Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E$_2$ synthase in human lung fibroblasts: implications for lung inflammation and cancer


A new concept indicates that chronic inflammation plays a crucial role in conditioning the regional environment and promoting development of certain cancers. This concept is best supported to date in colon cancer (39). Therapeutic approaches focusing on preventing the development of colon cancer include the administration of nonsteroidal anti-inflammatory drugs. Several studies have shown that there is benefit from administering anti-inflammatory agents to patients with colon cancer. This highlights the importance of inflammation in oncology (43). Importantly, these studies led to investigation of the role of cyclooxygenase (COX)-2 in cancer etiology. In colon cancer, the fibroblasts underlying the epithelial cells express COX-2 first (51, 55). The resulting eicosanoid mediators released by fibroblasts lead to damage of the epithelial cells, resulting in a predisposition to transformation. Similar to colon cancer, there are reports of upregulation of both COX-2 and microsomal prostaglandin E synthase (mPGES) in lung cancers (56, 65).

Little is known of the mechanisms by which cigarette smoke activates human lung fibroblasts, despite the fact fibroblasts are highly numerous in the lung. Fibroblasts were once considered to be relatively inert, producing only collagen and other extracellular matrix proteins that serve as scaffolding (27). However, the current paradigm is that they are tissue “sentinel” cells that play a key role in producing proinflammatory mediators associated inflammation induced by production of these mediators is central to the pathogenesis of chronic obstructive pulmonary disease. Little is known of the responses of normal lung fibroblasts to cigarette smoke, despite their abundance. We report herein that normal human lung fibroblasts, when exposed to cigarette smoke extract, induce COX-2 with concurrent synthesis of prostaglandin E$_2$ (PGE$_2$). The mechanisms by which cigarette-derived toxicants lead to increased COX-2 levels and PGE$_2$ synthesis include increases in steady-state COX-2 mRNA levels (approximately four- to fivefold), phosphorylation of ERK1/2, and nuclear translocation of the p50 and p65 subunits of the transcription factor NF-kB, which are important elements in COX-2 expression. Furthermore, there was a dramatic 25-fold increase in microsomal prostaglandin E synthase, the key enzyme involved in the production of PGE$_2$. We propose that normal human lung fibroblasts, when exposed to cigarette smoke constituents, elicit COX-2 expression with consequent prostaglandin synthesis, thus creating a proinflammatory environment. This chronic inflammatory state may act as one of the first steps towards epithelial transformation.

Lung disease; mitogen-activated protein kinases; eicosanoids

Address for reprint requests and other correspondence: P. J. Sime, Div. of Pulmonary and Critical Care Medicine, Univ. of Rochester, 601 Elmwood Ave., Box 692, Rochester, NY 14642 (E-mail: Patricia_Sime@urmc.Rochester.edu).

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Adrians (53) such as PGE2, which initiates inflammation (29); IL-8, a neutrophil chemoattractant (52); and IL-6 (50). Fibroblasts also produce other cytokines and chemokines that influence immunity and inflammation, such as macrophage chemoattractant protein (MCP)-1 (30). These mediators can stimulate epithelial and endothelial cells within the lung parenchyma, leading to prolonged inflammation and tissue derangement (15). Furthermore, proinflammatory mediators produced by cigarette smoke-exposed lung fibroblasts may predispose a smoker to lung cancer by inducing chronic synthesis of eicosanoids.

The purpose of this study was to determine the direct effects of cigarette smoke on primary strains of human lung fibroblasts. Information on the responses of normal human lung fibroblasts to smoke is virtually absent, as most studies use mouse cells or transformed epithelial cells. The key concept we investigated was whether cigarette smoke extract provoked an “inflammatory” phenotype in human lung fibroblasts. There are two isoforms of the COX enzymes. COX-1 is generally constitutively expressed and is typically considered a “housekeeping” gene, although it can be upregulated (28). COX-2 on the other hand is usually only expressed under inflammatory conditions such as occur in arthritis (32) or tumorgenic conditions such as colon cancer (57) and nonsmall cell lung cancer (56). We assayed for cigarette smoke-induced changes in key proteins involved in biosynthesis of eicosanoids, namely COX-2 and mPGES. Because chronic inflammation and COX-2 are now closely associated with the development of cancers of the colon, oral cavity, and other tissues, a new pathway is identified highlighting the mechanism by which cigarette smoke exposure may initiate chronic inflammation and predispose to the development of lung cancer.

