MAPK pathway mediates EGR-1-HSP70-dependent cigarette smoke-induced chemokine production

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Li CJ, Ning W, Matthay MA, Feghali-Bostwick CA, Choi AM. MAPK pathway mediates EGR-1-HSP70-dependent cigarette smoke-induced chemokine production. Am J Physiol Lung Cell Mol Physiol 292: L1297–L1303, 2007; doi:10.1152/ajplung.00194.2006.—Cigarette smoking, a major risk factor for chronic obstructive pulmonary disease, can cause airway inflammation, airway narrowing, and loss of elasticity, leading to chronic airflow limitation. In this report, we sought to define the signaling pathways activated by smoke and to identify molecules responsible for cigarette smoke-induced inflammation. We applied cigarette smoke water extract (CSE) to primary human lung fibroblasts and found that CSE significantly increased CXC chemokine IL-8 production. Meanwhile, 70-kDa heat shock protein (HSP70) was also induced by CSE in a dose- and time-dependent manner. CSE treatment stimulated HSP70 secretion by primary fibroblasts, which augmented IL-8 production. This was further confirmed by exogenously added recombinant HSP70. Using HSP70 small interfering RNA, we confirmed that CSE-induced chemokine production was dependent on heat shock protein expression. Further investigation showed that CSE could also stimulate early growth response-1 (EGR-1) in an ERK-dependent manner and that the expression of HSP70 was EGR-1 dependent. In view of these findings, we hypothesize that the MAPK-EGR-1-HSP70 pathway regulates the cigarette smoke-induced inflammatory process.

chronic obstructive pulmonary disease; cigarette smoke extract; early growth response-1; interleukin-8; 70-kDa heat shock protein

IT HAS BEEN GENERALLY ACCEPTED that cigarette smoking is clearly associated with the development of chronic airway obstruction (5, 6, 20, 37) and is responsible for 80–90% of cases of chronic obstructive pulmonary disease (COPD) in the United States (34). However, only 15–20% of heavy smokers develop clinically significant airflow obstruction, so, even in many heavy smokers, pulmonary function remains within normal limits. Besides the risk factors that are involved in airway obstruction such as smoke components, smoking habits, and passive smoke exposure (31), genetic predisposition (19) is also considered a key factor that affects the lung’s response to cigarette smoke inhalation and the development of airway obstruction secondary to tobacco smoke. In addition to smoke-induced emphysema, genetic susceptibility leading to α1-antitrypsin deficiency is associated with the propensity for the development of early-onset, familial emphysema (12). Thus both environmental and genetic factors contribute to the pathogenesis of emphysema.

The molecular basis for tobacco smoke-induced emphysema is poorly understood. To better understand the cellular and molecular events or signaling pathways that may contribute to the pathogenesis of smoke-induced emphysema or COPD, we have used the two most accepted methods of gene expression profiling, namely, serial analysis of gene expression and microarray analysis, to construct the global gene expression profiles of lung tissues from control smokers (GOLD-0) and moderate (GOLD-2) COPD smokers (25). By analyzing the comprehensive data produced by serial analysis of gene expression and microarray, we have identified numerous classes of genes, the expression of which is altered in COPD patients. This broad classification includes genes encoding molecules for signal transduction, receptor function, growth factor, nuclear chromatin and DNA binding, adhesion and cytoskeleton, metabolism, matrix, cell cycle, and oxidative stress such as HSP70 protein 8, heme oxygenase (decycling) 1 (HO-1), and hypoxia inducible factor 3 α-subunit. Some of these genes have not been previously associated with COPD. Among the genes differentially expressed between GOLD-2 and GOLD-0 smokers, early growth response-1 (EGR-1), a transcription factor, was identified with more robust expression in the lung tissues of COPD patients than tissues of control smokers. Further functional studies suggested that EGR-1 might play a key role in the development of cigarette smoke-related COPD by regulating matrix metalloproteinase activity and modulating the turnover of extracellular matrix proteins during COPD development (25).

It has long been accepted that exposure to cigarette smoke can cause airway inflammation, which subsequently leads to a series of pulmonary structure changes (17). EGR-1 has been thought to regulate chemokine secretion in pulmonary artery endothelial cells and is considered to be a proinflammatory molecule (24). However, whether EGR-1 involves the inflammatory response in lung and the potential function of EGR-1 in smoke-related COPD pathogenesis are poorly understood.

