Cigarette smoke extract induces COX-2 expression via a PKCα/c-Src/EGFR, PDGFR/P13K/Akt/NF-κB pathway and p300 in tracheal smooth muscle cells

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Yang C-M, Lee I-T, Lin C-C, Yang Y-L, Luo S-F, Kou YR, Hsiao L-D. Cigarette smoke extract induces COX-2 expression via a PKCα/c-Src/EGFR, PDGFR/P13K/Akt/NF-κB signaling in HTSMCs. CSE stimulated the phosphorylation of c-Src, EGFR, PDGFR, and Akt, which were inhibited by pretreatment with the inhibitor of PKCα (Gö6976 or Gö6983), c-Src (PP1), EGFR (AG1478), PDGFR (AG1296), or P13K (LY294002). Moreover, CSE induced a significant increase in COX-2 expression, which was reduced by pretreatment with these inhibitors or transfection with siRNA of PKCα, Src, or Akt. Furthermore, CSE-stimulated NF-κB p65 phosphorylation and translocation were also attenuated by pretreatment with Gö6976, PP1, AG1478, AG1296, or LY294002. CSE-induced COX-2 expression was also mediated through the recruitment of p300 associated with NF-κB in HTSMCs, revealed by coimmunoprecipitation and Western blot analysis. In addition, pretreatment with the inhibitors of NF-κB (helenalin) and p300 (garcinol) or transfection with p65 siRNA and p300 siRNA markedly inhibited CSE-regulated COX-2 expression. However, CSE-induced PGE2 generation was reduced by pretreatment with the inhibitor of COX-2 (NS-398). These results demonstrated that in HTSMCs, CSE-induced COX-2-dependent PGE2 generation was mediated through PKCα/c-Src/EGFR, PDGFR/P13K/Akt leading to the recruitment of p300 with NF-κB complex.

Cigarette smoking is a risk factor in the pathogenesis of chronic obstructive pulmonary disease (COPD), which is characterized by abnormal inflammatory responses in the lungs (34). Moreover, the detrimental effects of cigarette smoke (CS) in lung inflammation may result from enhancing cyclooxygenase (COX)-2 expression and prostaglandin (PG) generation in the lungs (33). Airway smooth muscle is considered as an end-response effector mediating regional differences in ventilation by contraction in response to various proinflammatory mediators and exogenous substances under homeostatic or pathological conditions (20). Although COX-2 has been shown to regulate airway inflammatory responses (21), the mechanisms of intracellular signaling pathways involved in CSE-induced COX-2 expression in human tracheal smooth muscle cells (HTSMCs) are not completely defined.

COX is the enzyme that converts arachidonic acid to PGH2, which can be further metabolized to prostanooids, including PGE2, prostacyclin (PGI2), and thromboxane A2 (TXA2). It has been shown that COX exists in at least two distinct isoforms. COX-2 has been shown to be induced by many proinflammatory stimuli, including CS (54). Furthermore, COX-2 is thought to be the main isoform responsible for the production of proinflammatory prostanooids in various inflammatory diseases (6). PKCs are important signaling intermediates that are implicated in airway inflammation, bronchospasm, and mucous production in chronic airways, such as asthma and COPD (11). Src protein tyrosine kinases (PTKs) are also one of the most important families for intracellular signal transductions related to acute inflammatory responses (41). Recent studies have suggested that numerous components of the P13K pathway play a crucial role in the expression and activation of inflammatory mediators, inflammatory cell recruitment, immune cell function, airway remodeling, and corticosteroid insensitivity in chronic inflammatory diseases (23). Moreover, several studies have indicated that the expression of COX-2 and synthesis of PGE2 induced by the proinflammatory mediators are mediated by the activation of PTKs, c-Src, EGFR, PDGFR, or P13K/Akt in various cell types (24, 56, 57).

Furthermore, CS has been implicated in initiating inflammatory responses in the airways through the activation of transcription factors, such as NF-κB, AP-1, and other signaling transduction pathways, such as MAPKs, leading to enhancing expression of inflammatory genes (44). The activation of NF-κB is a tightly regulated process that involves its translocation from the cytoplasm to the nucleus where it binds to cognate DNA sequences (4). NF-κB is composed of homo- and heterodimers of members of the Rel family of transcription factors and is normally sequestered in the cytoplasm through its interaction with the IκB (inhibitory of NF-κB) family of inhibitory proteins (4). In response to various stimuli, such as TNFα, IL-1, LPS, or PMA, IκB proteins undergo rapid phosphorylation of IκBα on serines 32 and 36 and IκBβ on serines 19 and 23, which facilitates their ubiquitination on neighboring lysine residues and thereby targets these proteins for rapid degradation by a proteasome (4). Following the degradation of IκB, NF-κB is released and is free to translocate into the nucleus and to activate target genes. In addition to CS, IL-1β, TNFα, LPS, and UV light are potent inducers of NF-κB, which stimulate expression of inflammatory genes (4). These findings imply that...
these signaling components might also be implicated in the expression of COX-2 induced by CSE in HTSMCs.

