Cigarette smoke extract induces endothelin-1 via protein kinase C in pulmonary artery endothelial cells

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Lee, Sang-Do, Dong-Soon Lee, Yong-Gam Chun, Tae-Sun Shim, Chae-Man Lim, Younysuck Koh, Woo-Sung Kim, Dong-Soon Kim, and Won-Dong Kim. Cigarette smoke extract induces endothelin-1 via protein kinase C in pulmonary artery endothelial cells. Am J Physiol Lung Cell Mol Physiol 281: L403–L411, 2001.—We examined the mechanism of endothelin (ET)-1 regulation by cigarette smoke extract (CSE) and the effect of platelets on CSE-induced stimulation of ET-1 gene expression in human and bovine pulmonary artery endothelial cells (PAECs). Our data suggest that CSE stimulates ET-1 gene expression and that CSE induced platelet aggregation and increased the expression of ET mRNA and ET biosynthesis in cultured endothelial cells. Our data suggest that CSE stimulates ET-1 gene expression via PKC in PAECs. CSE and platelets showed a synergistic effect on ET-1 gene expression, possibly through the activation of platelets by CSE.

CIGARETTE SMOKING HAS BEEN IMPLICATED in chronic obstructive pulmonary disease (COPD), chronic hypoxic cor pulmonale, atherosclerosis, and related cardiovascular dysfunction (18, 19, 38). The mechanism for the increased risk of vascular dysfunction is not well understood. It is presumed to be due to the absorption of tobacco constituents that affect endothelial cell function (6, 23, 29, 33, 36), but the true mediator of these vascular diseases associated with smoking is not known. Endothelin (ET)-1 is a potent vasoconstrictor and mitogenic agent released by endothelial cells (41) and has been implicated in the pathogenesis of pulmonary hypertension (2, 14, 28). In humans, cigarette smoking results in a significant increase in plasma ET-1 levels (15, 16), and regular cigarette smoking is associated with functional and morphological changes in the pulmonary arteries (6, 7, 11, 17, 23). The ETA receptor antagonist BQ-610 blocks cigarette smoke-induced mitogenes in rat airways and vessels (10). Pulmonary hypertension in patients with COPD is associated with the increased expression of ET-1 in vascular endothelial cells, suggesting that the local production of ET-1 may contribute to the vascular abnormalities associated with this disorder (14). Evidence obtained by a variety of approaches indicates that platelets are activated in the circulation of chronic smokers in vivo (13), and platelets stimulate expression of ET mRNA and ET biosynthesis in cultured endothelial cells (31). In the present study, we tested the hypothesis that cigarette smoke extract (CSE) stimulates ET-1 gene expression and that CSE and platelets show a synergistic effect on ET-1 gene expression, possibly through the activation of platelets by CSE, in human and bovine pulmonary artery endothelial cells (PAECs). Protein kinase C (PKC) has been implicated in the regulation of ET-1 production in various endothelial cells (12, 25, 40), and a recent study (21) has shown that cigarette smoke condensate induces PKC activity in endothelial cells. So the role of PKC in CSE-induced stimulation of ET-1 gene expression was also determined in this study.

MATERIALS AND METHODS

Reagents. The Rp diastereomer of adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS) was purchased from BIOMOL Research Laboratories (Plymouth, PA). Staurosporine, calphostin C, phorbol 12-myristate 13-acetate (PMA), actinomycin D, cycloheximide, prostacyclin, and ADP were purchased from Sigma (St. Louis, MO).