In this paper, we attempt to delineate the signaling pathway(s) by which cigarette smoke regulates chemokine production such as IL-8. We present data that cigarette smoke extract (CSE) can activate EGR-1 in an ERK MAPK-dependent man-

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ner, with EGR-1 regulating 70-kDa heat shock protein (HSP70) activation, which in turn helps to stimulate the synthesis and secretion of CXC chemokine IL-8.

MATERIALS AND METHODS

Reagents. Polyclonal rabbit anti-EGR-1 and anti-HSP70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-p44/42 MAPK (Thr202/Tyr204), p44/42 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), and SAPK/JNK antibodies were purchased from Cell Signaling Technology, New England Biolabs (Beverly, MA). The specific inhibitor of p38 MAPK SB-203580, the MAPK/ERK (MEK) inhibitor U-0126, and SAPK/JNK MAPK inhibitor SP-600125 were from Calbiochem (San Diego, CA). ELISA kits for human IL-8 and human recombinant HSP70 and recombinant HSP70 were from R&D Systems (Minneapolis, MN).

Primary lung fibroblast isolation and culture. Human lung fibroblasts (NL9) were cultured as previously described (27) from the explanted lung tissue of donor lung. Primary mouse lung fibroblasts were cultured from lung tissues of EGR-1 null (−/−) and littermate EGR-1 control (+/+; mice (gift of D. J. Pinsky, College of Physicians and Surgeons of Columbia University, New York, NY). Fibroblasts were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C with 5% CO2.

Preparation and treatment of CSE. Nonfiltered research reference cigarettes IIR3F were purchased from the University of Kentucky (Lexington, KY). CSE was prepared at a concentration of 1 cigarette/5 ml in serum-free DMEM as previously described (35) with modifications. This medium was defined as 100% CSE and was used after adjusting the pH to 7.4 and filtering through a 0.22-μm filter. Cells were grown to 95% confluence in 60-mm cell culture plates and rendered quiescent in medium containing 0.5% FBS before CSE treatment. For experiments that used MAPK inhibitors, cells were preincubated for 2 h with inhibitors. The concentrations used for the kinase inhibitors fall within the optimal range for inhibiting respective MAPK without cytotoxicity. All inhibitors were dissolved in DMSO as recommended by the manufacturer. The vehicle contained the same maximal concentration of DMSO as the treatment group, which contained the maximal concentration of DMSO. This maximal DMSO content did not exceed 0.1%.

Extracellular IL-8 and HSP70 analysis. Cell culture media were collected at different time points after CSE treatment as indicated. The production of IL-8 or HSP70 was analyzed by ELISA in accordance with the manufacturer’s recommendations. For the neutralizing antibody experiment, primary lung fibroblasts (NL9) in passage 2 were plated in six-well tissue culture plates at 1.5 × 10^5 cells/well in DMEM supplemented with 10% FBS. When cells reached 90% confluence, the medium was replaced with DMEM-0.5% FBS and treated with 1 μg/ml control antibody or anti-HSP70 antibody (Santa Cruz) with or without 20% CSE for 24 h. Culture medium was collected, and IL-8 levels were analyzed by ELISA (R&D Systems).

Western blot analysis. Protein samples were collected at various time points with cell lysis buffer. The protein concentration was measured by the Bradford method. The same amount of protein was separated on a 4–12% gradient SDS-PAGE in Tris-glycine-SDS running buffer and then transferred to nitrocellulose membranes. The membranes were incubated with the appropriate primary antibody as indicated. The primary antibodies were rabbit anti-EGR-1 antibody (1:500), rabbit anti-HSP70 antibody (1:500), and phospho-specific and nonphospho-specific rabbit polyclonal antibodies to p38 MAPK, p44/42 MAPK, or SAPK/JNK (1:1,000). Signals were visualized after incubation with horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. All immunoblots were repeated in triplicate.

Synthesis of small interfering RNA and transient transfection of NL9 cells. Human EGR-1 small interfering RNA (siRNA), targeting the coding region 1237–1258 relative to the start codon of the EGR-1 gene, and a control scrambled duplex (scEGR-1 siRNA) were obtained as previously described (13). Human HSP70 siRNA targeting the coding region 245–265 (accession no. NM 021979; 5’T’-AAGAACCAGGTGGCCATGAA-3’T’) of the HSP70 sequence was designed with Dharmacon software, and a control nonspecific siRNA was purchased from Dharmacon (Lafayette, CO). The siRNA was transiently transfected into NL9 cells (24 h) with TransIT-TKO (Mirus, Madison, WI) following the manufacturer’s protocol. The medium was replaced, and the cells were exposed to CSE.