Materials and Methods

Cell cultures. Primary human lung fibroblast strains were established as previously described (13). Cells were cultured in MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 50 U/ml gentamicin (Life Technologies) and incubated in humidified 5% CO2/95% air at 37°C as previously described (52). Fibroblasts were used at early passage (P6–12).

Preparation of aqueous cigarette smoke extract. Research-grade cigarettes (1R3F) with a filter from the Kentucky Tobacco Research and Development Center at the University of Kentucky were smoked in 10-cm plates (9/11011/H9260) in 400 μg/ml aprotinin, 10 μM pepstatin, and PMSF; Sigma). Lysates were centrifuged (14,000 g, 4°C, 10 min) to remove debris, and protein quantitation was done by bicinchoninic acid method (Pierce, Rockford, IL). Typically, 20 μg of total cellular protein were fractionated on 10% or 12% SDS-PAGE gels, electrophoresed onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA), and blocked with 10% nonfat dry milk in Tris-buffered saline with TWEEN overnight at 4°C. Analysis for COX-1, COX-2, mPGES, cystolic prostaglandin E synthase (cPGES) (Cayman Chemical), and phosphorylated ERK1/2 (Cell Signaling Technologies, Beverly, MA) was accomplished as previously described (29). Proteins were visualized by enhanced chemiluminescence (NEN Life Science Products, Boston, MA).

Immunocytocchemistry. Fibroblasts in eight-well chamber slides (~5 × 10^5 cells/well) were cultured in serum-free MEM for 48 h before treatment with either IL-1β (1 ng/ml) or 1% CSE for 8 h. Cells maintained in serum-free MEM were used as controls. For NF-κB inhibition studies, cells were treated with 10 μM KPbPV before CSE treatment. Previously published methods for COX and NF-κB staining were used (52). Briefly, after treatments, cells were washed 1× with PBS and fixed with 3% H2O2/methanol for 15 min before being blocked with 5% normal horse serum. COX antibodies described in the Western blotting protocol were diluted in PBS/BSA buffer and incubated overnight at 4°C. Biotinylated anti-mouse IgG antibody was used for secondary binding and incubated for 2 h at 4°C before incubation with streptavidin-horseradish peroxidase for 1 h at room temperature. Antibody binding was visualized with the horseradish peroxidase substrate aminoethyl-carbachol (Zymed, South San Francisco, CA), which stains red.

EMS. Lung fibroblasts strains were grown to confluent monolayers in 10-cm plates (~6 × 10^5 cells/plate) and then serum starved for 48 h before stimulation with 1% CSE for times as shown in Figs. 1–9. Control treatments were with serum-free MEM only. Nuclear extracts were prepared as previously described (50). Briefly, cells were washed two times with ice-cold PBS, scraped from the culture dishes, and centrifuged (1,000 g, 10 min). The cell pellets were resuspended in 400 μl of hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 0.5 mM phenylmethyl sulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM DTT, and 10 mM sodium molybdate) and allowed to swell on ice for 15 min. NP-40 was then added to a final concentration of 0.5%. After rapid mixing for 15 s, the nuclei were centrifuged (4,000 g, 1 min), and the supernatants containing cytosolic proteins were collected. The pellet was resuspended in 50 μl of nuclear extract buffer B (hypotonic buffer A supplemented with 20% glycerol and 0.4 M KCl) and shaken vigorously at 4°C for 30
a 3-fold increase in PGE$_2$ production was observed in 1 h in a reaction mix containing 10 $\mu$g of nuclear extract were incubated with 1 ng (~50,000 counts/\mu l) of $^{32}$P-labeled NF-\kappa B probe (5'-AGTTGAGGGGACTTTCCCA-GGC-3'; Promega, Madison, WI) at room temperature for 15 min in a reaction mix containing 10 $\mu$g of BSA and 1 $\mu$g of poly(dI/dC) in binding buffer [7.5% glycerol, 1 mM MgCl$_2$, 0.05 mM EDTA, 0.5 mM DTT, 35 mM NaCl, and 7.5 mM HEPES (pH 8)] in a total volume of 25 $\mu$l. The DNA/protein complex was separated on 4% native polyacrylamide gels. Binding specificity was determined by competition using excess (100X) unlabeled oligonucleotide. Radioactive bands were detected by autoradiography.

