Cigarette smoke condensate-induced adhesion molecule expression and transendothelial migration of monocytes

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Shen, Yamin, Vinod Rattan, Chand Sultana, and Vijay K. Kalra. Cigarette smoke condensate-induced adhesion molecule expression and transendothelial migration of monocytes. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H1624–H1633, 1996.—Cigarette smoking is clearly linked with increased incidence of atherosclerosis and cardiovascular disease. The adherence of blood monocytes to the endothelium, followed by their migration beneath the endothelium, are initiating events in the formation of foam cells, promoting atherogenesis. We show that cigarette smoke condensate (CSC)-induced surface expression of a subset of cell adhesion molecules (CAM) [intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule 1 (VCAM-1)] in human umbilical vein endothelial cells (HUVEC) is associated with an increase in the binding activity of nuclear transcription factor NF-κB to the consensus motif common to the CAM genes. Furthermore, CSC (25 μg/ml) both increases the rate of transendothelial migration of vitamin D_3-differentiated monocyte-like cells across the HUVEC monolayer by 200% and causes an approximately 10-fold increase in the phosphorylation of platelet endothelial CAM (PECAM-1), an adhesion molecule located at intercellular junctions and involved in endothelial cell-cell adhesion. Our results show that CSC-induced activation of protein kinase C in endothelial cells initiates a signaling pathway, leading to heightened binding of NF-κB to specific DNA sequences, which in turn increases surface expression of the subset of CAMs. Furthermore, our studies demonstrate a link between the phosphorylation of PECAM-1 and the migration of blood monocytes across vascular endothelium.

endothelial cells; cell adhesion molecules; platelet endothelial cell adhesion molecule-1; phosphorylation

EPIDEMIOLOGICAL STUDIES have established that cigarette smoking is a major risk factor associated with accelerated atherosclerosis and coronary artery disease (18). The adherence of circulating monocytes to the endothelium, transmigration into the subendothelium, and subsequent formation of foam cells are the principal initial events in the pathogenesis of atherosclerosis (25). Although cigarette smoke can damage various tissues and organs, one feature that is common to the cigarette smoke-mediated diseases is the presence of increased numbers of adherent leukocytes in the vascular endothelium (15), along with leukocyte-mediated tissue damage (29) and endothelial injury (22). However, relatively little information is available regarding the cellular mechanisms by which cigarette smoke initiates atherosclerosis.

Because cigarette smoke is known to contain >4,000 constituents [92% gaseous components, 8% particulate (9)], it is difficult to predict which components individually, or in combination, are potential stimuli for atherogenesis. A second problem is discovering which components are able to pass through lung tissue into the circulation to effect changes at the site of atherosclerosis, i.e., the vasculature. Although relatively little is known about how particulate-phase components of cigarette smoke (polycyclic aromatic hydrocarbons, nitrosamines, and phenolic compounds) enter the circulation, recent studies (26) have shown that components of cigarette smoke condensate (CSC), e.g., polycyclic aromatic hydrocarbons, are absorbed from the lungs into the circulation. Thus we chose to investigate the effect of total CSC, i.e., the nongaseous phase of cigarette smoke, rather than individual components. We have studied the effects of CSC, as one can easily obtain the preparation from commercial sources. The condensate is produced from standard 2R1 cigarettes by the use of an automatic smoking machine (13), thus allowing reproducible data on the mix of components present in cigarette smoke, i.e., CSC.

Cigarette smoke has been shown in vivo to be a cause of increased adherence of leukocytes to vascular endothelium (16). Because the initial events in the process of atherogenesis involve a cascade of adhesive interactions between monocyte and vascular endothelium, followed by the migration of monocytes across the endothelial cell monolayer, we examined the molecular mechanism by which CSC is involved. In our previous study (13) we showed that CSC causes augmented adherence of monocytes to cultured endothelial cells by means of an upregulation of both the CD11b ligand on monocytes and counterreceptors [cell adhesion molecules (CAM)] intercellular adhesion molecule 1 (ICAM-1) and endothelial leukocyte adhesion molecule 1 (ELAM-1) on endothelial cells.

We show that CSC causes an increase in the surface expression of a subset of CAMs [ICAM-1, ELAM-1, and vascular cell adhesion molecule 1 (VCAM-1)] and, additionally, an increase in the binding activity of transcription factor NF-κB in endothelial cell nuclear extracts. The activation of NF-κB binding to consensus sites in the regulatory regions of genes encoding CAMs (4, 10, 30) contributes to increased surface expression of ICAM-1, ELAM-1, and VCAM-1 in response to CSC. Additionally, we present evidence that CSC accelerates the transmigration of monocyte-like cells across the endothelial cell monolayer by 1.8- to 2-fold. Previous studies (1, 12, 20) have established that platelet endothelial cell adhesion molecule 1 (PECAM-1), concentrated at intercellular junctions of endothelial cells, is involved in cell-cell adhesion. Furthermore, PECAM-1, in both in vitro and in vivo studies, appears to be directly involved in mediating the transmigration of monocytes and neutrophils across the endothelial cell monolayer (19, 28). We show that CSC causes a several-fold increase in the phosphorylation of PECAM-1 as a result of activation of protein kinase C (PKC) in endothelial cells. Furthermore, CSC-mediated transendothelial migration of monocytes is inhibited 60 ± 8% by an antibody to PECAM-1 and 80 ± 10% by an inhibitor of PKC, indicating that PECAM-1 phosphorylation may affect cellular adhesiveness between endothelial cells, thus facilitating the migration of monocytes and/or leukocytes across the endothelial cell monolayer.