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Cell culture and experimental design. Bovine PAECs were obtained from American Type Culture Collection (CCL 209) and grown in DMEM supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin for 4 or 5 days before they were subcultured. Experiments were performed on confluent contact-inhibited cells that had been kept in serum-free DMEM for 24 h to induce quiescence. For experiments designed to measure ET-1 peptide in conditioned medium, the medium was replaced by serum-free DMEM immediately before the studies began. CSE was added to the cell suspension as indicated. Controls consisted of either untreated or vehicle-treated cells. Human PAECs were obtained from Stratagene (La Jolla, CA) and grown in endothelial cell growth medium (Clonetics). After achieving confluence, the cells were washed with MCDB-131 alone and incubated with MCDB-131 containing human albumin (1 mg/ml) and test reagents. Rp-cAMPS, staurosporine, calphostin C, PMA, actinomycin D, and cycloheximide were added in concentrations and time intervals indicated in RESULTS and Figs. 1–5. None of the compounds used caused significant cytotoxic effects. Humatoid smoke passed through a glass fiber Cambridge filter (Fisher, Omaha, NE) that retained 99% of all particulate matter were then extracted for 15 min at 37°C in PBS. The Cambridge filters with the absorbed particulate matter were then aspirated into another centrifuge tube, and prostacyclin (0.8 μM) was added to prevent platelet aggregation. PRP was then centrifuged for 10 min at 800 g, and the pellet was resuspended in 15 ml of Ca2+-free Tyrode buffer of the following composition (in mM): 137 NaCl, 2.7 KCl, 1.0 MgCl2, 0.35 NaH2PO4, 11.9 NaHCO3, and 5.5 glucose, pH 7.35, with 0.8 μM prostacyclin. The mixture was centrifuged for 10 min at 600 g, and the pellet was resuspended in 15 ml of Ca2+-free Tyrode solution with the addition of prostacyclin (0.8 μM final concentration). This mixture was then centrifuged for 10 min at 800 g, the pellet was resuspended in 2 ml of Ca2+-free Tyrode buffer, and CaCl2 was added for a final concentration of 1.8 mM (31). The pellet number was adjusted to 2.5 × 106 cells/ml with the use of a Coulter counter (Coulter, Hialeah, FL).

RNA isolation and Northern blot analysis. RNA from PAECs was extracted by the method described by Chomczynski and Sacchi (8). Total RNA (20 μg) was fractionated by electrophoresis on a 1% agarose-6% formaldehyde denaturing gel and transferred onto a positively charged nylon membrane (Hybond-Blot, Ontex, Gocheshurg, MD). The RNA was cross-linked to the filter by ultraviolet light. The membrane was prehybridized in Rapid-Hyb buffer (Amersham, Arlington Heights, IL) for 20 min at 65°C. A plasmid containing the ovine preproET-1 cDNA probe (550 bp) was kindly provided by Dr. S. Abman (University of Colorado, Denver, CO). The probes were labeled with [α-32P]dCTP with a random-primer labeling kit (Amersham) and added to the prehybridization solution at ~1 × 106 dpm/ml. After hybridization for 2 h at 65°C, the filters were washed three times for 15 min at room temperature in 2× SSC-0.1% SDS and twice for 30 min at 65°C in 1× SSC-0.1% SDS followed by autoradiography and scanning densitometry. A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control for RNA loading. The quantification of preproET-1 mRNA was determined by scanning densitometry. Briefly, films containing the hybridization signals were developed after 24 h and 2–3 days of exposure. The 24-h exposure signal had a lighter signal, and it was compared with a 2- to 3-day exposure to ensure that saturation of the radiographic film had not occurred. The film with the best signal-to-noise ratio was then scanned with a Molecular Dynamics series 400 phosphorimager and ImageQuant software (Sunnyvale, CA). The preproET-1 mRNA signal was then normalized to the GAPDH signal.

Nuclear transcription run-off analysis. Confluent cultures of bovine PAECs were incubated for 2 h in the presence and absence of CSE (1%). At the end of the incubation, bovine PAECs were lysed in ice-cold 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, and 0.5% (vol/vol) Nonidet P-40, and the nuclei were isolated with a Dounce homogenizer as described by Kavanagh et al. (22). Aliquots of nuclear suspension were incubated with 0.5 mM each CTP, ATP, and GTP and 250 μCi of [α-32P]labeled UTP (3,000 Ci/mmol; New England Nuclear, Boston, MA). The samples were phenol-chloroform extracted, precipitated, and resuspended at equal counts per minute (cpm) per milliliter in hybridization buffer (10–20 × 106 cpm/ml). Hybridization to denatured probes (1 μg) dot blotted on nitrocellulose filters was performed at 40°C for 3 days in the presence of 50% formamide. cDNA probes for ovine preproET-1 gene were used.