Therefore, CSE may play a potential role in regulation of expression of inflammatory genes, such as COX-2, and thereby promote inflammatory responses. We report in this study for the first time that in HTSMCs, CSE-induced COX-2 expression was mediated through a PKCa/Src/EGFR, PDGFR/P38/Akt/ NF-kB and p300-dependent pathway, associated with PGE2 synthesis.

MATERIALS AND METHODS

Materials. G60976, G60983, PP1, AG1478, AG1296, LY294002, garcinol, and helenalin were from Biomol (Plymouth Meeting, PA). Quick Change Site-Directed Mutagenesis Kit and GeneJammer transfection reagent were from Stratagene (La Jolla, CA). Metafetene transfection reagent was from Biontex (Munich, Germany). Luciferase assay kit was from Promega (Madison, WI). Anti-COX-2, anti-p65, and anti-PKCa antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-c-Src, anti-phospho-EGFR, anti-phospho-PDGFR, anti-phospho-Akt, and anti-phospho-p65 antibodies were from Cell Signaling (Danvers, MA).

Preparation of cigarette smoke extract. Cigarette smoke extract (CSE) was prepared by a method described previously with some modifications (36, 39, 45). In brief, 10 cigarettes (0.9 mg nicotine/cigarette, Long Life; Taiwan Tobacco and Liquor Production, Taipei, Taiwan) were combusted, and the smoke was sucked through a standard glass-fiber Cambridge filter with a constant flow (0.3 l/min) by an air compressor. The Cambridge filters have been reported to trap 99% of the smoke particulate matter (36). The smoke filter was dissolved by soaking the filter in DMSO for 30 min at room temperature. The solution containing CSE was centrifuged, and the supernatant was collected and filtered using a 0.22-μm filter column (Millipore, Bedford, MA). The CSE stock solution (20 mg/ml of CSE containing 0.36 mg/ml of nicotine) was kept in microtubes (each 15 μl) and was immediately stored in a –80°C freezer. Before each experiment, the frozen CSE stock solution was defrosted and further diluted to the desired concentration with cell medium. The quality of the CSE solution was assessed based on the absorbance at 302 nm, which is the specific absorption spectrum of peroxynitrite (38). Our preliminary study indicated that DMSO less than 1% had no effect on HTSMCs (8). Therefore, the concentration of DMSO in the testing solution was always <1% to prevent possible cytotoxicity or other effects.

Cell culture. HTSMCs were purchased from ScienCell Research Lab (San Diego, CA) and grew as previously described (29). Experiments were performed with cells from passages 3–8.

Transient transfection with siRNAs. SMARTpool RNA duplexes corresponding to human PKCa, Src, Akt, p300, p65, and scrambled #2 siRNA were from Dharmacon Research (Lafayette, CO). Transient transfection of siRNAs was performed using Metafetene transfection reagent. siRNA (100 nM) was formulated with Metafetene transfection reagent according to the manufacturer’s instructions. The transfection efficiency (~60%) was determined by transfection with EGFP.

Western blot analysis. Growth-arrested HTSMCs were incubated with CSE at 37°C for the indicated time intervals. The cells were washed, scraped, collected, and centrifuged at 45,000 g at 4°C for 1 h to yield the whole cell extract, as previously described (29). Samples were denatured, subjected to SDS-PAGE using a 12% running gel, and transferred to nitrocellulose membrane. Membranes were incubated with a primary antibody for 24 h and then with an anti-mouse horseradish peroxidase antibody for 1 h. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

RNA isolation and real-time RT-PCR. Total RNA was extracted using a TRIzol reagent (Invitrogen). mRNA was reverse-transcribed into cDNA and analyzed by real-time RT-PCR (Applied Biosystems, Branchual, NJ). Real-time PCR was performed using SYBR Green PCR reagents (Applied Biosystems) and primers specific for COX-2 and GAPDH mRNAs. The forward and reverse primers used for PCR were as follows: 5’-CGGATGCCTTCAAATCAA-3’ (sense) and 5’- TTCACCAGAACACAT-3’ (antisense) for GAPDH; 5’-GGGATCTTTTGCCCAAGCT-3’ (sense) and 5’-AAAGGCGGACTG-3’ (antisense) for COX-2. The levels of COX-2 expression were determined by normalizing to GAPDH expression.