METHODS

Cell cultures. Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cord veins by collagenase digestion, as previously described (13). These cells exhibited cobblestone morphology, binding of factor VIII-related antigen, and uptake of acetylated low-density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Biomedical Technologies, Stoughton, MA). HUVEC passaged from two to six times were used. Bovine aortic endothelial cells (BAEC) were derived from the aorta of 2- to 3-day-old calves (Shamrock Meats, Vernon, CA) as previously described (11). The promyelocytic cell line HL-60 (American Type Culture Collection, Rockville, MD) was cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS). The HL-60 cell line (5×10^5 cells) was terminally differentiated to monocyte-like cells by 3–4 days of culture in the presence of 1×10^{-7} M 1α,25-dihydroxyvitamin D_3 (Biomol Research Laboratories, Plymouth Meeting, PA) as described (14).

Enzyme-linked immunosorbent assay for surface expression of CAMs. HUVEC were grown to confluence in 24-well plates, rinsed with phosphate-buffered saline (PBS), and incubated for different amounts of time with fresh RPMI-1640 medium containing 10% FCS (1 ml) in the presence and absence of 25 μg/ml CSC at 37°C. At the end of the incubation period (15 min to 8 h) the medium was aspirated, and the wells were washed twice with PBS. Paraformaldehyde (2.5%, 500 μl) was added to each well to fix the cells. The cell surface expression of ICAM-1, ELAM-1, VCAM-1, and P-selectin was assayed by incubation with monoclonal antibodies to ICAM-1, ELAM-1, VCAM-1 (AMAC, Westbrook, MN), and P-selectin (Chemicon, Temecula, CA). Incubation was carried out for 120 min at room temperature at saturating conditions of antibody (1:500 dilution in 0.5 ml PBS). Cells were washed, then incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Sigma Chemical, St. Louis, MO) diluted 1:1,000 in PBS for 1 h at room temperature, and then washed three times with 1 ml of PBS. Binding of the secondary antibody was determined by the addition of 200 μl of *p*-nitrophenyl phosphate substrate (Sigma). The 24-well plate was incubated in the dark for 30 min, and the reaction was terminated by the addition of 50 μl of 3 N NaOH. Absorbance was read at 405 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (model 7520, Cambridge Technology, Watertown, MA) interfaced with an IBM PC. The surface expression of CAMs is shown as mean ± SD of the optical density after subtracting the blank value, which is the optical density in the absence of primary antibody.

Measurement of PKC activity. Fresh RPMI-1640 medium was added to either HUVEC or BAEC grown to confluence (100-mm dish; 2–3 × 10^6 cells) before the addition of CSC. Cells were kept in a tissue culture incubator in a 5% CO_2 atmosphere at 37°C for various lengths of time. The cells were then washed twice with ice-cold Ca^{+2}- and Mg^{+2}-free PBS. To each plate, 3 ml of *buffer A* [20 mM tris(hydroxymethyl)aminomethane (Tris)·HCl (pH 7.4), 1 mM EDTA, 0.1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, and 300 nM pepstatin A] were added. Cells were then collected by scraping and homogenized by 25 strokes in a glass Dounce homogenizer. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant (cytosolic) and particulate (membrane) fractions were assayed for PKC activity as described (6). Briefly, the membrane fraction was suspended in 3 ml of *buffer B* [*buffer A* with 1% Nonidet P-40 (NP-40)], incubated for 30 min at 4°C followed by homogenization in a Dounce homogenizer, and centrifuged a second time at 10,000 *g* for 20 min. The cytosolic and detergent-solubilized membrane fractions were applied to a 0.5-ml (diethylamino)ethyl ether (DE-52, Whatman Laboratory, Maidstone, UK) cellulose column previously equilibrated with *buffer A*. The column was washed with 2 ml of *buffer A* followed by elution with 1 ml of *buffer A* containing 0.1 M NaCl. The 1-ml fractions were divided in half: 0.5 ml was used for estimation of PKC activity [50 μg of bovine serum albumin (BSA) was added; samples were stored at −80°C for ≤1 wk], and total protein was measured in the remaining 0.5-ml aliquot by the method of Lowry et al. (17).

PKC activity was assayed in eluted fractions of both cytosolic and detergent-solubilized membrane fraction by determining the incorporation of ^{32}P from [γ-^{32}P]ATP into histone IIIS (Sigma). The reaction mixture (125 μl) consisted of (in mM) 50 Tris·HCl (pH 7.5), 25 $MgCl_2$, 0.8 $CaCl_2$, and 0.25 [γ-^{32}P] ATP (containing ~120 cpm/pmol ATP), and 12.5 μg histone IIIS, 0.1 μM leupeptin, 25 μl of sample, 0.7 μCi/50 μl of [^{32}P]ATP, and either 50 μl of sonicated lipid mixture (100 μg/ml of L-α-phosphatidyl-L-serine, and 10 μg/ml of diolein) or 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pH 7.5, as described (6). After a 5-min incubation at room temperature, the reaction was terminated by the addition of 75 μl ice-cold 70% trichloroacetic acid (TCA). The acid-precipitable material was collected on a 0.45-μm membrane filter (Millipore/Continental Water Systems, Bradford, MA). Filters were washed three times with 0.5 ml of ice-cold 25% TCA. The filters were dried, and radioactivity was counted in a Beckman LS-8000 scintillation counter. PKC activity was calculated by subtracting the basal activity in the presence of EGTA from the calcium- and phospholipid-stimulated activity.