Radioimmunoassay of ET-1. Levels for ET-1 peptide secretion in endothelial cell culture supernatants were quantified by radioimmunoassay (RIA; Peninsula Laboratories, Belmont, CA) in triplicate. Cell supernatants were collected, lyophilized, and suspended in RIA buffer consisting of 100 mmol/l of NaH2PO4, 0.05 mol/l of NaCl, 0.1% bovine serum albumin, and 0.05% sodium azide.
albumin, 0.1% Triton X-100, and 0.01% NaN₃. The rabbit anti-ET-1 that was used showed 100% specificity for ET-1, 17% cross-reactivity to Big ET-1, and 7% reactivity to ET-2 and ET-3. Antiserum (100 μl) was added in equal amounts to 100 μl of either ET standards or reconstituted cell supernatants and incubated for 24 h before 100 μl of 125I-ET-1 at a final concentration of 3,500 cpm were added for 24 h. ET bound to the antibody was immunoprecipitated with 100 μl of anti-rabbit serum before being separated from unbound antibody by centrifugation. Finally, the amount of radioactivity in the immunoprecipitants was determined by gamma counting. ET-1 standard curves revealed 50% displacement by 8–12 pg of ET-1. ET concentrations were calculated by computer-aided processing of the counting data using a log-log transformation of the calibration curve and were corrected for protein concentration per dish with a Bradford protein assay (4).

Platelet aggregation tests. An 18-ml sample of blood obtained from a healthy nonsmoker was collected into a plastic syringe containing 2 ml of sodium citrate (129 mM adjusted to pH 7.4 with citric acid) and was centrifuged at 150 g for 15 min and adjusted with platelet-poor plasma (PPP) to 0.3 × 10⁹ platelets/ml. ADP (1.0 μM) was used as an inducer. Ex vivo PRP aggregometry was performed in an automated platelet aggregation analyzer (PA-3220 Aggregometer II, Daichi), according to the method of Born and Cross (3). Aggregation was quantified as the extent (intensity) of light transmission in stimulated PRP calibrated as 100% light transmission for PPP and 0% for nonstimulated PRP.

Quantitative analysis of platelet membrane glycoprotein expression. Murine monoclonal antibodies to Gp IIb/IIIa (CD41a), Gp Ib (CD42b), Gp IIIa (CD61), and P-selectin (CD62P); isotypic control; fluorescein isothiocyanate (FITC)-labeled F(ab)² fragments of human Ig-absorbed sheep anti-mouse IgG antibodies; and calibration beads with four different known amounts of antibody per bead were provided by Dr. M. Canton (Biocytex, Marseille, France).

For the ligand binding studies, 9 parts of blood from healthy nonsmokers who had not taken aspirin or any other antiplatelet agent in the previous 7 days were collected to 1 part of sodium citrate (3.8%). This was centrifuged at 150 g for 10 min, and the PRP was aspirated. The PRP was diluted to 1 × 10⁹ platelets/ml with PBS and incubated with CSE (1%) or PBS for 10 min at room temperature. Aliquots of each dilution were incubated with monoclonal antibodies (10 μg/ml) at room temperature for 20 min. Antibody binding was determined with FITC-labeled F(ab)² fragments of human Ig-absorbed sheep anti-mouse IgG (heavy plus light) antibodies. The samples were fixed with 1 ml of 1% formaldehyde after 10 min of incubation and analyzed by flow cytometry (FACScan, Becton Dickinson) at 488-nm excitation. Platelet populations were gated according to their forward and side light scatter. Histograms were generated with 10,000 counts, and geometric mean fluorescence was calculated with the CELLQUEST software of the FACScan system (Becton Dickinson). The binding of an isotypic control antibody was taken as nonspecific binding and was subtracted from the observed geometric mean fluorescence.

Calibration beads consisting of a mixture of four different populations of 2-μm-diameter latex beads, each with a different defined amount of murine antibody per bead, were used to estimate the number of antibodies bound per platelet, similar to the method described by Ponec et al. (32) The beads were analyzed in parallel with the samples, with the same FITC reagent and the same settings as the samples. The single bead populations were gated according to their geometric mean fluorescence. Histograms of the geometric mean fluorescence intensity of 10,000 events were recorded and used to plot a log-log graph of the mean fluorescence intensity versus the number of antibodies attached to each bead. The number of platelet-bound monoclonal antibody molecules was estimated from this graph on the basis of the geometric mean fluorescence intensity of the sample.