**Statistical analysis.** Statistical significance was determined by using Student’s paired two-tailed test where $P < 0.05$ indicates statistical significance between tested samples.

**RESULTS**

**CSE stimulates PGE$_2$ synthesis in human lung fibroblasts.** To determine whether or not CSE stimulated production of PGE$_2$ in normal human lung fibroblast strains, three fibroblast strains derived from three different human subjects were incubated with 1% CSE. Supernatants were harvested and analyzed for PGE$_2$ content by enzyme immunoassay. A CSE dose-dependent increase in PGE$_2$ production was observed in strain 1 fibroblasts treated for 24 h (Fig. 1A). Relative to the control media-treated cells, fibroblasts exposed to 1% CSE produced significant PGE$_2$ in a time-dependent manner. A threefold induction over basal levels of PGE$_2$ was observed after 8 h of incubation with 1% CSE, and a sevenfold increase in PGE$_2$ levels was noted after 48 h (Fig. 1B). IL-1$\beta$, a known potent inducer of COX-2 protein and PGE$_2$ synthesis, was used as a positive control for comparison (Fig. 1B). CSE had no toxic effects at the concentrations used, as determined by trypan blue exclusion and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyloxazolium bromide assays (data not shown). Differences in PGE$_2$ production are therefore not related to changes in fibroblast cell number after exposure to CSE.

To ascertain whether or not the increased PGE$_2$ synthesis observed in CSE-treated human lung fibroblasts was unique to a single strain of fibroblasts, other human lung fibroblast strains were analyzed for increased PGE$_2$ synthesis after CSE treatment. Two other normal human lung fibroblast strains also exhibited increased PGE$_2$ synthesis in response to CSE treatment (Fig. 1B). Differences in PGE$_2$ levels in the fibroblasts are a reflection of fibroblast strain differences. These data indicate that normal human lung fibroblasts respond to components found in CSE by producing PGE$_2$, and this inflammatory phenotype is not strain specific and occurs in fibroblasts from different human beings.

**NS-398, a selective COX-2 inhibitor, blocks CSE-induced PGE$_2$ synthesis.** Induction of COX-2 is associated with increased PGE$_2$ production in inflammation (54). A COX-2-selective inhibitor, NS-398 (10 $\mu$M), was used to determine whether COX-2 was responsible for the observed increases in PGE$_2$ synthesis by CSE-treated fibroblasts (62). Two different strains of human lung fibroblasts were pretreated with NS-398 for 1 h before treatment with 1% CSE for 24 h, and supernatants were analyzed for PGE$_2$. As shown in Fig. 2, the fibroblast strains incubated with 1% CSE had a three- to fourfold induction in PGE$_2$ synthesis over basal levels after 24 h, depending on the strain. However, cells treated with both CSE