Statistical analysis. Data are expressed as means ± SE. Differences in measured variables between experimental and control groups were assessed by Student’s t-test. Results were considered statistically significant at P < 0.05.

RESULTS

CSE stimulates the production of IL-8 by human primary lung fibroblasts. Our group (25) has previously identified a group of transcripts related to inflammatory processes, such as growth factors, cytokines, and chemokines, which changed significantly in the lungs of patients with COPD compared with normal smokers. Because cigarette smoke is known to induce neutrophil recruitment in the airway lumen in smokers with mild to moderate chronic airway obstruction (11, 14), we examined the release of a CXC chemokine IL-8, a powerful neutrophil chemoattractant, by human primary lung fibroblasts exposed to CSE. CSE markedly stimulated IL-8 release in a dose- and time-dependent manner (Fig. 1). CSE treatment (20%) induced human lung fibroblasts to release sevenfold more IL-8 than untreated cells (Fig. 1A). We therefore chose 20% CSE to examine the time course of IL-8 production in response to CSE. The results showed that CSE (20%) treatment significantly induced IL-8 release from 16 to 24 h (Fig. 1B).

Fig. 1. Cigarette smoke water extract (CSE) can stimulate the production of IL-8 by NL9 cells in a dose- and time-dependent manner. NL9 cells were incubated in the absence (control; CTL) or presence of CSE for the indicated concentrations and times. IL-8 production was analyzed by ELISA. A: dose response of IL-8 production in NL9 culture medium after 24 h of CSE exposure. B: time course of IL-8 production in NL9 culture medium treated with 20% CSE. *P < 0.05 vs. CTL cells.
CSE induces stress response HSP70 in fibroblasts. A group of stress response genes, including HO-1, HSP70 protein 8, catalase, and thioltransferase, was differentially expressed between GOLD-2 and GOLD-0 smokers (25). It has been reported that tobacco smoke exposure could induce the expression of the inducible HSP70 and HO-1 in both monocytes and endothelial cells (3). Here we found that CSE can also induce stress response genes in primary lung fibroblasts in response to CSE. Both HSP70 (Fig. 2) and HO-1 (data not shown) were activated by CSE treatment in a dose- and time-dependent manner. CSE stimulated HSP70 expression in a dose-dependent manner (Fig. 2A). HSP70 was highly expressed from 4 to 16 h and peaked at 8 h. The expression level returned to almost baseline levels at 24 h (Fig. 2B).

HSP70 regulates the production of IL-8 of human primary lung fibroblasts in response to CSE. Because the heat shock response can regulate NF-κB activation and NF-κB-dependent proinflammatory gene expression such as IL-8 (21), we investigated the relationship between HSP70 and IL-8 production in human primary lung fibroblasts in response to CSE. siRNA duplexes (HSP70 siRNA) were used to block HSP70 protein production. Figure 3 shows that the production of IL-8 was decreased when fibroblasts were transiently transfected with HSP70 siRNA. These results suggest that CSE-induced HSP70 activation mediates IL-8 production.

CSE induces HSP70 secretion into the medium, which stimulates IL-8 production. Next we examined how HSP70 regulates the production of IL-8 in CSE-treated fibroblasts. It has been reported that, although heat shock proteins have typically been regarded as being exclusively intracellular proteins, they can be released into the extracellular environment by damaged tissue and provide the “danger” signal, which activates innate and proinflammatory immune responses (33). Secreted heat shock protein binds to Toll-like receptors (TLRs) similarly to LPS and activates the induction of the proinflammatory cytokines IL-1β, IL-6, and TNF-α (1, 2). Marked induction of HSP70 was observed in fibroblast culture medium in response to CSE (Fig. 4A). The concentration of HSP70 in the medium was as high as 200 ng/ml after 24-h treatment with CSE (Fig. 4B). Upon exogenous administration of recombinant HSP70 to CSE-treated fibroblasts, IL-8 production increased (Fig. 4C). These results suggest that HSP70 is secreted into the extracellular environment via an unidentified mechanism and stimulates the production of IL-8 in primary lung fibroblasts. To examine whether it is exclusively the extracellular HSP70 that leads to CSE-induced IL-8 production, we determined CSE-induced IL-8 production in the presence or absence of neutralizing antibodies against HSP70 in the medium. Figure 5 demonstrates that fibroblasts subjected to neutralizing antibody to HSP70 in the medium exhibited marked reduction of CSE-induced IL-8 production but did not completely abrogate the response. These data suggest that the extracellular HSP70 plays a critical role in mediating CSE-induced IL-8 production but also point to an HSP70-independent pathway of IL-8 production by CSE stimulus.