Data analysis. For Northern analysis, densitometry values for ET-1 were first divided by the values for the internal control to correct for any gel loading errors. ET-1 control values were then normalized to a value of 1; the multiple of induction of ET-1 levels from stimulated samples is displayed relative to these values. Statistical comparisons for each stimulus versus control were calculated with a paired two-tailed t-test. RIA results are reported as nanograms of ET-1 per milligram of total protein and were analyzed for significance with an unpaired t-test. Platelet aggregation results are reported as the intensity (in percent) of light transmission and were analyzed for significance with an unpaired t-test. The value of platelet membrane glycoprotein expression is expressed as the number of platelet-bound molecules and was analyzed for significance with an unpaired t-test. All data are expressed as means ± SE, and significance was set at P < 0.05 or P < 0.01 as noted.

RESULTS

Induction of ET-1 by CSE in bovine PAECs. CSE caused an increase in preproET-1 mRNA in the tested range of 0.1–10%, with its maximal effect at 1% (Fig. 1A). CSE (1%) led to an increase in preproET-1 mRNA that was time dependent. Quantitative densitometric analysis of this experiment indicated a significant difference in preproET-1 mRNA levels compared with the control level after 60 min of exposure to CSE. The peak induction occurred after 2 h, and transcript levels remained significantly elevated through 4 h (Fig. 1B). The noted increase in preproET-1 mRNA in response to CSE may depend on newly synthesized transcripts, the heightened stability of preproET-1 transcripts, or a combination of both. To identify which mechanisms may be responsible for the increase in transcript level, an actinomycin D chase experiment and a nuclear run-off analysis were done. Actinomycin D (10 μg/ml) was used to arrest new RNA synthesis, thus allowing quantification of the rate of disappearance of preproET-1 mRNA. PreproET-1 mRNA disappeared with a half-life of <15 min in both vehicle- and CSE-treated cells (Fig. 2A). Nuclear run-off analyses were carried out to determine the transcriptional rate of preproET-1 gene after 2 h of exposure of the cells to CSE (1%). Compared with the transcriptional rate of preproET-1 in the control cells, there was an average 3.4-fold increase in preproET-1 gene transcription in cells treated with CSE (range 1.9- to 4.8-fold; Fig. 2B). To elucidate whether new protein synthesis is required to elicit the CSE-induced increase in preproET-1 mRNA, cells were treated with cycloheximide in the absence and presence of CSE (1%), and preproET-1 mRNA levels were compared with those in control or CSE-treated cells not exposed to cycloheximide (Fig. 2C). When the cells were exposed to either CSE or cycloheximide for 2 h, an increase in preproET-1 mRNA was observed. In cells pretreated with cycloheximide, CSE...
induced a further increase in preproET-1 mRNA. To evaluate the time course of ET-1 production by unstimulated and 1% CSE-stimulated bovine PAECs, cell culture supernatants were withdrawn at 1, 4, 8, and 24 h (Table 1). Extracellular ET-1 production increased from 0.49 ± 0.04 to 2.61 ± 0.10 ng/mg protein in control cultures and from 0.51 ± 0.04 to 4.80 ± 0.21 in CSE-stimulated cultured cells. After 4 h of incubation, ET-1 production in the group stimulated with CSE was significantly higher than that of unstimulated cells.

Effect of PPE, GPE, and nicotine on preproET-1 mRNA level in bovine PAECs. To investigate which component of cigarette smoke is responsible for the induction of preproET-1 mRNA, cigarette smoke was separated into PPE and GPE. Incubation of bovine PAECs with PPE (1%) or GPE (1%) resulted in an increase in preproET-1 mRNA similar to that observed with CSE (1%). In contrast, incubation of bovine PAECs with nicotine (0.1 and 1 μmol/l) did not change the level of preproET-1 mRNA (Fig. 3).

Role of PKC in the CSE-induced ET-1 gene expression in human and bovine PAECs. Activation of PKC by PMA, PKC depletion before agonist stimulation,
Table 1. Extracellular ET-1 produced by bovine PAECs cultured without and with CSE

<table>
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<th>Time, h</th>
<th>n</th>
<th>Control</th>
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<th>P Value</th>
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<tr>
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<td>0.51 ± 0.04</td>
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<tr>
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<td>3</td>
<td>2.61 ± 0.10</td>
<td>4.80 ± 0.21</td>
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Values are means ± SE; n, no. of experiments. ET-1, endothelin-1; PAEC, pulmonary artery endothelial cell; CSE, cigarette smoke extract; NS, not significant.