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**Fig. 1.** Cigarette smoke extract (CSE) induces PGE$_2$ production in normal human lung fibroblasts. PGE$_2$ production was assayed by enzyme immunoassay. A: CSE induces PGE$_2$ synthesis in human lung fibroblasts (strain 1) in a dose-dependent manner. Cells were treated with increasing concentrations of CSE (0.001–1%) for 24 h, and supernatants were harvested and analyzed for PGE$_2$ levels. Results are presented as PGE$_2$ pg/ml ± SD. Experiments were done in triplicate and repeated at least 3 times. *$P < 0.05$ indicates statistical significance between cells treated with CSE relative to cells treated with media alone. B: fibroblast strains were serum-starved for 48 h before treatment with 1% CSE (prepared as described in MATERIALS AND METHODS) for the indicated times, and the supernatants were analyzed for PGE$_2$. Values for PGE$_2$ thus represent total PGE$_2$ accumulating over the time periods indicated. Cells treated with IL-1$\beta$, a potent inducer of PGE$_2$ synthesis, were used as a positive control. *$P < 0.05$ indicates statistical significance between cells treated with CSE or IL-1$\beta$ relative to cells treated with media alone.
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and NS-398 had significantly decreased PGE\(_2\) levels relative to fibroblasts treated with CSE alone, supporting the idea that the elevated PGE\(_2\) levels are a result of COX-2 activity. In contrast, treatment of fibroblasts with 10 \(\mu\)M NS-398 did not affect their basal production of PGE\(_2\). This fact supports the concept that basal production of PGE\(_2\) from the fibroblasts is derived from activity of the COX-1 enzyme. Together, these data indicate that COX-2 is crucial for the increased PGE\(_2\) synthesis resulting from CSE exposure.

**CSE induces COX-2 protein in normal human lung fibroblasts.** The fact that the COX-2-selective inhibitor NS-398 blocked the CSE-induced increase in PGE\(_2\) accumulation suggests that COX-2 protein levels were upregulated. To test this hypothesis, we analyzed COX-2 protein levels by Western blot analysis with extracts obtained from normal human lung fibroblasts (*strain 1*) treated with 1% CSE. Kinetics studies indicate that COX-2 protein levels were increased by 4 h and peaked by 8 h of CSE treatment (Fig. 3A), after which the levels decreased. Fibroblasts exposed to media alone for 24 h had nominal COX-2 expression; however, cells exposed to IL-1\(\beta\) for 24 h showed elevated COX-2 expression (Fig. 3A), indicating the ability of these cells to express COX-2. The appearance of an additional band \(\sim 74\) kDa in the COX-2 Western blot (Fig. 3A) likely reflects variable phosphorylation or glycosylation of the protein product. COX-1 levels remained unchanged over the course of treatment (Fig. 3A). Lung fibroblast production of COX-2 protein was dose dependent following CSE treatment (0.1, 0.5, and 1%) for 8 h (Fig. 3B). Elevated COX-2 protein levels were also observed in other human fibroblasts strains that were treated with CSE for 8 h (Fig. 3C). In agreement with the data from *strain 1*, COX-1 levels remain unaffected. We next analyzed fibroblasts for elevated COX-2 expression and localization after CSE exposure by immunocytochemistry (Fig. 4, A and B). IL-1\(\beta\)-treated cells were used as a positive control for COX-2 induction in the fibroblasts (Fig. 4, A and B). There was increased COX-2 staining in 1% CSE-treated cells relative to the cells treated with media alone for both fibroblast strains (Fig. 4). The majority of the COX-2 staining was observed in the cytosol with some nuclear envelope and nuclear staining as well. COX-1 staining in fibroblasts was the same whether or not the cells were exposed to media, IL-1\(\beta\), or CSE (Fig. 4, A and B). These findings are consistent with the Western blot data described above. Isotype controls showed only weak background staining.

**CSE increases COX-2 steady-state mRNA levels in human lung fibroblasts.** Increased COX-2 protein induced by CSE suggests that there was a coordinate increase in steady-state COX-2 mRNA levels. Therefore, total cellular RNA was isolated and reverse transcribed to convert mRNA to cDNA, which was then amplified by both semiquantitative and real-time PCR to determine any relative increases in COX-1 and COX-2 mRNA. Interestingly, there was a dramatic, dose-dependent increase in COX-2 steady-state mRNA levels in CSE (0.5 and 1.0%)-treated cells after only 4 h (Fig. 5A). We next verified semiquantitative results by performing real-time
PCR analysis (Fig 5B). These data revealed that following fibroblast exposure to 1% CSE there was approximately a four- to fivefold increase in steady-state COX-2 mRNA levels. There was a small, but not significant, increase in COX-1 mRNA. These observations support the Western blot (Fig. 3) and immunohistochemistry (Fig. 4) showing increases in COX-2, but not COX-1, protein after CSE exposure. These data support that COX-2 is the key enzyme responsible for the increase in PGE2 after CSE exposure.