Isolation of cell fractions. Cells were harvested, sonicated for 10 s at output 4 with a sonicator (Ultrasonics), and centrifuged at 8,000 rpm for 15 min at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 14,000 rpm for 60 min at 4°C to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

Measurement of COX-2 luciferase activity. For construction of the COX-2-luc plasmid, human COX-2 promoter, a region spanning –453 to +9 bp was cloned into a pGL3-basic vector (Promega). COX-2-luc activity was determined using a luciferase assay system as previously described (30) (Promega). Firefly luciferase activities were standardized for β-gal activity.

Measurement of PGE2 generation. HTSMCs were cultured in six-well culture plates. When reaching confluence, cells were treated with CSE (50 μg/ml) for the indicated time intervals at 37°C. The medium was collected and stored at –80°C until being assayed. PGE2 was measured using a PGE2 enzyme immunosassay kit (Cayman).

Coimmunoprecipitation assay. Cell lysates containing 1 mg of protein were incubated with 2 μg anti-p300 antibody at 4°C for 24 h, and then 10 μl of 50% protein A-agarose beads was added and mixed at 4°C for 24 h. The immunoprecipitates were collected and washed three times with lysis buffer without Triton X-100. Laemmli buffer (5×) was added, subjected to electrophoresis on 12% SDS-PAGE, and then blotted using an anti-p65 or anti-p300 antibody.

Immunofluorescence staining. Cells were plated on six-well culture plates with cover slips, shifted to serum-free DMEM/F-12 medium for 24 h, and then treated with CSE. Cells were fixed, permeabilized, and stained using an anti-p65 antibody as previously described (30). The images were observed under a fluorescence microscope (Zeiss, Axiovert 200M).

Analysis of data. Concentration-effect curves were made, and E50 values were estimated using the GraphPad Prism Program (San Diego, CA). Data were expressed as means ± SE and analyzed by one-way ANOVA followed with the Tukey post hoc test. P < 0.05 was considered significant.

RESULTS

Requirement of PKCa for CSE-induced COX-2 expression and PGE2 generation. PKCa has been shown to regulate COX-2 expression in human lung epithelium (38). In addition, CS has been shown to activate PKC isoforms, such as PKCa (42). To investigate whether CSE-induced COX-2 expression and PGE2 production were mediated through PKCa, HTSMCs were pretreated with G66976 or G66983 (an inhibitor of PKCa) (61) for 1 h and then incubated with CSE for 24 h. As illustrated in Fig. 1, A and B, CSE-induced COX-2 expression was inhibited by pretreatment with G66976 or G66983 in a concentration-dependent manner. There was no change in the level of COX-1 in these cells. G66976 has been shown to enhance agonist-induced tyrosine phosphorylation of the EGFr-receptor, possibly through inhibition of protein tyrosine phosphatase (PTP), since a PTP inhibitor, sodium orthovanadate, mimicked the effects of G66976 (49). We found that pretreat-
CSE induces COX-2 expression in HTSMCs.

CSE-induced COX-2 expression and its regulation by PKC and c-Src.

Cigarette smoke extract (CSE) stimulates cyclooxygenase-2 (COX-2) accumulation and PGE2 release through a PKCα-dependent pathway. A–C: cells were pretreated with Gö6976, Gö6983, or sodium orthovanadate, and then incubated with CSE for 24 h. The expression of COX-2 or COX-1 was determined by Western blot analysis. D: cells were pretreated with Gö6976 (1 μM) or Gö6983 (1 μM) and then incubated with CSE for 24 h. The RNA samples were analyzed by real-time RT-PCR for the levels of COX-2 mRNA expression. E: cells were transiently transfected with COX-2-luc reporter gene, pretreated with Gö6976 (1 μM), and then challenged with CSE for 24 h. The COX-2 promoter activity was determined in the cell lysates. F: cells were pretreated with Gö6976 (1 μM) or NS-398 (10 μM) and then incubated with CSE for 24 h. The medium was collected and analyzed for PGE2 release. G: cells were transfected with scrambled siRNA or PKCα siRNA and then challenged with CSE for 24 h. The levels of PKCα and COX-2 expression were determined by Western blot analysis. H: cells were treated with CSE for the indicated times. The membrane (ME) and cytosolic (CE) extracts were prepared and subjected to Western blot analysis using a PKCα antibody. PMA was a positive control for PKCα translocation. Data are expressed as means ± SEM of 3 independent experiments. *P < 0.05; #P < 0.01 compared with the cells exposed to CSE alone.