and inhibition with PKC antagonists was used to determine the role of PKC in CSE-induced stimulation of ET-1 gene expression. The PKC inhibitors staurosporine (at 10^{-8} mol/l) and calphostin C (at 10^{-7} mol/l) abolished the induction of preproET-1 mRNA and ET-1 peptide by CSE (1%; Fig. 4, A, B, and D). In bovine PAECs, short-term activation of PKC with PMA (0.1 μmol/l) showed a modest increase in preproET-1 mRNA at 30 min, which declined rapidly to below the basal level over 12 h of incubation with PMA. PKC depletion by PMA pretreatment for 12 h also reduced the CSE-mediated preproET-1 mRNA induction (Fig. 4C). These findings suggest that PKC is involved in CSE-induced ET-1 gene expression in PAECs. On the other hand, Rp-cAMPs (at 5 × 10^{-4} mol/l), which blocks the activation of PKA by cAMP, had no effect on CSE-induced ET-1 gene expression in bovine PAECs (Fig. 4, A and D).

Effects of washed human platelets on CSE-induced ET-1 gene expression in human and bovine PAECs. The addition of washed human platelets (10^7 cells/ml) to bovine PAECs led to an increase in preproET-1 mRNA and ET-1 peptide, whereas a lower concentration of platelets (10^6 cells/ml) did not significantly alter preproET-1 mRNA and ET-1 peptide levels. Incubation of platelets (10^6 cells/ml) with CSE (1%) and bovine PAECs produced a significant increase in preproET-1 mRNA and ET-1 peptide compared with the values in the presence of CSE (1%) alone (Fig. 5, A and C). In human PAECs, platelets (10^7 cells/ml) did not significantly alter preproET-1 mRNA and ET-1 peptide levels, whereas incubation of platelets (10^7 cells/ml) with CSE (1%) and human PAECs produced a significant increase in preproET-1 mRNA and ET-1 peptide compared with the values in the presence of CSE (1%) alone (Fig. 5, B and C).

Effects of CSE on platelet aggregation and platelet membrane glycoprotein expression. To investigate whether the observed synergistic effect of CSE (1%) and platelets on ET-1 gene expression in PAECs was due to activation of platelets by CSE, we used a platelet aggregation test and a flow cytometric method, which permit the detection of a spectrum of activation-dependent modifications in the platelet surface membrane. In the presence of platelet activation agonists, a quantitative variation of the membrane glycoprotein expression is observed. In stimulated platelets, surface expression of Gp IIb/IIIa, Gp IIIa, and P-selectin increases, whereas expression of Gp Ib decreases (27). In this study, we used a panel of monoclonal antibodies directed against different platelet surface glycoproteins (Gp IIb/IIIa, Gp Ib, Gp IIIa, and P-selectin). CSE induced platelet aggregation, which was concentration dependent in the tested range of 0.1–10%, and enhanced the aggregation induced by ADP (Table 2). CSE (1%) also increased the expression of GP IIb/IIIa, Gp IIIa, and P-selectin and decreased the expression of Gp Ib ex vivo (Table 3).