CSE induces mPGES in human lung fibroblasts. There is no published information as to whether or not cigarette smoke induces mPGES protein in human lung fibroblasts. mPGES is the inducible form of prostaglandin E synthase, whereas cPGES is the constitutively expressed isoform (58). Kinetics studies for mPGES synthesis were completed on human lung fibroblasts treated with 1% CSE for the indicated times (Fig. 6A). Five- to sixfold increases in mPGES protein levels were observed after 16–24 h, which correlates with an approximately fivefold increase in PGE2 at the 24-h time point (see Fig 1B). Greater than 25-fold increases of mPGES were noted after 48 h of treatment (Fig. 6A). cPGES levels remained unchanged throughout the course of treatment (Fig. 6A). COX-2 protein levels peaked at 8 h, but the key to a significant increase in PGE2 is the increase in mPGES, which is downstream of
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We next treated CSE-exposed fibroblasts with PD-98059, a MAP kinase inhibitor, to determine whether MAP kinases played any role in CSE-induced fibroblast activation. We noted a 50% inhibition in mPGES protein induction in the presence of PD-98059 in strain 1 fibroblasts treated with 1% CSE for 48 h (Fig. 6B). A densitometric representation of three separate experiments (see Fig. 6C) using the PD-98055 inhibitor supports the Western blot data.

**CSE induces ERK1/2 phosphorylation.** ERK1/2 are members of the MAP kinase family and are important upstream signals for COX-2 gene expression (11). Therefore, the effect of CSE on ERK1/2 was analyzed. Kinetics studies using 1% CSE treatments on normal human lung fibroblasts showed a dramatic increase in both ERK1/2 phosphorylation at 5 min after incubation, with optimal phosphorylation observed at 30 min (Fig. 7A). Phosphorylation levels were decreased by 1 h.

To determine ERK 1/2-specific activation, we used PD-98059 (10 µM), an inhibitor of tyrosine kinase phosphorylation. No phosphorylation was observed in lung fibroblasts treated with media, DMSO, or PD-98059, (Fig 7B). However, fibroblasts treated with 1% CSE (30 min) exhibited substantially increased phosphorylation (Fig. 7B). Increased ERK1/2 phosphorylation was observed in cells that were first pretreated with PD-98059 (10 µM, 1 h) before cotreatment with 1% CSE for 30 min.

To further show the importance of ERK1/2 phosphorylation in CSE-induced PGE$_2$ synthesis, we treated fibroblasts with CSE in the presence or absence of PD-98059 to determine the effect on COX-2 protein expression. Cells were first preincubated with PD-98059 (10 µM, 1 h) and then cotreated with 1% CSE for either 8 h for COX-2 protein analysis (Fig. 7C) or 24 h for PGE$_2$ synthesis (Fig. 7D). Fibroblasts that were first pre-treated with PD-98059 did not produce either COX-2 or PGE$_2$ to the same levels as those fibroblasts treated with 1% CSE.

**COX-2.** The medium controls were from the last time point (48 h).

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alone (Fig. 7, C and D). These findings support the critical role of ERK1/2 phosphorylation in CSE-induced COX-2 expression and PGE2 production.