^{32}P labeling and immunoprecipitation of PECAM-1. HUVEC or BAEC grown to confluence in 100 × 15 mm tissue culture dishes were washed with PO_4-free RPMI-1640 (GIBCO BRL/Life Technologies, Grand Island, NY) and radiolabeled in 3 ml of PO_4-free medium with 0.25 mCi of ^{32}Pi (carrier free; ICN Biomedicals, Irvine, CA) for 4 h at 37°C. The monolayer of radiolabeled BAEC or HUVEC was washed with PO_4-free medium and incubated in 2 ml of the same medium with CSC (25 μg/ml) for 5, 15, 30, and 60 min. The medium was then aspirated, and 2 ml of fresh PO_4-free medium were added. Cells were scraped and centrifuged. The cell pellets were washed three times with PO_4-free medium and then suspended in 1 ml of lysis buffer [50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium orthovanadate, 10 mM EDTA, 50 mM sodium fluoride, 100 μg/ml PMSF, 1 μg/ml pepstatin, and 1 μg/ml leupeptin] and kept at 4°C for 15 min. The lysate was centrifuged at 3,000 *g* for 10 min. A 4% (5 μl; wt/vol) slurry of protein A agarose (Sigma) was added to the

supernatant. To the precleared supernatant, 5 μl of monoclonal antibody to bovine PECAM-1, (XVD_2, purified ascites fluid; Ref. 11) was added followed by a 60-min incubation at 4°C. Protein A agarose (20 μl of 4% slurry) was then added, and the mixture was incubated for an additional 60 min. The immunocomplex was collected and washed three times with 300 μl of lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). The pellet was solubilized in electrophoresis sample buffer (50 mM Tris·HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.1% bromphenol blue) by boiling in a water bath for 5 min. Samples were then electrophoresed on 10% SDS-polyacrylamide gels. The gels were dried, exposed to Kodak XR-5 X-ray film at −80°C, and developed. The radioactivity incorporated in the 130-kDa PECAM-1 gel band was estimated by scanning with Ambis Radioanalytic Imaging Systems (San Diego, CA).

Preparation of nuclear extracts. Confluent HUVECs (6 × 10^6 cells) were incubated at 37°C in the presence and absence of CSC (25–30 μg/ml) for times ranging from 15 to 20 min. At the end of the incubation period, the medium was aspirated, and cells were washed twice with 3 ml ice-cold PBS. Cells were scraped in 1 ml PBS then centrifuged, and nuclear extracts were prepared as described (2). Briefly, cells were suspended in 400 μl of *buffer C* [10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 0.3 μg/ml leupeptin, and 0.7 μg/ml pepstatin] and incubated on ice for 10 min. Cells were resuspended in 100 μl of *buffer D* (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM Na-EDTA, 1 mM DTT, 0.5 mM PMSF, 0.3 μg/ml leupeptin, and 0.7 μg/ml pepstatin) after pelleting. After a 20-min incubation at 4°C, the homogenate was centrifuged at 8,000 *g* for 5 min. The supernatant, containing nuclear proteins, was transferred to a microfuge tube and stored at −80°C. Protein concentration was measured by the Lowry method (17). The nuclear extract was used for assay of NF-κB activity within 10 days of storage.

Electrophoretic mobility shift assay for transcription factor NF-κB. A double-stranded oligonucleotide containing a tandem repeat of NF-κB DNA-binding consensus sequence -GGGACTTTCC- was obtained (GIBCO/BRL-Life Technologies, Gaithersburg, MD). The sequence was as follows

5′-GATCCAAGGGGACTTTCCATGGATCCAAGGGGACTTTCCATG-3′

3′-GTTCCCCTGAAAGGTACCTAGGTTCCCCTGAAAGGTACCTAG-5′

The double-stranded oligonucleotide (5 ng) was end labeled with 100 μCi [γ-^{32}P]ATP (ICN Biomedicals, Irvine, CA), using T4 polynucleotide kinase as suggested in the manufacturer's kit (GIBCO/BRL-Life Technologies). The labeled oligonucleotide probe was purified by passing over a Sephadex G-50 spin column. A DNA-binding reaction mixture containing 3–5 μg nuclear extract, 10 mM Tris (pH 7.6), 50 mM NaCl, 1 mM DTT, 0.02 μM ATP, 5 μg BSA, and 10% (vol/vol) glycerol in a total volume of 25 μl was incubated in the presence and absence of excess unlabeled oligonucleotide. The mixture was preincubated on ice for 15 min, followed by the addition of ^{32}P-labeled probe (1 × 10^5 counts/min), and the binding reaction was allowed to proceed for 20 min at room temperature. Then 2 μl of 0.1% bromphenol blue dye was added to each sample. The samples were subjected to electrophoresis on 6% nondenaturing polyacrylamide gel, using 0.25× TBE running buffer (25 mM Tris, pH 8.0, 22.5 mM borate, and 0.025 mM EDTA) at 150 V for 2–3 h. The gels were dried and exposed to X-ray film (Kodak X-Omat AR). After development, films were scanned for absorbance at 630 nm, using an LKB Ultrascan XL laser densitometer.

Assay for transendothelial migration of monocytes. HUVEC monolayers were grown to confluence on fibronectin-coated porous membranes (Biocoat cell culture inserts, 3.0 μm; Collaborative Biomedical Products, Bedford, MA) for 5–6 days. The transendothelial resistance was measured at room temperature on these filters, using an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL). Daily resistance measurements were made with the electrode after alcohol sterilization. Calculations for ohms per square centimeter were made by subtracting a blank filter and multiplying by the monolayer area. At *days 5–6* endothelial cells exhibited maximal resistance and were used at that time for migration studies. The confluent monolayer of HUVEC was incubated in duplicate with aliquots (1 ×10^5 cells/well) of vitamin D_3-differentiated HL-60 cells in the presence and absence of CSC (25 μg/ml) for time periods ranging from 30 min to 8 h at 37°C. The lower compartment of the Transwell chamber contained 1 ml of RPMI-1640 medium with 20% FCS, whereas the upper compartment contained 0.5 ml of the same medium containing HL-60 cells. At the indicated time period, a 50-μl aliquot was removed from the bottom compartment of the well and stained with 0.2% trypan blue for counting of transmigrated monocyte cells, using a hemocytometer grid (Olympus 1MT-2 microscope). To keep the volume constant in the lower compartment, an equal amount (50 μl) of medium was added at removal of monocytes. In inhibition experiments, 100 μl of either PECAM-1 monoclonal antibody (XVD_2; antibody to bovine counterpart of PECAM-1; 1:500 dilution of purified ascites fluid) or the PKC inhibitor bisindoylmaleimide (GF 109203X; 20 nM; Calbiochem, La Jolla, CA) were added 45 min before the addition of CSC to the upper compartment of HUVEC cells.