DISCUSSION

In the studies reported here, CSE caused a concentration- and time-dependent increase in preproET-1 mRNA and an increase in ET-1 peptide in bovine PAECs. The induction of preproET-1 mRNA by CSE was a result of an increased transcriptional rate. The preproET-1 mRNA induction was not dependent on de novo protein synthesis as evidenced by the cycloheximide experiments. In fact, treatment with cycloheximide alone produced a dramatic increase in preproET-1 mRNA levels, and cotreatment with CSE and cycloheximide potentiated the induction over either treatment alone. The cycloheximide effect could result from stabilization of the transcript by interference with a translation-dependent ribonuclease or increased transcription by inhibition of a labile repressor protein. A number of AUUUA motifs known to be present in the 3'-untranslated region of several mRNA species with short half-lives (5) are found in the 3'-untranslated region of preproET-1 mRNA. Destabilization of these other AUUUA-containing mRNA species has been shown to be translationally dependent. This corroborates our cycloheximide evidence, suggesting that destabilization of bovine preproET-1 mRNA is translationally dependent. CSE (10%) caused a decrease in preproET-1 mRNA, which seems to be due to its toxic effect because CSE (10%) induced significant apoptosis of bovine PAECs in our experiment (data not shown).
Cigarette smoke is known to contain ≈4,000 different constituents distributed in gas and particulate phases (20). To determine whether the component(s) responsible for CSE-induced stimulation of ET-1 gene expression was confined to either the gas or particulate phase, we separated CSE into a GPE and a PPE. Both GPE and PPE solutions induced comparable increases in preproET-1 mRNA expression, suggesting that the components in CSE responsible for stimulation of ET-1 gene expression exist in both the gas and particulate phases. Our results demonstrate that nicotine is unlikely to be responsible for the stimulation of ET-1 gene expression by CSE (1%) because nicotine (10⁻⁷ to 10⁻⁶ mol/l) in comparable concentrations to those in CSE (1%) did not affect ET-1 gene expression in our bovine PAECs. This is supported by the study that showed that ET-1 is upregulated in the plasma of smokers but not in the plasma of patients administered transdermal nicotine (15). In vivo, pulmonary endothelial cells are not the only source of plasma ET-1. ET-1 is also produced by airway epithelial cells, neurons and astrocytes in the central nervous system, endometrial cells, hepatocytes, kidney mesangial cells, Sertoli cells, breast epithelial cells, neutrophils, and macrophages (24). Aside from the direct effects of the components of cigarette smoke, several factors related to smoking could also stimulate ET-1 production in vivo. For example, it is well established that cigarette smoking acutely increases catecholamine levels in blood (9), which, in turn, could stimulate ET-1 production (24). So the effect of cigarette smoking on ET-1 production in vivo is complicated, and this area needs further investigation.

In vascular endothelial cells, PKC seems to mediate ET-1 gene induction caused by thrombin, angiotensin II, or hemodynamic shear stress (37), and a recent

Fig. 4. A: inhibition of CSE-stimulated preproET-1 mRNA induction by staurosporine and calphostin C in bovine PAECs. Total RNA was isolated from control bovine PAECs (lane 1) or from cells treated with CSE for 2 h (lane 2), 10⁻⁸ mol/l of staurosporine for 30 min before CSE (lane 3), 10⁻⁷ mol/l of calphostin C for 30 min before CSE (lane 4), or 5 × 10⁻⁴ mol/l of the Rp diastereomer of adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS) for 30 min before CSE (lane 5). Data are representative of 3 independent experiments. Ratios are averages from 3 separate experiments. *P < 0.05 compared with CSE alone.

B: inhibition of CSE-stimulated preproET-1 mRNA induction by staurosporine and calphostin C in human PAECs. Total RNA was isolated from control human PAECs (lane 1) or from cells treated with CSE for 2 h (lane 2), 10⁻⁸ mol/l of staurosporine for 30 min before CSE (lane 3), or 10⁻⁷ mol/l of calphostin C for 30 min before CSE (lane 4). Data are representative of 3 independent experiments. Ratios are averages from 3 separate experiments. *P < 0.05 compared with CSE alone.

C: inhibition of CSE-stimulated preproET-1 mRNA induction by protein kinase C (PKC) depletion with phorbol 12-myristate 13-acetate (PMA). Representative Northern blot analysis shows total cellular RNA hybridized with ET-1 and GAPDH cDNAs in bovine PAECs exposed to PMA for 12 h and after 12 h of incubation with PMA before exposure to CSE for 2 h. Ratios are averages from 3 separate experiments. Note the initial preproET-1 mRNA increase at 30 min followed by a steady decline to below the control level. **P < 0.05 compared with control. **P < 0.05 compared with CSE alone.