**CSE induces NF-kB activation in human lung fibroblasts.** To examine additional mechanism by which smoke induces COX-2, we tested whether or not NF-kB was activated by smoke. Nuclear extracts isolated from normal human lung fibroblasts treated with 1% CSE for varying times (15 min–4 h) were analyzed for NF-kB activation by EMSA analysis (Fig. 8A). TNF-\(\alpha\) (5 ng/ml, 30 min) treatment was used as a positive control. A time-dependent increase in binding with a \(^{32}\)P-NF-kB probe was observed, with optimal binding seen after 30 min. Binding started to decrease after 60 min, with basal levels reached by 120 min. Specific NF-kB binding was competed out with excess unlabeled probe (Fig. 8B). Specific localization of the p50 and p65 subunits of the NF-kB complex involved in promoter binding was analyzed by immunocytochemistry. Fibroblasts in eight-well chamber slides were treated with 1% CSE for 1 h and then stained for p50 and p65 (Fig. 8C). Fibroblasts treated with medium alone had a diffuse p50 and p65 staining in the cytoplasm, and only weak staining was observed in the nucleus, explaining NF-kB basal binding observed during EMSA analysis. CSE treatment led to translocation of both subunits observed as increased staining in the nucleus (shown by arrows) and decreased cytoplasmic staining (Fig. 8C). These data support a role for NF-kB in CSE-induced COX-2 production.

**Blockade of NF-kB translocation inhibits CSE-induced COX-2 protein and PGE2 synthesis.** K\(_D\)PV is a tripeptide found in the NH\(_2\) terminus of the \(\alpha\)-melanocyte stimulating hormone that has been shown to block the nuclear localization signal on the p50 subunit of NF-kB (20). We utilized this tripeptide to further probe the link between NF-kB activation and PGE2 synthesis in human lung fibroblasts. Cells treated with K\(_D\)PV (10 ng/ml, 30 min) before 1% CSE treatment were analyzed for COX-2 upregulation (Fig. 9A) and PGE2 synthesis (Fig. 9B). K\(_D\)PV inhibited CSE-induced COX-2 protein in a dose-dependent manner (Fig. 9A). A decrease in IL-1\(\beta\)-induced COX-2 expression was also noted in fibroblasts pretreated with K\(_D\)PV. Furthermore, PGE2 synthesis decreased to background levels in cells pretreated with K\(_D\)PV (10 ng/ml) before 1% CSE treatment (Fig. 9B). IL-1\(\beta\) treatment was used as a positive control, and there was greater than a 60% decrease in PGE2 production in fibroblasts pretreated with K\(_D\)PV before IL-1\(\beta\) (Fig. 9B). These data support the requirement for NF-kB...
activation for optimal CSE-induced COX-2 and PGE2 synthesis.

DISCUSSION

Cigarette smoking is linked to the development of inflammatory diseases including COPD, asthma, and sarcoidosis. Furthermore, epidemiological studies implicate cigarette smoking as crucial to the development of lung cancer (45). Overexpression of COX-2 is a cardinal feature of lung inflammatory diseases characterized by tissue destruction (33), altered vasculature (48), impaired wound healing (49), and even changes in airway remodeling (34), all of which are also associated with elevated PGE2 levels. PGE2 also plays an important role in the inhibition of cellular immune responses, natural killer function, and type-1 immune responses (14, 17).

Our findings reported herein demonstrate a role for cigarette smoke in inducing COX-2 and mPGES synthesis and in increasing PGE2 production in normal human lung fibroblasts. Our data clearly show that CSE induces proinflammatory COX-2 protein in human lung fibroblasts. This has important implications for understanding how cigarette smoking may incite and exacerbate inflammatory diseases such as COPD and asthma and promote tumorigenesis. We speculate that the peroxidase function of COX-2 may play a significant role in metabolizing various constituents (~6,000) found in cigarettes (21), of which carcinogens such as benzanthracenes, benzopyrenes, and other polycyclic hydrocarbons comprise 25% (21). Carcinogens such as benzo[a]pyrene (B[a]P) have been shown to increase COX-2 protein (25) and PGE2 synthesis in epithelial cell lines and vascular smooth muscle cells (63). There is evidence of COX-2-mediated activation of B[a]P to the more genotoxic B[a]P-7,8-dihydrodiol, a metabolite that has been shown to bind DNA (9, 60). Byproducts of arachidonic acid metabolism such as malondialdehyde can also alkylate DNA, therefore leading to mutations (7). Toxic volatile smoke components such as acrolein and acetaldehyde also lead to increased PGE2 and COX-2 production (24). Increased PGE2 levels abrogate activation of tumor suppressors such as p53 and Rb. PGE2 also leads to increased activity of oncogenic proteins such as K-ras, thus increasing the probability of tumorigenesis (45). In the study reported herein, we did not analyze which components of CSE lead to the observed effects on PGE2 and COX-2 production. However, our preliminary data suggest that acrolein increases PGE2 synthesis in normal human lung fibroblasts (data not shown).