Involvement of c-Src in induction of COX-2 and PGE2 by CSE in HTSMCs. c-Src has also been shown to regulate COX-2 expression in human gastric epithelial cells (7). Animal studies have demonstrated that pharmacological inhibitors for Src attenuate tissue injury and improve survival from a variety of pathological settings related to acute inflammatory responses (41). To investigate whether CSE-induced COX-2 expression and PGE2 production were mediated through c-Src, cells were pretreated with PP1 (an inhibitor of c-Src) for 1 h and then incubated with CSE for 24 h. As shown in Fig. 2A, CSE-induced COX-2 expression was inhibited by pretreatment with PP1 in a concentration-dependent manner. In addition, pretreatment with PP1 also reduced CSE-induced COX-2 mRNA expression, promoter activity, and PGE2 release (Fig. 2, B–D). To further ensure that CSE-induced COX-2 expression was mediated via c-Src, cells were transfected with Src siRNA and then incubated with CSE for 24 h. As shown in Fig. 2E, transfection of HTSMCs with Src siRNA downregulated the expression of total c-Src protein and inhibited COX-2 expression induced by CSE. Activation of c-Src occurs as a result of multiple phosphorylation sites on specific residues, including Tyr416 and Tyr527 (47). The major phosphorylation site of c-Src at the Tyr416 residue results in activation due to c-Src autophosphorylation (47). We further examined whether c-Src...
phosphorylation at Tyr416 was stimulated by CSE in HTSMCs using an anti-phospho-c-Src (Tyr416) antibody. As shown in Fig. 2F, CSE time dependently stimulated c-Src phosphorylation at Tyr416 with a maximal response within 10 min. Moreover, pretreatment of HTSMCs with Go6976 or PP1 attenuated c-Src phosphorylation stimulated by CSE during the period of observation (Fig. 2F). These results suggested that CSE-induced COX-2 expression was mediated through a PKCα/c-Src-dependent c-Src signaling pathway in HTSMCs.

Involvement of EGFR and PDGFR in CSE-induced COX-2 expression and PGE2 generation in HTSMCs. There is evidence indicating that EGFR plays a role in COX-2 gene expression (19). In rat renal mesangial cells, PDGF has been shown to induce a rapid and transient increase of COX-2 mRNA and protein expression (17). To investigate whether CSE-induced COX-2 expression and PGE2 production were mediated through EGFR or PDGFR phosphorylation in HTSMCs, selective inhibitors of EGFR (AG1478) and PDGFR (AG1296) were used. We found that CSE-enhanced COX-2 protein and mRNA expression, promoter activity, and PGE2 generation were inhibited by pretreatment with either AG1478 or AG1296 (Fig. 3, A–D). Furthermore, whether CSE stimulated EGFR and PDGFR phosphorylation involved in CSE-induced COX-2 expression, CSE-stimulated EGFR or PDGFR phosphorylation was determined by Western blot analysis using an anti-phospho-EGFR or anti-phospho-PDGFR antibody. CSE stimulated EGFR and PDGFR phosphorylation in a time-dependent manner (Fig. 3E), which were inhibited by pretreatment with Go6976, PP1, AG1478, AG1296, or Go6983 (Fig. 3F). Moreover, pretreatment with 10 μM sodium orthovanadate markedly enhanced CSE-regulated EGFR or PDGFR phosphorylation (Fig. 3F). These data demonstrated that CSE-induced COX-2 expression and PGE2 generation were mediated through PKCα/c-Src-dependent EGFR and PDGFR activation in HTSMCs.

CSE-induced COX-2 expression and PGE2 release mediated through PI3K/Akt signaling. A previous finding has demonstrated that PI3K plays a key role in corticosterone-induced COX-2 expression (52). Several studies have shown that EGFR kinase-mediated activation of the PI3K/Akt pathway is required for Zn2+–induced COX-2 expression (55). To determine whether CSE-induced COX-2 expression was mediated through PI3K/Akt, a selective PI3K inhibitor, LY294002, was used. Pretreatment with LY294002 reduced CSE-induced COX-2 expression in a concentration-dependent manner (Fig. 4A). This downregulation of COX-2 protein expression was further confirmed by reduction of COX-2 mRNA, promoter activity, and PGE2 release in HTSMCs pretreated with LY294002 (Fig. 4, B–D). To further ensure that CSE-induced COX-2 expression was mediated via Akt, cells were transfected with Akt siRNA and then incubated with CSE for 24 h. As shown in Fig. 4E, transfection of HTSMCs with Akt siRNA downregulated the expression of total Akt protein and markedly inhibited COX-2 expression induced by CSE. To determine whether
CSE-induced responses were mediated through Akt phosphorylation. CSE-stimulated Akt phosphorylation was determined by Western blot analysis using an anti-phospho-Akt antibody. As shown in Fig. 4, CSE time dependently stimulated Akt phosphorylation, which was attenuated by pretreatment with Go6976, PP1, AG1478, AG1296, or LY294002. These results suggested that exposure of HTSMCs to CSE induces COX-2 expression and PGE2 synthesis, which were mediated through a PI3K/Akt-dependent signaling, downstream of PKC/EGFR and PDGFR.