CSC and chemicals. The whole CSC derived from standard 2R1 nonfilter cigarettes was obtained from the Tobacco and Health Research Institute, Lexington, Kentucky. The whole CSC is obtained by the puffing of cigarettes by an automatic smoking machine, with a yield, according to the manufacturer, of ~26 mg/cigarette (13). The CSC contains 1.32 mg of nicotine per cigarette. The CSC was kept at −20°C and used within 30 days. Fresh CSC stock solution (12.5 mg/ml) was prepared by dissolving CSC in absolute ethanol. The corresponding amount of ethanol was used as a control in all experiments. The amount of CSC reagent (10–60 μg/ml) used in all experiments was tested for the presence of endotoxin by the *Limulus* amebocyte lysate assay (E-Toxate kit, Sigma). The CSC reagent was free of endotoxin at a detection limit of 0.0015 EU/ml (13). All other chemicals, unless indicated, were obtained from Sigma.

Statistical analysis. For experiments run at one time period, the controls and experimental samples were analyzed with one-way analysis of variance (ANOVA) and Student's *t*-test, using an Instat software program (GraphPad, San Diego, CA). Values were considered significant if *P* values were <0.05.

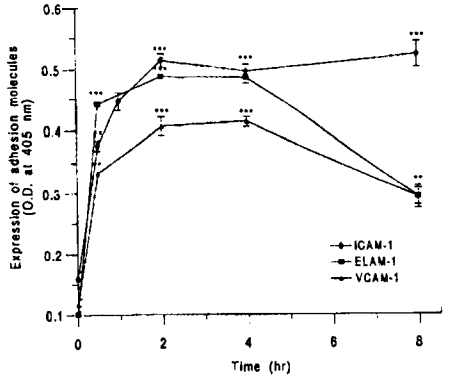

Fig. 1. Time course of cell adhesion molecule (CAM) expression in human umbilical vein endothelial cells (HUVEC) in response to treatment with cigarette smoke condensate (CSC). HUVEC were incubated with CSC (25 μg/ml) for indicated times. Cells were processed for intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), and vascular cell adhesion molecule 1 (VCAM-1) expression by enzyme-linked immunosorbent assay (ELISA), utilizing corresponding antibodies as described in METHODS. Data are presented as means ± SD of measurements of samples run in triplicate (n = 3). O.D., optical density. *** Significant difference between untreated time (0 min) and CSC treatment (P < 0.001); ** P < 0.01.

RESULTS

Effect of CSC on surface expression of adhesion molecules in cultured endothelial cells. Previous studies (13) indicate that a concentration of 25–30 μg/ml of CSC is not toxic for BAEC or HUVEC and is optimal for the adherence of monocytes to cultured endothelial cells and the surface expression of CD11b molecule on monocytes. Thus this concentration of CSC was used in our studies. Treatment of HUVEC with CSC (25 μg/ml) resulted in a time-dependent increase in the surface expression of ICAM-1, ELAM-1, and VCAM-1 (Fig. 1) but had no effect on the expression of P-selectin (data not shown), as determined by ELISA assay. However, nicotine, also present in CSC, at a concentration of 10–25 μg/ml did not increase the surface expression of any of these CAMs (data not shown). As shown in Fig. 2, PKC inhibitor GF 109203X (27) reduced surface expression of ICAM-1 by 92 ± 4%, ELAM-1 by 93 ± 4%, and VCAM-1 by 95 ± 6% (P < 0.01). Another PKC inhibitor, chelerythrine, also reduced CAM expression in the range of 70–80% (data not shown). The addition of the protein synthesis inhibitor cycloheximide reduced CSC-induced surface expression of ICAM-1 by 90 ± 6%, ELAM-1 by 86 ± 7%, and VCAM-1 by 93 ± 3% (P < 0.01). As shown in Fig. 2, the addition of actinomycin D, a transcription inhibitor, reduced CSC-induced surface expression of ICAM-1 by 95 ± 7%, ELAM-1 by 84 ± 9%, and VCAM-1 by 87 ± 7% (P < 0.01).

CSC-induced activation of NF-κB binding activity in HUVEC. The transcription factor NF-κB is known to induce transcription of a variety of cellular genes by binding to specific sequence motifs in their upstream regulatory elements (7). The NF-κB consensus binding sequence is present in the upstream regulatory region of the genes for ICAM-1, ELAM-1, and VCAM-1 (4, 10, 30, 31). NF-κB is constitutively expressed in the cytoplasm, bound to the inhibitor protein IκB. This is activated on phosphorylation of IκB, which disrupts the binding; NF-κB then translocates to the nucleus (7). The binding of NF-κB to specific DNA sequences and its interaction with other DNA-binding proteins affects transcription. We measured the level of NF-κB binding activity in nuclear extracts of HUVEC cells before and after treatment with CSC. Untreated HUVEC exhibited low NF-κB activity (Fig. 3). Treatment of HUVEC with 25 μg/ml CSC resulted in a time-dependent increase over a 15- to 60-min time range in NF-κB

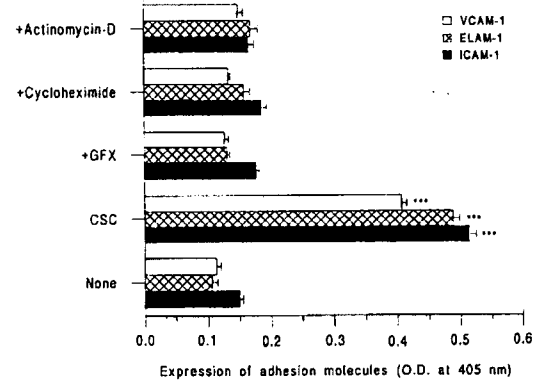

Fig. 2. Effect of inhibitors on CAM expression. HUVEC confluent monolayers in 24-well plates were preincubated with inhibitors for 30 min, where indicated, before treatment with CSC. CAM expression was assayed by ELISA by measuring absorbance at 405 nm. CSC, 25 μg/ml; GF 109203X [bisindoylmaleimide (GFX)], 20 nM; cycloheximide 10 μg/ml; and actinomycin D, 5 μg/ml. Data are presented as means ± SD (n = 3, in triplicate). *** Significant difference between CSC-treated and untreated HUVEC (none) and HUVEC incubated with indicated inhibitors (P < 0.001).