Wide use of selective COX-2 inhibitors has provided convincing data linking this enzyme to inflammation and tumorigenesis (reviewed in Ref. 54). In our study, we demonstrated that the COX-2-selective inhibitor NS-398 blocked CSE-induced PGE2 synthesis in normal human lung fibroblasts (Fig. 2). This is an important observation because of the link between PGE2 overproduction and bronchogenic carcinoma (31, 35). Interestingly, the COX-2 inhibitor NS-398 has been shown to restore tumor cell apoptosis and to reduce microvascular

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**Fig. 8.** The transcription factor NF-κB is induced by CSE stimulation of normal human lung fibroblasts. A: human lung fibroblasts (strain 1) in 10-cm plates were treated with TNF-α (5 ng/ml, 2 h), a potent inducer of NF-κB activation, and 1% CSE for times as shown. Nuclear extracts (5 μg) prepared as described in MATERIALS AND METHODS were incubated with 32P-labeled NF-κB oligonucleotides, and the complex was resolved in a 4% native PAGE gel. B: excess unlabeled probe was incubated with sample (1% CSE, 30 min treatment) to determine specific binding. These data are representative of 4 separate experiments. C: NF-κB localization of the specific subunits was determined by immunocytochemistry using specific monoclonal antibodies to p50 and p65. TNF-α (5 ng/ml) was used as a positive control. Fibroblasts show nuclear translocation of NF-κB p50 and p65 subunits after both TNF-α and 1% CSE treatment (arrows). Isotype controls included using mouse IgG1 antibody for background staining. Original magnification ×400. Data are representative of 4 experiments.
density and tumor growth of PC-3 prostate carcinoma cells xenografted into nude mice (reviewed in Ref. 12). Furthermore, NS-398 upregulates p27KIP1, a cyclin-dependent kinase inhibitor, leading to G1 growth arrest in cancer cells (22). Studies have shown that 70% of nonsmall cell lung cancer tumors express lower p27KIP1 levels (4, 10) due to enhanced degradation of this cyclin-dependent kinase inhibitor. We were thus excited by our findings that NS-398 abrogated COX-2 and PGE2 synthesis in fibroblasts, demonstrating the possible utility of COX-2-specific inhibitors to ameliorate cigarette smoke-induced inflammation and/or tumorigenesis.

The striking effect that CSE had on mPGES, the enzyme responsible for the conversion of PGH2 to PGE2, was very interesting. We observed a significant increase in mPGES levels (Fig. 6) in CSE-treated fibroblasts after 24 h, making this the first study to show that CSE affects mPGES levels in human lung fibroblasts. mPGES is an inducible enzyme whose expression can be markedly increased in the lung following proinflammatory stimuli such as LPS (38). Overexpression of mPGES has been noted in nonsmall cell lung cancers (65) with elevated levels of PGE2 in bronchoalveolar lavage and lung tissue from patients with bronchogenic carcinoma (31, 37). mPGES acts in concert with COX-2, under proinflammatory conditions, whereas cPGES and COX-1, the constitutive isoforms of these enzymes, are important in homeostasis (65). The increase in mPGES protein expression in normal human lung fibroblasts after CSE treatment (Fig. 6) supports a role for fibroblasts in mediating lung inflammation. Cigarette smoke components increase not only COX-2 expression, but also mPGES, thus enhancing PGE2 synthesis, which we propose would lead to an inflammatory phenotype in the lung. Furthermore, the E-prostanoid (EP) 2 receptor, which mediates some PGE2 effects (5), is important in tumor progression, as noted by abrogated tumor growth in EP2-/- mice compared with their wild-type littermates (64).

