation of Bcl-2 [27] or enhancement of cytochrome c release from the mitochondria [18] has also been involved in JNK-mediated cell death. Further studies are needed to reveal the mechanisms activated by JNK in the cytotoxic effects of CSE.

Our results demonstrated that other MAP Kinases, ERK and p38, were also activated by CSE. In general, the ERK cascade has been shown to communicate growth factor-induced proliferation signals[16]. Recent evidence has also suggested that the ERK pathway is anti-apoptotic [28]; in our study, however, PD98059, a commonly used specific ERK inhibitor, neither suppressed nor enhanced CSE-induced endothelial cell injury. On the other hand, the p38 MAPK pathway is regarded as a key regulator of inflammation [29]; also, the involvement of p38 in cell damage is recognized [30]. Our study demonstrated that SB203580 did not affect the cytotoxicity of CSE. These observations suggested that neither the ERK nor the p38 cascade was requisite for the cytotoxic effect of CSE on vascular endothelial cells.

We demonstrated that CSE-induced endothelial cell injury depends on the JNK cascade. Several reports have noted the relation between cigarette smoke and JNK signaling: the JNK pathway is up-regulated in whole lung of ferrets exposed to cigarette smoke for 6 months [31]; CSE regulates fra-1 induction through JNK signaling in lung epithelial cells [32]; and mucin production in lung cells induced by tobacco smoke is also controlled through the AP-1 and JNK pathways [33]. There are, however, no reports describing the cytotoxic effects of CSE through the JNK pathway, and to the best of our knowledge, this report is the first to demonstrate that CSE injures vascular endothelial cells through JNK activation.

Oxidative stress generated by CSE is recognized as one of the mechanisms of CSE-induced damage to cells [13]. Also, the reactive oxygen species (ROS) activate MAP kinase pathways [15]. We, therefore, examined the involvement of ROS in the effects of CSE. Our results with antioxidants demonstrated that oxidative stress was involved, at least partially, in the up-regulation of the JNK pathway and the cytotoxic effect induced by CSE; also that JNK was activated not immediately but gradually after exposure to CSE, suggesting that oxidants generated intracellularly by

chemical components of CSE, rather than by oxidant components of CSE such as H_2O_2, are responsible for this oxidative stress. It is also likely that other ROS-independent signal cascades are involved in the mechanisms of JNK activation and its cytotoxicity. Future studies need to be directed to disclose the chemical components involved in the cytotoxic effects of CSE.

Atherosclerosis and COPD are critical human diseases associated with cigarette smoking, yet the precise mechanisms of their development are still unclear; nevertheless, vascular endothelial injury is now recognized as one of the most critical steps in their development [6,13]. In determining prophylactic and/or therapeutic strategies against these diseases, it is reasonable to regard the signal molecules that regulate their mechanisms as appropriate targets. The key finding of this study is that CSE induces vascular endothelial cell injury through the JNK pathway; consequently, blocking JNK activation would provide a new therapeutic approach. JNK inhibitors have already shown promise in the treatment of rheumatoid arthritis in animal models [34]. Further studies are needed to confirm the involvement of the JNK pathway in cigarette smoke-induced human diseases and to develop strategies for clinical application.

Acknowledgment

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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