CSE-induced COX-2 and PGE2 upregulation mediated via NF-κB signaling. The transcription factor NF-κB is a key regulator of immune and inflammatory responses through the induction of numerous genes, including those encoding for cytokines, chemokines, and adhesion molecules (31). Moreover, NF-κB has been shown to be involved in COX-2 expression and PGE2 release in various cell types (38, 59). In addition, PKC and c-Src have been shown to mediate NF-κB activation, which leads to IL-6 expression (9). Therefore, the involvement of NF-κB in CSE-induced COX-2 expression was investigated using the selective NF-κB inhibitor helenalin. We found that CSE-enhanced COX-2 protein and mRNA expression, promoter activity, and PGE2 generation were inhibited by pretreatment with helenalin (Fig. 5, A–D). To further confirm that CSE-induced COX-2 expression was mediated via NF-κB, cells were transfected with p65 siRNA and then incubated with CSE for 24 h. As shown in Fig. 5E, transfection of HTSMCs with p65 siRNA downregulated the expression of total NF-κB (p65) protein and markedly reduced CSE-regulated COX-2 expression. In addition, CSE also stimulated an increase in NF-κB promoter activity (Fig. 5F) and NF-κB (p65) translocation (Fig. 5G), which were inhibited by pretreatment with Go6976, PP1, AG1478, AG1296, or helenalin (Fig. 5, F and G). To further ensure the translocation of NF-κB (p65), nuclear translocation of NF-κB (p65) was observed by immunofluorescence staining. As illustrated in Fig. 5H, CSE-stimulated translocation of NF-κB (p65) was inhibited by pretreatment with Go6976, PP1, AG1478, AG1296, LY294002, or helenalin. These data demonstrated that the CSE-induced COX-2 expression and PGE2 generation were mediated via sequential activation of PKCα/c-Src/EGFR, PDGFR/PI3K/Akt/NF-κB pathway in HTSMCs.
CSE increases the recruitment of p300 with NF-κB in HTSMCs. NF-κB transcriptional competence requires interaction with the transcription coactivator p300 (16). Therefore, the involvement of p300 in CSE-induced COX-2 expression was investigated using a selective p300 inhibitor garcinol. As shown in Fig. 6, A–D, CSE-enhanced COX-2 protein and mRNA expression, COX-2 promoter activity, and PGE2 release were inhibited by pretreatment with garcinol. To further confirm that CSE-enhanced COX-2 expression was mediated via p300, cells were transfected with p300 siRNA and then incubated with CSE for 24 h. As shown in Fig. 6E, transfection of HTSMCs with p300 siRNA downregulated the expression of total p300 protein and markedly reduced CSE-induced COX-2 expression. Furthermore, we presented evidence that p300 formed a complex with NF-κB (p65) in HTSMCs stimulated by CSE, which was blocked by pretreatment with G66976, PP1, AG1478, AG1296, LY294002, helenalin, or garcinol (Fig. 6F). These data indicated that interaction between p300 and NF-κB was involved in CSE-induced COX-2 transcription.

DISCUSSION

COPD is a complex chronic inflammatory disease involving a wide variety of cells and inflammatory mediators. CS is the major cause of COPD in humans, the fifth leading cause of death worldwide, and predicted to be the third leading cause by 2020 (37). Moreover, COX metabolites have diverse effects in the lungs and are known to modify airway tone and inflammatory responses. The levels of prostanoids in bronchoalveolar lavage fluid are increased in asthma, which may be mediated through upregulation of both COX-1 and COX-2 in the airways of asthmatics (5). COX-1 is constitutively expressed in most tissues and considered to be the “housekeeping” isoform that produces PGs, which maintain normal cell and organ functions. In contrast, COX-2 is primarily an inducible isoform whose expression can be upregulated by cytokines, endotoxin, or CS in many cell types. It is highly expressed in inflamed tissues and believed to produce PGs involved in inflammatory processes (5). However, the molecular mechanisms by which CSE induced COX-2-dependent PGE2 generation are not fully understood in HTSMCs. The present study further demonstrates that CSE-induced COX-2 expression is mediated through a PKC/c-Src/EGFR, PDGFR/P3K/Akt/NF-κB signaling pathway. Genetic silencing through transfection with siRNA of PKCα, c-Src, Akt, p65, or p300 and pretreatment with the inhibitors of PKCα, c-Src, EGFR, PDGFR, P3K, NF-κB, and p300 abrogated CSE-induced COX-2 expression and PGE2 synthesis. Moreover, activation of PKCα, c-Src, EGFR, PDGFR, and P3K/Akt leading to transactivation of NF-κB and p300 is involved in CSE-induced COX-2 expression in HTSMCs.