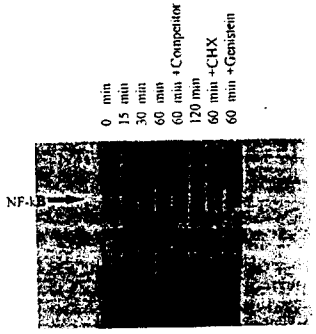

Fig. 3. Effect of CSC and inhibitors on NF-κB binding activity in HUVEC nuclear extracts by gel shift assay. HUVEC were treated with CSC (25 μg/ml) for indicated time period (15–120 min) in the presence and absence of inhibitors [genistein, 25 μg/ml; cycloheximide (CHX), 10 μg/ml]. Nuclear extracts were prepared and incubated with NF-κB oligonucleotide-labeled probe as described in METHODS. Where indicated, 100-fold excess cold competing oligonucleotide was added to nuclear extracts and incubated for 10 min before addition of radiolabeled DNA probe. Data are representative of 3 independent experiments.

activity (Fig. 3). The effect was optimal at 60 min, as demonstrated by the decrease in NF-κB activity observed after a 120-min incubation. Addition of excess competitor oligonucleotide reduced >90% of the NF-κB activity, indicating specific incorporation in NF-κB band. Treatment of HUVEC with CSC for 60 min in the presence of protein synthesis inhibitor cycloheximide (10 μg/ml) did not affect NF-κB binding activity. However, the addition of genistein (25 μg/ml), an inhibitor of protein tyrosine kinase (23), to the CSC incubation mixture reduced NF-κB activity (Fig. 3), indicating the significance of tyrosine phosphorylation in the activation of NF-κB.

Phosphorylation of PECAM-1 in CSC-treated endothelial cells. Because PECAM-1, in platelets, has been shown to undergo phosphorylation that is augmented severalfold by thrombin (21), experiments were performed to determine whether the cell-cell adhesion molecule, PECAM-1, in endothelial cells was phosphorylated by CSC. As shown in Fig. 4 (*top*), PECAM-1 exhibited phosphorylation in its basal state. Treatment with CSC for 30 min to 4 h resulted in a time-dependent increase in PECAM-1 phosphorylation. There was an approximately 10-fold increase in ^{32}P incorporation into PECAM-1 at 2 h. Similarly, in BAEC, CSC caused a threefold increase in the phosphorylation of bovine PECAM-1 (Fig. 4, *bottom*), although the kinetics of phosphorylation were slightly different from those of PECAM-1 in HUVEC. The optimal phosphorylation of PECAM-1 in BAEC occurred after incubation for 30 min (Fig. 4, *bottom*).

Effect of inhibitors on phosphorylation of PECAM-1. Because serine/threonine residues in the cytoplasmic tail of PECAM-1 have been shown to undergo phosphorylation in platelets (21), we studied the effect of PKC inhibitors on the CSC-mediated phosphorylation of PECAM-1 in BAEC. As shown in Fig 5, GF 109203X, a selective PKC inhibitor, (27) reduced the CSC-mediated incorporation of ^{32}P into bovine PECAM-1 by 60 ± 5%. As shown in Fig. 5, other PKC inhibitors (staurosporine and chelerythrine at concentrations of 100 nM and 0.66 μM, respectively) inhibited CSC-mediated phosphorylation of PECAM-1 in BAEC by >90%. Similarly,

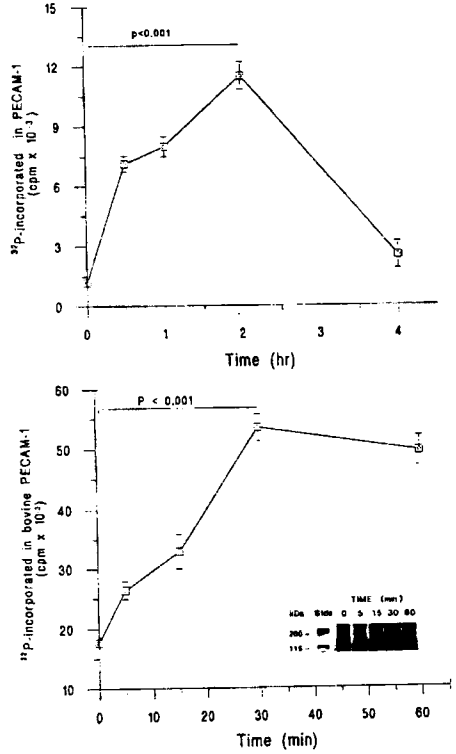

Fig. 4. Effect of CSC on phosphorylation of PECAM-1 in HUVEC (*top*) and bovine aortic endothelial cells (BAEC; *bottom*). Confluent HUVEC or BAEC (equal no. of cells) were washed with phosphate-free RPMI-1640 medium, labeled with orthophosphate ^{32}P for 4 h, and incubated with CSC (25 μg/ml) for indicated time periods. *Inset*: cells were washed, lysed, and immunoprecipitated with antibody to PECAM-1 followed by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Quantitation of counts in protein band of PECAM-1 in SDS gel was performed on an Ambis Radioanalytic Imaging Systems gel scanner. Results are expressed as means ± SD of triplicates and are representative of 3 independent experiments. Stds, standards (molecular weight markers). $P < 0.001$ indicates significant difference between untreated and CSC-treated at 2 h in HUVEC and at 30 min in BAEC.