CSE-induced HSP70 production is dependent on EGR-1. Copland et al. (7) reported that, when rat lung was exposed to high tidal volume, EGR-1, c-Jun, IL-1β, and HSP70 were significantly upregulated after as short as 30-min ventilation.
and before any discernible lung injury appeared. Temporal studies demonstrate that EGR-1 and c-Jun were increased early and before the induction of HSP70 and IL-1β. The authors suggested that linkage of EGR-1 and c-Jun and production of IL-1β might be the potential mediators for stress responses. Thus we investigated the relationship between EGR-1 and HSP70 during the stress response induced by cigarette smoke.

Our group (25) previously demonstrated that EGR-1 is activated by CSE treatment. EGR-1 protein levels were quickly increased within 1 h. The EGR-1 mRNA levels and DNA binding activity were also activated by CSE as quickly as 30 min (25). Here, we exposed lung fibroblasts from EGR-1 null (−/−) and control (+/+ ) mice to CSE and examined the expression of HSP70. The results shown in Fig. 6 A demonstrate that HSP70 can be markedly induced in lung fibroblasts isolated from EGR-1(+/+ ) mice but not in EGR-1(−/−) fibroblasts. IL-8 production was also dependent on EGR-1. When EGR-1 expression was blocked by siRNA, IL-8 production was significantly inhibited (Fig. 6B).

**CSE-induced EGR-1 and IL-8 expression are dependent on ERK MAPK.** Recent studies have shown that CSE exerts its biological effects via the MAPK signaling pathway in several cell culture systems (22, 28). We also found that 20% CSE caused significant increases in the phosphorylation of all three MAPK members (ERK1/ERK2, p38, and JNK) within 15 min of exposure (Fig. 7A). Herein, we used MAPK inhibitors to determine the role of the MAPK pathway in mediating EGR-1 and IL-8 production. We pretreated fibroblasts with SB-203580, a specific inhibitor of p38, U-0126, a MEK inhibitor, and the JNK MAPK inhibitor SP-600125 before exposing cells to CSE. As shown in Fig. 7, CSE-induced EGR-1 expression (Fig. 7B) and IL-8 production (Fig. 7C) are significantly decreased by the MEK inhibitor U-0126. These results suggest that the ERK MAPK pathway may mediate the activation of...
the transcription factor EGR-1 in response to CSE and down-stream CSE-induced IL-8 production. In addition, fibroblasts subjected to MEK inhibitor also demonstrated reduced CSE-induced HSP70 production and secretion (Fig. 8).

DISCUSSION

It is accepted that genomic variations or genetic factors are important determinants of susceptibility to COPD because only ~20% of smokers develop this disease (5). Guerassimov et al.’s report (15) that the development of emphysema in cigarette smoke-exposed mice is strain dependent supports this perspective. Based on our previous (25) and present findings, we hypothesize that EGR-1 is possibly a critical upstream signaling transcription factor that may regulate the CSE-induced stress response and inflammatory process.

There are several hypotheses that attempt to explain the development of cigarette smoke-related COPD: the inflammatory response, protease-antiprotease imbalance, oxidant-antioxidant imbalance, and apoptosis hypothesis (30). Together, these closely related hypotheses may account for both the bronchial injury and emphysema seen in cigarette smokers with COPD. As a major immediate early-response transcription factor, EGR-1 is potentially activated by a variety of cellular stressors and alters the expression of its target genes, including transcription factors such as Id1 and Myc, repair enzyme systems such a thymidine kinase, angiogenic factors,
cigarettes (TNF-α), apoptotic factors (Fas), cell cycle regulators (p21, p53), metabolic factors, proteases (MT1-matrix metalloproteinase), and stress response genes (HO-1) (4, 16, 38). We find that these EGR-1-regulated genes might involve physiological processes that relate to smoke-associated COPD pathogenesis. However, whether EGR-1 is involved in COPD and the potential function of EGR-1 in smoke-related COPD pathogenesis are poorly understood.