Fig. 4. Involvement of PI3K/Akt in CSE-enhanced COX-2 expression and PGE2 generation. A: cells were pretreated with LY294002 and then incubated with CSE for 24 h. The expression of COX-2 was determined by Western blot analysis. B: cells were pretreated with LY294002 (30 μM) and then incubated with CSE for 6 h. The RNA samples were analyzed by real-time RT-PCR for the levels of COX-2 mRNA expression. C: cells were transiently transfected with COX-2-luc reporter gene, pretreated with LY294002 (30 μM), and then challenged with CSE for 2 h. The COX-2 promoter activity was determined in the cell lysates. D: cells were pretreated with LY294002 (30 μM) and then incubated with CSE for 24 h. The medium was collected and analyzed for PGE2 release. E: cells were transfected with scrambled siRNA or Akt siRNA and then challenged with CSE for 24 h. The levels of Akt and COX-2 expression were determined by Western blot analysis. F: cells were treated with CSE for the indicated times or pretreated with G66976 (1 μM), PP1 (10 μM), AG1478 (10 μM), AG1296 (10 μM), or LY294002 (30 μM) for 1 h and then incubated with CSE for 30 min. The cell lysates were subjected to Western blot analysis using an anti-phospho-Akt antibody. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05; **P < 0.01 compared with the cells exposed to CSE alone.
CS is a complex aerosol that can be separated into gas and particulate phases, whereas many substances are partitioned between these two phases (53). Both phases are harmful, containing high concentrations of toxic and carcinogenic compounds and high levels of reactive components and radicals, and are both associated with diverse pulmonary disorders, including cancer (51). In the gaseous phase, high levels of ROS are found. The gaseous phase components of CS are freely transported during inhalation, by simple diffusion, towards the bronchopulmonary epithelial cells (51). Their toxic activity on the cells begins immediately, with the first puff, simply following the laws of gases. The toxic components of the gaseous phase harm the cilia of the bronchial epithelium and impair the movement of the mucous by altering its viscosity and by disturbing or paralyzing the ciliary beat (46, 50). In addition, the gaseous phase of CS has been shown to cause DNA damage in lung cells, albeit less than whole smoke (1), and affects molecular pathways within the cells, such as the unfolded protein response pathways (25). In the particulate phase of CS, lipophilic components are present. Lipophilic components are able to pass the lipid bilayer. Recent reports have shown that components present in CSE, which does not contain ROS, are able to pass through the membranes of cells (53). Highly reactive components like polycyclic aromatic hydrocarbons, aldehydes, phenols, heavy metals, and amines are lipophilic candidates that easily enter the cell. For example, acrolein, a known toxin in tobacco smoke, has been shown to induce COX-2 and prostaglandin production in human umbilical vein endothelial cells via p38 MAPK (43). In addition, acrolein and crotonaldehyde contained in CS also have been shown to elicit IL-8 release in pulmonary cells through MAPKs (35). Thus, exposure to CS may induce COX-2, PGs, or IL-8 production, and this may contribute to airway inflammation in smokers. Although whole smoke can be divided into two
phases and both phases are harmful, in our study, we critically evaluated the effects of the particulate phase of CS on COX-2 expression in HTSMCs.

PKC activation appears to be essential for late responses, such as gene expression (40). PKC is a predominant component in the kinase cascades initiating by ligand binding to both G protein-coupled receptors and receptors containing intrinsic tyrosine kinase activity. In this study, we investigated the molecular mechanisms involved in CSE-induced COX-2 expression and PGE2 synthesis by PKC. Our results demonstrated that the major molecular mechanism triggered by CSE is mediated through the PKC isoforms (particular PKCα), since pretreatment with a selective PKCα inhibitor (Gö6976 or Gö6983) (61) interrupted CSE-mediated responses, suggesting that PKCα activation is required for the CSE-mediated COX-2 expression and PGE2 synthesis in HTSMCs. To confirm this hypothesis, the translocation of PKCα was characterized by Western blot analysis, indicating that exposure to CSE resulted in a rapid translocation of PKCα from the cytosol to the plasma membrane. The involvement of PKCα in CSE-induced responses was further confirmed by transfection with PKCα siRNA, which almost completely inhibited COX-2 expression induced by CSE. These data were consistent with the findings that PKCα is responsible for COX-2 expression induced by various stimuli (24, 38).