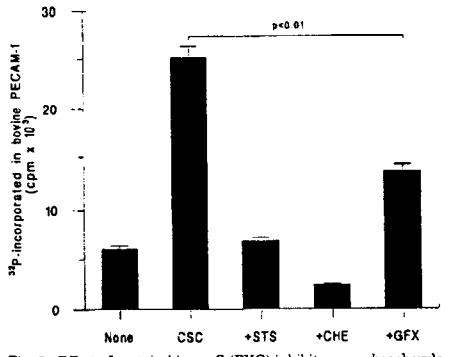

Fig. 5. Effect of protein kinase C (PKC) inhibitors on phosphorylation of bovine PECAM-1 in BAEC. BAEC (equal no. of cells) were labeled with ^{32}P for 4 h, washed, and preincubated, where indicated, with inhibitors [staurosporine (STS, 100 nM), chelerythrine (CHE, 0.66 μM), or GFX; 20 nM] for 10 min before treatment with CSC (25 μg/ml) for 30 min. Cells were washed, lysed, and immunoprecipitated with antibody to bovine PECAM-1 and processed for SDS-PAGE. Radioactivity in SDS-PAGE in lane corresponding to PECAM-1 was counted by an Ambis Radioanalytic Imaging Systems gel scanner. Results are expressed as means ± SD of triplicates and represent 1 of 4 independent experiments with similar results.

in HUVEC, GF 109203X reduced CSC-mediated phosphorylation of PECAM-1 by 50 ± 6% (n = 3; data not shown).

Effect of CSC on PKC activity in BAEC and HUVEC. Because PKC inhibitors inhibited the phosphorylation of PECAM-1 seen in response to CSC treatment of HUVEC and BAEC, we next examined the direct effect of CSC on PKC activation. As shown in Fig. 6 (*left*), treatment of BAEC with 25 μg/ml CSC resulted in an increase in membrane-associated PKC activity, with a modest decrease in cytosolic PKC activity. The effect of CSC on PKC activity was time dependent: PKC activity

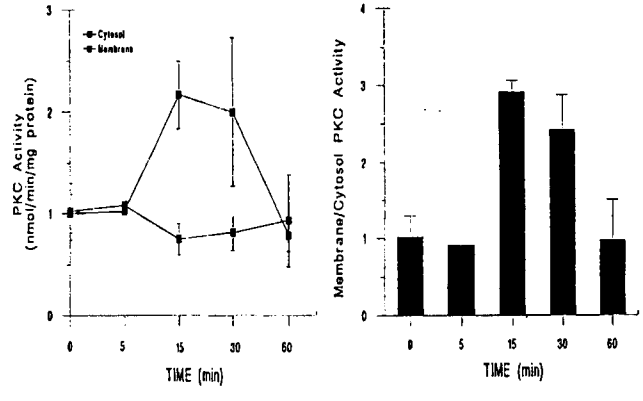

Fig. 6. Effect of CSC on time course of activation of PKC. BAEC were treated with CSC (25 μg/ml) for time period (5, 15, 30, and 60 min) at 37°C. Cells were washed, homogenized, and assayed for PKC activity in the cytosol and membrane (*left*) and ratio of membrane to cytosol activity (*right*). Data represent means ± SD of 5 independent experiments.

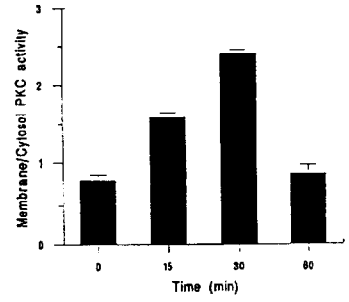

Fig. 7. Effect of CSC on activation of PKC in HUVEC. HUVEC (3 × 10^6 cells) were treated with CSC for indicated times and processed for estimation of PKC activity as described in Fig. 6. Data are means ± SD of experiment run in triplicate.

in the membrane pool increased by twofold at 30 min with a concomitant 25% decrease in the PKC activity in the cytosol, whereas PKC activity in the membrane after a 60-min CSC treatment was similar to basal level. As illustrated in Fig. 6 (*right*), the ratio of membrane-associated to cytosolic PKC activity at 15 min of treatment with CSC was ~2.5 times that of untreated BAEC. Similarly, CSC treatment of HUVEC resulted in a twofold increase in membrane-associated PKC activity at 30 min, with a concomitant decrease of 35–40% in cytosolic PKC activity. Figure 7 shows the increase in the ratio of membrane-associated to cytosolic activity at 30 min after treatment of HUVEC with CSC. Treatment with nicotine (10 μg/ml), a concentration similar to that present in cigarette smoke (13), caused a modest (30%) increase in membrane-associated PKC activity. In BAEC, the ratio of membrane-associated to cytosolic PKC activity was ~2.5 for 25 μg/ml CSC, 3.9 for 100 nM 12-*O*-tetradecanolyphorbol 13-acetate (TPA), and 1.4 for 10 μg/ml nicotine (Fig. 8).

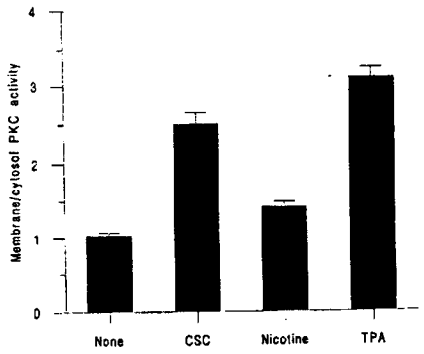

Fig. 8. Effect of CSC, nicotine, and phorbol ester (TPA) on activation of PKC in BAEC. Confluent BAEC (3×10^6 cells) in fresh RPMI-1640 medium were treated with CSC (25 μg/ml), nicotine (10 μg/ml), and TPA (10^{-7} M) for 30 min as described in METHODS. Data are expressed as ratio obtained by dividing PKC activity in the membrane by cytosolic activity. Data represent means ± SD of duplicates of 2 independent experiments.