EGR-1 has been thought to regulate chemokine secretion in pulmonary artery endothelial cells and is considered a proinflammatory molecule (24). Cigarette smoke-induced chronic inflammation has also long been viewed as central to the pathogenesis of COPD. Airway inflammation could result in the oxidant-antioxidant imbalance and protease-antiprotease imbalance. These lead to a series of pulmonary structure changes (airway remodeling, parenchymal destruction, and loss of alveolar attachments), which consequently cause airway narrowing and loss of elasticity and finally the development of chronic airflow limitation (5). This led us to investigate the association between EGR-1 and inflammation in smoke-related COPD pathogenesis. We found that CSE could induce lung fibroblasts to secrete the chemokine IL-8, a powerful chemoattractant for neutrophils, in an EGR-1-dependent manner. The synthesis of IL-8 in human lung fibroblasts was also blocked when EGR-1 was knocked down by specific siRNA. These results suggest that EGR-1 might be a key regulator of cigarette smoke-induced inflammation. A recent report demonstrated that cigarette smoke-induced EGR-1 upregulates proinflammatory cytokines in lung epithelial cells, corroborating our findings in fibroblasts in lung epithelial cells (29).

COPD is characterized by chronic inflammation in the airway or alveoli involving mononuclear cell infiltration, consisting predominantly of T lymphocytes and macrophages in patients with mild disease. On the other hand, neutrophils and macrophages are prevalent in patients with more severe stages of the disease (9). Migration and activation of inflammatory cells are regulated by cytokines and chemokines, which have chemotactic activity for macrophages and monocytes (monocyte chemoattractant protein-1), neutrophils (IL-8 and growth-related oncogene-α), and macrophages and neutrophils (TNF-α), the predominant inflammatory cells associated with COPD (8). Cytokines and chemokines can be secreted by a variety of structural cells, such as epithelial, endothelial, and smooth muscle cells and fibroblasts, as well as by inflammatory cells. Our findings demonstrate that fibroblasts secrete IL-8 when exposed to CSE. IL-8 is a powerful neutrophil attractor. IL-8 activates neutrophils by increasing degranulation and neutrophil elastase release, which result in the alteration of pulmonary structure. Recent reports have also demonstrated that cigarette smoke can be a potent inducer of various cytokines and chemokines in lung epithelial cells, smooth muscle cells, and macrophages (18, 23, 26, 36, 39). Furthermore, the NF-κB pathway has been shown to be important in regulating CSE-induced cytokines in lung epithelial cells (23, 39).

We have identified a novel early molecular pathway that mediates chemokine IL-8 release by human primary fibroblasts after cigarette smoke exposure. A key factor in cigarette smoke-related COPD, EGR-1, can mediate the synthesis of HSP70. HSP70 is then secreted into the extracellular environment and activates proinflammatory molecule (such as IL-8) production. Heat shock proteins are abundant, soluble intracellular proteins, which are thought to be molecular chaperones with a principal function in intracellular repair processes (32). In acute myocardial infarction patients, HSP70 was released into the circulation and correlated with the increase of inflammatory markers, including IL-6 and IL-8 and myocardial damage (10). The released HSP70 may activate cells through TLR-2, TLR-4, and CD14, thereby mediating proinflammatory cytokine production (1, 2).

In summary, the present study demonstrates that EGR-1 mediates cigarette smoke-induced chemokine release in primary human pulmonary fibroblasts in an ERK1/2 MAPK-dependent manner. This increase in chemokine synthesis is due, at least in part, to a transient activation of signaling, leading to activation of the transcription factor EGR-1. The activation of EGR-1 results in an intracellular increase of HSP70, which is secreted into the extracellular environment and acts as a proinflammatory molecule to stimulate cells to release IL-8. Proteases secreted by inflammatory cells as well as by structural cells such as epithelial cells and fibroblasts result in degradation of lung tissue and the development of COPD. Our findings indicate that EGR-1 might be one of the key genetic factors that control the development of cigarette smoke-related COPD.

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