The role of signal transducers such MAP kinases and NF-κB reinforces the importance of the COX-2 pathway in this CSE-induced inflammation model. Our data suggest that CSE induces the MAP kinases ERK1/2, followed by NF-κB mobilizer and COX-2 mRNA and protein production. Increased PGE2 production may then enhance COX-2 mRNA stability and translation by activating p38 MAP kinase (11). Our unpublished preliminary data show that CSE induces p38 MAPK activation in CSE-exposed human lung fibroblast (data not shown). One may speculate that p38 MAPK is perpetually being turned on in smokers’ lungs, thus providing a scenario in which chronic inflammation feeds itself by a positive feedback loop via PGE2 and COX-2 induction. PGE2 has also been shown to activate ERK2 in a human colon carcinoma cell line (12), whereby PGE2 can induce epithelial cell proliferation by

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activating the epidermal growth factor receptor (EGFR) via the ERK2 pathway. These findings suggest that fibroblasts expressing high levels of PGE2 may also be able to activate the EGFR on the adjacent epithelial cells leading to uncontrolled cell proliferation and consequent tumorigenesis in the presence of DNA damage.

Cigarette smoke induces NF-κB mobilization to the nucleus in human lung fibroblasts. Significant nuclear activation of NF-κB was observed by EMSA analysis after a 30-min incubation with 1% CSE (Fig. 8A). A dramatic influx of both p50 and p65 subunits into the nucleus was observed by immunocytochemistry (Fig. 8C). Our data show basal levels of NF-κB in the nucleus, which we conclude to be important for maintaining homeostasis in the lung fibroblasts. The ability to block COX-2 upregulation and PGE2 synthesis with K2PV (Fig. 9), an inhibitor of NF-κB nuclear translocation, further delineates the importance of the NF-κB pathway in CSE-mediated PGE2 induction in normal human lung fibroblasts. Our studies corroborate findings on the effect of cigarette smoke components on COX-2 and PGE2 induction in lung cells. The tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces COX-2 expression in rat lung cells (8). Studies on type II alveolar lung cells obtained from A/J mouse lung exposed to NNK had elevated COX-2 expression. However, when mice were exposed to NNK had elevated COX-2 expression. However, on type II alveolar lung cells obtained from A/J mouse lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces COX-2 expression in rat lung cells (8). Studies on type II alveolar lung cells obtained from A/J mouse lung exposed to NNK had elevated COX-2 expression. However, when mice were treated with pyridoline diethiocarbamate (PDTC), a nonspecific NF-κB inhibitor shown to inhibit COX-2 transcription, decreased COX-2 mRNA synthesis and protein induction were noted in the cells (59). There was also a significant decrease in tumor formation in the lungs of mice treated with PDTC before NNK challenge compared with the sham-treated groups (59).

In conclusion, our data support the hypothesis that CSE leads to increased PGE2 production via activation of the COX-2 pathway. Importantly, we have demonstrated that fibroblasts from different human beings respond to components of cigarette smoke by increasing COX-2, mPGES, and PGE2. This smoke-induced fibroblast activation allows these lung structural cells to initiate and promote inflammation. Chronic smoking, possibly coupled to exposure to other respiratory toxicants, likely initiates chronically high COX-2/mPGES/ PGE2 levels in the lung. This environment may lead to changes in the overlying epithelial cells, which may eventually become malignant (65). Moreover, the observed overexpression herein of COX-2, mPGES, and PGE2 in normal human lung fibroblasts is likely important in driving inflammatory and possibly fibrotic conditions of the lung (47).

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REFERENCES