Effect of CSC on transendothelial migration of monocytes. It has been suggested that the adhesion of monocytes to endothelium, followed by their transendothelial migration, initiates atherosclerotic lesion development. The possibility that CSC effects on monocyte-endothelium adhesion might also influence monocyte migration across the endothelial cell monolayer was investigated. As shown in Fig. 9, addition of CSC to confluent monolayers of HUVEC cultured in Transwell chambers resulted in stimulation of migration of vitamin D_3-differentiated HL-60 cells. We observed a 150–200% increase in the number of monocytes migrating across the endothelial cell monolayer at 4 h after treatment with CSC. Because PECAM-1 is localized at endothelial cell-cell junctions and thought to mediate the transendothelial migration of leukocytes (19, 28), we investigated the effect of PECAM-1 antibody. We observed that a PECAM-1 antibody from BAEC (11), which cross-reacts with PECAM-1 in HUVEC, inhibited (60 ± 8%) the transmigration of vitamin D_3-differentiated HL-60 cells (Fig. 10). Moreover, the addition of the PKC inhibitor GF 109203X before treatment with 25 μg/ml CSC reduced (80 ± 10%; $n = 3$) the transendothelial migration of monocytes. It is pertinent to mention that treatment of confluent monolayers of HUVEC with CSC for 2 h reduced the transendothelial electrical resistance from ~60 to <30 Ω/cm².

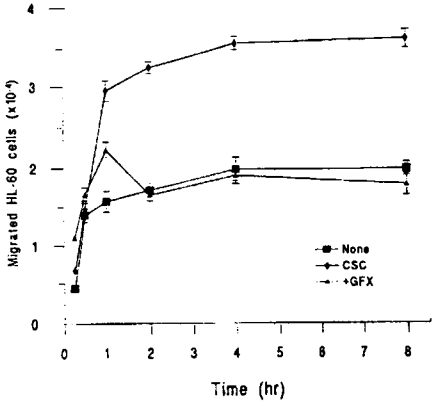

Fig. 9. Time course of transmigration of HL-60 vitamin D_3-differentiated monocytes across HUVEC monolayer. HUVEC were grown to confluency on fibronectin-coated porous membranes (Transwell biocoat cell culture inserts, Becton-Dickinson) and incubated with vitamin D_3-differentiated HL-60 cells (1×10^6 cells/well) in presence and absence of CSC (25 μg/ml) for indicated times. PKC inhibitor GFX (20 nM) was added 45 min before addition of CSC. Transmigrated monocytes in lower compartment of Transwell chamber were recovered and counted in a hemocytometer. Data are expressed as means ± SD of 3 independent experiments.

DISCUSSION

The adhesion of circulating blood monocytes to vessel walls and the subsequent infiltration of monocytes just beneath the endothelium, where they form foam cells, are thought to be important early events in the formation of fatty-streak lesions in the pathogenesis of atherosclerosis (25). This hypothesis is supported by the observation (5) that shortly after feeding animals an atherogenic diet, an increase occurs in the rate of adherence of circulating monocytes to the arterial endothelium followed by entry into the subendothelium space, where they accumulate cholesteryl ester and form foam cells. Because cigarette smoking is known to

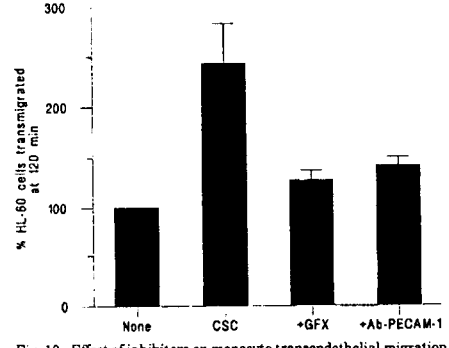

Fig. 10. Effect of inhibitors on monocyte transendothelial migration. HUVEC grown to confluency on Transwell chambers were incubated with GFX (20 nM) or an antibody to bovine PECAM-1 for 45 min before addition of CSC and vitamin D_3-differentiated HL-60 cells as described in METHODS. Transmigrated monocyte cells at 2-h time period were counted. Data are expressed as means ± SD of 3 independent experiments.

increase the incidence of atherosclerosis (18), we examined the mechanism by which cigarette smoke might cause such a phenomenon. Because of the presence of >4,000 constituents in cigarette smoke, in both the gaseous and the particulate phase, and the difficulty of predicting which component or combination of components could promote atherogenesis, we chose to examine the global effects of CSC, the nongaseous phase of cigarette smoke.

In our previous study (13) we showed that CSC augments the adherence of human monocytes to cultured endothelial cells as a result of increased expression of CD11b ligand on monocytes and the counter receptors ICAM-1 and ELAM-1 on endothelial cells. In the present study we show that CSC, in HUVEC at a concentration of 25 μg/ml (shown previously to be optimal for the adherence of monocytes), selectively induces the surface expression of ICAM-1, ELAM-1, and VCAM-1 but not P-selectin. CSC-induced surface expression of these subsets of CAMs in HUVEC is reduced by an inhibitor of PKC, indicating that PKC activation is involved in CSC-mediated surface expression of CAMs. These results are supported by the direct measurement of PKC activity in CSC-treated endothelial cells. Our studies show that CSC causes an approximately twofold increase in membrane-associated PKC activity in HUVEC and BAEC; such activation is known to involve translocation of cytosolic PKC to the membrane (6).