It has been demonstrated that Src family kinase plays a key role in the transduction of signals by GPCRs and growth factor receptors, which are involved in cell migration and proliferation (28). In addition, Src also plays a major role in the expression of inflammatory genes, such as COX-2 in various cell types (7, 24). In HTSMCs, CSE-induced COX-2 expression was significantly blocked by the c-Src inhibitor PP1 and transfection with Src siRNA. Moreover, CSE has been shown to stimulate the phosphorylation of Src, which was also attenuated by the inhibitors of PKC (Go6976) and c-Src (PP1), but not by the inhibitors of EGFR (AG1478) and PDGFR (AG1296) (Supplemental data for the article is available online at the AJP-Lung web site.). The ability of the c-Src kinase inhibitor PP1, but not AG1478 and AG1296, to inhibit c-Src activation indicated that CSE-stimulated c-Src activation precedes transactivation of EGFR and PDGFR in HTSMCs. These results were consistent with our previous study showing that in rat vascular smooth muscle cells, Src kinases phosphorylation is required for the activation of receptor tyrosine kinase, Akt, and p42/p44 MAPK in response to bradykinin, since pretreatment with the c-Src kinase inhibitor PP1 attenuated the phosphorylation of these signaling proteins (60). These results suggested that CSE induces COX-2 expression in a PKCα/c-Src-dependent manner in HTSMCs.

Fig. 6. Cooperation of NF-κB with p300 in CSE-induced COX-2 expression and PGE2 release. A: cells were pretreated with garcinol and then incubated with CSE for 24 h. The expression of COX-2 was determined by Western blot analysis. B: cells were pretreated with garcinol (10 μM) and then incubated with CSE for 6 h. The RNA samples were analyzed by real-time RT-PCR for the levels of COX-2 mRNA expression. C: cells were transiently transfected with COX-2-luc reporter gene, pretreated with garcinol (10 μM), and then challenged with CSE for 2 h. The COX-2 promoter activity was determined in the cell lysates. D: cells were pretreated with garcinol (10 μM) and then incubated with CSE for 24 h. The medium was collected and analyzed for PGE2 release. E: cells were transfected with scrambled siRNA or p300 siRNA and then challenged with CSE for 24 h. The levels of p300 and COX-2 expression were determined by Western blot analysis. F: cells were treated with CSE for the indicated times or pretreated with Gö6976 (1 μM), PPI (10 μM), AG1478 (10 μM), AG1296 (10 μM), LY294002 (30 μM), HLN (1 μM), or garcinol (10 μM) for 1 h and then incubated with CSE for 1 h. The cell lysates were subjected to immunoprecipitation using an anti-p300 antibody, and then the immunoprecipitates were analyzed by Western blot analysis using an anti-p65 or anti-p300 antibody. Data are expressed as means ± SE of 3 independent experiments. *P < 0.05; #P < 0.01 compared with the cells exposed to CSE alone.
It has been reported that several GPCRs initiate the Akt pathway through transactivation of the EGFR or PDGFR in various cell types (18, 32). In this model, Akt activation often involves sequential transactivation of these growth factor receptors in a c-Src kinase-dependent manner (15). However, little was known about the mechanisms of CSE that initiate the expression of COX-2 mediated through c-Src-dependent transactivation of EGFR and PDGFR in HTSMCs. Our results demonstrated that CSE-stimulated COX-2 expression and PGE$_2$ synthesis were inhibited by pretreatment with either AG1478 or AG1296, confirming the involvement of EGFR and PDGFR in COX-2 expression. Furthermore, CSE-stimulated phosphorylation of EGFR or PDGFR was also inhibited by pretreatment with Gö6976, PP1, AG1478, or AG1296, indicating that PKCα/c-Src is an upstream component of EGFR and PDGFR. In this regard, CSE shares the same mechanism as GPCRs for lysophosphatidic acid, bradykinin, and endothelin (13), which promotes the association of Src with proline-rich tyrosine kinase 2, and subsequently the Src/proline-rich tyrosine kinase 2 complex activates the growth factor receptors, such as EGFR and PDGFR (3). Our results were also consistent with the reports showing that activation of EGFR and PDGFR lead to COX-2 expression in various cell types (17, 56, 57). Gö6976 has been shown to enhance agonist-induced tyrosine phosphorylation of the EGFR, possibly through inhibition of PTP, since a PTP inhibitor, sodium orthovanadate, mimicked the effects of Gö6976 (49). Moreover, in HTSMCs, we found that pretreatment with sodium orthovanadate, but not Gö6976, markedly enhanced CSE-regulated EGFR or PDGFR phosphorylation. In addition, pretreatment with sodium orthovanadate further enhanced CSE-mediated COX-2 expression. Thus, we suggested that Gö6976 attenuates CSE-stimulated EGFR or PDGFR phosphorylation and thus inhibition of COX-2 expression in HTSMCs. These results suggested that PKCα/c-Src kinase activation is required for CSE-stimulated transactivation of EGFR and PDGFR leading to COX-2 expression in HTSMCs.