D: inhibition of CSE-stimulated ET-1 secretion by staurosporine and calphostin C in bovine PAECs. Bovine PAECs at confluence were incubated for 16 h with vehicle (control), CSE, 10⁻⁸ mol/l of staurosporine for 30 min before CSE, 10⁻⁷ mol/l of calphostin C for 30 min before CSE, and 5 × 10⁻⁴ mol/l of Rp-cAMPS for 30 min before CSE. ET-1 levels were measured in the culture supernatants of triplicate experiments. *P < 0.05 compared with control. **P < 0.05 compared with CSE alone.
study (21) has shown that cigarette smoke induces PKC activity in endothelial cells. In this study, the PKC inhibitors staurosporine and calphostin C abolished the induction of ET-1 gene expression by CSE in human and bovine PAECs. Prolonged exposure of cells to activators of PKC can result in downregulation of the enzyme (30). When cells are exposed to PMA, activation of PKC in the membrane fraction continues for up to 8 h, after which downregulation is apparent for 16–24 h (42). In our study in bovine PAECs, short-term activation of PKC with PMA (0.1 μmol/l) showed a modest increase in preproET-1 mRNA at 30 min, which declined rapidly to below basal level over 12 h of incubation with PMA. PKC depletion by PMA pretreatment for 12 h reduced the CSE-mediated preproET-1 mRNA induction in bovine PAECs. From these data, it seems likely that the CSE effects on ET-1 gene expression in PAECs were mediated by PKC. The 5′-flanking region of the human preproET-1 gene contains octanucleotide sequences for the 12-O-tetradecanoylphorbol 13-acetate-responsive element (TRE; activator protein-1 (AP-1)/c-Jun binding element). The synthesis and TRE binding activity of c-Jun protein, which is a major component of the transcription factor AP-1, is increased by activation of PKC (37). Cigarette smoke condensate exposure increased DNA binding of AP-1 in human type II alveolar epithelial cells (34), and ferrets given a β-carotene supplement and exposed to tobacco smoke had elevated expression of the c-jun and c-fos genes (39).

Evidence obtained by a variety of approaches indicates that platelets are activated in the circulation of chronic smokers (13). Platelets stimulate expression of preproET mRNA and ET biosynthesis in cultured endothelial cells, and activation of platelets results in a further enhancement of ET release (31). In this study, we examined the effect of platelets on CSE-induced stimulation of ET-1 gene expression in human and bovine PAECs. The addition of platelets (10^7 cells/ml)
to bovine PAECs led to an increase in preproET-1 mRNA and ET-1 peptide, whereas a lower concentration of platelets (10^6 cells/ml) did not significantly alter preproET-1 mRNA and ET-1 peptide levels. Incubation of platelets (10^6 cells/ml) with CSE (1%) and bovine PAECs produced a significant increase in preproET-1 mRNA and ET-1 peptide compared with the values in the presence of CSE (1%) alone. Similar results were obtained in experiments with human PAECs. We investigated whether the synergism of CSE and platelets was due to the activation of platelets by CSE. To evaluate the CSE-induced alterations in platelet function, we used a platelet aggregation test and a flow cytometric method, which permit the detection of a spectrum of activation-dependent modifications in the platelet surface membrane. In our study, CSE induced platelet aggregation and enhanced the aggregation induced by ADP. This result is in agreement with the study (1) that showed that platelet aggregation to thrombin and ADP increased 10 min after smoking compared with that before smoking. CSE also increased the expression of platelet membrane Gp IIb/IIIa, Gp IIIa, and P-selectin and decreased the expression of Gp Ib. These data suggest that CSE induces platelet activation ex vivo.

Regular cigarette smoking is associated with morphological changes in the muscular pulmonary arteries that evolve in parallel with small-airway disease and emphysema (17), and endothelial-dependent vasorelaxation is diminished in cigarette smokers and in the lungs of individuals with COPD and hypoxic cor pulmonale (6, 7, 11, 23). The cellular mechanism responsible for these pulmonary vascular alterations is not well understood. It is presumed to be due to the absorption of tobacco smoke constituents that affect endothelial cell function (6, 23, 29, 33, 36), but the true mediator of these vascular diseases associated with smoking is not known. ET-1 is a potent vasoconstrictor and has been implicated in the pathogenesis of pulmonary hypertension (2, 14, 28). Cigarette smoking is associated with a decrease in endothelial NO synthase (eNOS) protein and eNOS mRNA contents as well as in eNOS activity in PAECs (36). A reduction in NO production by cigarette smoke is presumed to be responsible, at least in part, for the increased risk of systemic and pulmonary vascular disease and dysfunction in cigarette smokers (33). Taken together, CSE causes an upregulation of ET-1 gene expression and downregulation of eNOS expression in PAECs, and the local imbalance in the release of these mediators may play a part in the development or maintenance of pulmonary vascular disease and dysfunction associated with smoking.

In summary, our study demonstrates that CSE stimulates ET-1 gene expression via PKC in PAECs. Platelets and CSE showed synergism in the stimulation of ET-1 gene expression, possibly through the activation of platelets by CSE. Further studies are needed to find the components of CSE that are responsible for this stimulating effect and for the mechanisms of synergism between platelets and CSE in the stimulation of ET-1 gene expression.

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