The CSC-induced surface expression of CAMs is inhibited by the protein synthesis inhibitor cycloheximide, indicating that CSC induces new protein synthesis. To ascertain how CSC causes increased biosynthesis and surface expression of CAMs, we investigated the effect of CSC on the binding activity of transcription factor NF-κB in HUVEC nuclear extracts. NF-κB, constitutively expressed in the cell cytosol, induces the transcription of a number of cellular genes (interleukin-2, interleukin-2 receptor, β-interferon, TNF-α and -β, granulocyte-macrophage colony-stimulating factor, immunoglobulin κ light chain, ICAM-1, ELAM-1, and VCAM-1) by binding to specific sequence motifs in the upstream regulatory elements of these genes (4, 7, 10, 30). NF-κB, inactive in the cytosol because it is bound to the inhibitory protein IκB, is activated on IκB phosphorylation, whereupon NF-κB is released from IκB and moves to the nucleus (7). Our studies show that CSC causes a time-dependent increase in NF-κB binding activity in HUVEC nuclear extracts, with optimal NF-κB binding activity observed at 60 min. Furthermore, the presence of cycloheximide during treatment with CSC did not reduce NF-κB activity, indicating that new synthesis of NF-κB is not required. However, CSC-induced NF-κB binding activity was significantly inhibited in the presence of the protein tyrosine kinase inhibitor genistein, indicating the importance of some tyrosine-phosphorylated substrate(s) in this activation, as has been observed for the TNF-activated NF-κB activation (23). The type of kinase involved in the CSC-induced activation of NF-κB remains to be determined. Recent studies (31) have shown that agents such as lipopolysaccharide, TNF-α, and IL-1β induce the synthesis of ICAM-1, ELAM-1, and VCAM-1 in Kaposi's sarcoma cells (KS) and also show increased NF-κB binding activity in the nuclear extracts from KS cells. However, there is no correlation between the levels of CAM mRNA expression and the induction of NF-κB binding activity in KS nuclear extracts, indicating that NF-κB activation is unlikely to be the only mechanism contributing to the increased expression of CAMs in response to these agents (31). We are presently investigating the possible correlation between the levels of CAM mRNA expression and the induction of NF-KB binding activity in CSC-treated HUVEC.

In the present study we used vitamin D_3-differentiated HL-60 cell lines, which were established as a reliable model for in vivo monocyte function and transendothelial migration studies (8, 14), to investigate the transendothelial migration of monocytes. We found that CSC causes a twofold increase in the transendothelial migration of differentiated HL-60 monocytes across the endothelial cell monolayer. Because previous studies have shown both in vitro and in vivo that PECAM-1, localized at endothelial intercellular junctions and involved in endothelial cell-cell adhesion, is involved in mediating the transendothelial migration of monocytes and neutrophils (19, 28), we added antibody to PECAM-1 to our monocyte migration experiment. Addition of antibody to bovine PECAM-1 reduced CSC-induced transendothelial migration of monocytes mediated by CSC by ~70%. These results support the participation of PECAM-1 in the transendothelial migration of monocytes mediated by CSC. However, we did not observe complete (100%) inhibition by addition of the antibody. It is possible that the antibody was not fully accessible to all PECAM-1 binding sites in the intercellular junctions of HUVEC or that other adhesion molecule(s), such as cadherins and integrins, known to be involved in endothelial intercellular adhesion (3) may also participate in this process.

Finally, our studies have uncovered a link between PECAM-1 phosphorylation and the transendothelial migration of monocytes. Previous studies have shown that the adhesion molecule PECAM-1, a member of the immunoglobulin supergene family, is a transmembrane protein of molecular mass 130 kDa (11, 20). The cytoplasmic tail of the PECAM-1 molecule contains 118 amino acid residues, of which 5 are tyrosine, 5 are threonine, and 12 are serine residues (20, 21), and all are potential targets for phosphorylation in response to CSC. Our studies show that, in HUVEC and BAEC, CSC causes a severalfold increase in the phosphorylation of PECAM-1. The phosphorylation of PECAM-1 mediated by CSC is decreased by inhibitors of PKC, indicating that CSC-mediated phosphorylation of PECAM-1 in endothelial cells presumably occurred on serine and/or threonine residues, because PKC is a serine/threonine-specific kinase. We did not perform phosphoamino acid analysis of HUVEC PECAM-1, because PKC data implies that phosphorylation of tyrosine residues does not occur. Other investigators have observed phosphorylation of serine but not tyro-

sine residues in PECAM-1 on activation of either platelets (21) or T lymphocytes (32).

Cigarette smoke could affect the barrier properties and intercellular junctions in the endothelial monolayer in vivo as a result of increased state of phosphorylation of PECAM-1. Because PKC inhibitors blocked the phosphorylation of PECAM-1, we investigated whether inhibition of CSC-mediated phosphorylation of PECAM-1 affected transendothelial migration of monocytes. The addition of an inhibitor of PKC, GF 109203X, before treatment with CSC reduced the migration of HL-60 monocytic cells by >80%. We suggest that phosphorylation events in PECAM-1 or another PKC substrate protein in HUVEC are important in regulating migration of monocytes across the endothelial cell monolayer. In support of our suggestion we note that phosphorylation/dephosphorylation events in tight junction protein ZO-1 of mammary epithelial cells affect junctional permeability (24). Specifically, it has been shown that treatment of mammary epithelial cells with dexamethasone caused an increase in transepithelial electrical resistance (TER), whereas treatment with okadaic acid, an inhibitor of protein phosphatase type 1 and type 2A, reduced the TER of dexamethasone-treated monolayers of epithelial cells. These results, obtained in epithelial cells, led us to speculate that increased phosphorylation of PECAM-1 in endothelium may similarly result in vivo in a decrease in the transendothelial resistance and a concomitant increase in the junctional permeability of the endothelial cell monolayer. As a consequence of the change in the adhesive function, the transmigration of monocytes in the endothelium is accelerated.

In conclusion, our studies show that CSC causes activation of the transcription factor NF-κB, which binds to consensus sequence motifs present in a subset of CAM genes, and stimulates increased surface expression of adhesion molecules, ICAM-1, ELAM-1, and VCAM-1, involved in the adhesion of blood leukocytes to vascular endothelium. CSC also activates PKC in the cell membrane, causing increased phosphorylation of PECAM-1, in turn signaling an increased migration of monocytes across the confluent endothelial monolayer. We suggest that phosphorylation/dephosphorylation events in PECAM-1 molecule in the vascular endothelium play an important role in the complex regulation of the transendothelial migration of monocytes.

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