Recent studies have suggested that numerous components of the PI3K pathway play a crucial role in the expression and activation of inflammatory mediators, inflammatory cell recruitment, immune cell function, and airway remodeling in chronic inflammatory respiratory diseases (23). It has been established that growth factors, such as EGF and PDGF, stimulate Akt activation, which is inhibited by wortmannin, LY294002, or overexpression of the DN mutant of PI3K (2, 14, 60). Akt has also been implicated in the pathogenesis of inflammatory responses (27). Akt plays a pivotal role in COX-2 expression. For example, in vascular smooth muscle cells, Akt phosphorylation is required for sphingosine-1-phosphate-induced COX-2 expression (22). Our results are consistent with these studies demonstrating that Akt is a key regulator in CSE-induced COX-2 expression and PGE$_2$ synthesis, which were attenuated by the selective PI3K inhibitor LY294002. This notion was further supported by the results showing that transfection of HTSMCs with Akt siRNA knocked down Akt protein expression and subsequently reduced CSE-induced COX-2 expression in HTSMCs. The involvement of Akt in COX-2 expression may be due to phosphorylation of Akt stimulated by CSE, which was further confirmed by the results indicating that pretreatment with Gö6976, PP1, AG1478, AG1296, or LY294002 attenuated CSE-stimulated Akt phosphorylation. These results suggested that CSE-induced COX-2 expression and PGE$_2$ synthesis is mediated via a PKCα/c-Src/EGFR, PDGFR/P13K/Akt-dependent signaling in HTSMCs.

NF-κB has been shown to be an inducible and ubiquitously expressed transcription factor responsible for the expression of several genes involved in various cellular functions, such as inflammation (10). Moreover, NF-κB has been shown to regulate COX-2 expression and PGE$_2$ release in chicken thrombocytes (48). In addition, PKCα and c-Src have been shown to mediate NF-κB activation (9). This is also confirmed by our observation that CSE-enhanced COX-2 expression and PGE$_2$ generation was attenuated by pretreatment with helenalin or transfection with p65 siRNA. Moreover, CSE-stimulated NF-κB translocation was mediated through a PKCα/c-Src/EGFR, PDGFR/P13K/Akt signaling pathway in HTSMCs. On the other hand, AP-1 may be also activated by PKC, PTK, and MAPKs, which are stimulated by various cytokines, including TNFα and IL-1β (26). Thus, the role of AP-1 involved in CSE-induced COX-2 expression and PGE$_2$ release is an important issue that needs to be investigated in the future. CBP (CREB-binding protein) and p300 are versatile coactivators that link transcriptional activators to the basal transcriptional apparatus (45). CBP and p300 act as coactivators of p65-driven gene activation and may play an important role in the cytokine-induced expression of various inflammatory genes (45). The interaction between the p65 subunit of NF-κB with either CBP or p300 increases the transcriptional activity of the NF-κB complex through both modification of chromatin structure and the direct acetylation of p65 (12, 34). p300 has been shown to increase COX-2 transcription (12), suggesting an important role of p300 in bridging the multiple DNA-bound transactiva-

Fig. 7. Schematic diagram illustrating the proposed signaling pathway involved in CSE-induced COX-2 expression and PGE$_2$ generation in human tracheal smooth muscle cells (HTSMCs). CSE activates the PKCα/c-Src/EGFR, PDGFR/P13K pathway to enhance Akt phosphorylation, which in turn initiates the activation of NF-κB and the recruitment of p300 with NF-κB, and ultimately induces COX-2-dependent PGE$_2$ generation in HTSMCs.
tors with transcription factors to initiate COX-2 transcription. Moreover, in this study, we also presented evidence that p300 formed a complex with NF-κB in HTSMCs stimulated by CSE. In the future, we expect to further determine which domains of NF-κB and p300 are involved in protein-protein interactions caused by CSE.

In summary, as depicted in Fig. 7, our results showed that in HTSMCs, CSE induces NF-κB and p300 activation through a PKCa/c-Src/EGFR, PDGFR/P38/Akt signaling pathway. Activated NF-κB and p300 were recruited to the promoter region of COX-2 leading to an increase of COX-2 expression associated with PGE2 synthesis. Therefore, blockade of these pathways may reduce PGE2 release via COX-2-dependent mechanisms and attenuate the inflammatory responses in airway diseases.

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