Cigarette smoke extract inhibits angiogenesis of pulmonary artery endothelial cells: the role of calpain

Yunchao Su, Wengang Cao, Zhaosheng Han, and Edward R. Block

Department of Medicine, University of Florida College of Medicine; and Research Service, Malcom Randall Veterans Medical Center, Gainesville, Florida 32608-1197

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Am J Physiol Lung Cell Mol Physiol 287: L794–L800, 2004. First published June 4, 2004; 10.1152/ajplung.00079.2004.—Angiogenesis is an integral part of normal tissue development and repair and is an essential part of the repair process of lung tissue and is implicated in a number of processes in COPD. For example, partial pneumonectomy, chronic hypoxia, and airway infection stimulate angiogenesis in the pulmonary circulation (11, 13, 14). It is not clear whether cigarette smoke inhibits angiogenesis and thus impairs the repair processes in lung.

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Angiogenesis is a complex process involving endothelial cell migration, proliferation, and differentiation, as well as tube formation. The cytoskeletal signaling in these processes is mediated by calpain (4, 16). Calpain is a family of calcium-activated, nonlysosomal neutral cysteine endopeptidases that are ubiquitously distributed in all mammalian cells including lung endothelial cells. Calpain acts via limited proteolysis of substrate proteins (6, 7). Calpastatin functions as a specific endogenous inhibitor for calpain (19). Calpain is involved in the signal transduction of cell migration and differentiation in cells including endothelial cells (2, 24, 33). The mechanism is related to calpain-induced alteration in the architecture of cell adhesion and cytoskeletal components (24). Inhibiting calpain in endothelial cells, lymphocytes, fibroblasts, and NIH 3T3 cells inhibits the ability of the cells to spread and migrate. Focal adhesions are larger and located at the periphery of the cell after calpain inhibition. Actin stress fibers also display a peripheral cortical distribution when calpain is inhibited (5). Calpain plays an important role in the activation of the Rho signaling pathway, which is a critical mediator of endothelial motility (17). In the present study, we examined the effect of cigarette smoke extract (CSE) on endothelial angiogenesis in pulmonary artery endothelial cells (PAEC) and evaluated the role of calpain in CSE-induced effects on endothelial angiogenesis.

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MATERIALS AND METHODS

Cell culture. Endothelial cells were obtained from the main pulmonary artery of 6- to 7-mo-old pigs. Third- to sixth-passage cells in monolayer culture were maintained in RPMI 1640 medium containing 4% FBS and antibiotics (10 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamicin, and 2 μg/ml Fungizone) and were used 2 or 3 days after confluence. All monolayers were initially identified as endothelial cells by phase-contrast microscopy. Selected dishes of cells were further characterized by electron microscopy or by indirect immunofluorescent staining for factor VIII antigen or both. By use of these techniques, monolayer cultures were estimated to be pure endothelial cells.

Preparation of CSE. CSE was prepared as described by us (31). It has been used to study the effects of cigarette smoke on isolated
vessels (21) and cultured cells (10, 23, 25) and is a good model for defining the effects of cigarette smoke on cell and tissue function. Commercial cigarettes (Marlboro brand) were smoked, and the mainstream smoke was passed through 30 ml of RPMI 1640, which was prewarmed to 37°C, by the driving of a vacuum. Each cigarette was smoked for 5 min. Three cigarettes were used to generate 30 ml of CSE solution. The CSE was diluted with RPMI 1640. Final concentration is expressed as %vol/vol. The control medium was prepared with the same protocol except that the cigarettes were unlit.

**Determination of angiogenesis.** Endothelial monolayer wound repair, tube formation, cell migration, and proliferation have been widely used to evaluate endothelial angiogenesis (1, 26).

To measure endothelial monolayer wound repair, we created a cell-free wound zone by scraping the monolayer with a sterile pipette tip. The wound width of monolayers (in mm) was measured under the microscope. Then monolayers were washed and incubated with 2.5–10% CSE in 5% CO2 at 37°C. Because the endothelial monolayer required at least 16 h to repair the wound a measurable distance, the wound width was measured again after 16 h. Endothelial monolayer wound repair distance is expressed as the difference between width of the wound before CSE exposure and after CSE exposure (mm).

To do the tube formation assay, we coated 96-well culture plates with 100 μl of Matrigel (BD Biosciences Discovery Labware, Bedford, MA) per well and then allowed them to polymerize for 30 min at 37°C. PAEC were seeded on coated plates at a density of 2 × 10^4 cells per well in RPMI 1640 containing 4% FBS and CSE at 37°C. Endothelial cells started to form tubes at 4 h. The tube formation was optimal after 8 h, started to fade after 10 h, and disappeared after 16–20 h. Therefore, the images of tubes were taken at 8 h in ×40 magnification with a digital output camera (Olympus) attached to an inverted phase-contrast microscope. Total tube length (in mm/mm²) in each well was measured.

Migration assays were performed using a Boyden chamber (Neuroprobe) with an 8-μm-pore polycarbonate filter. PAEC were digested with 0.05% trypsin and dispersed into homogeneous cell suspensions. Cell suspensions containing 2 × 10^4 PAEC with or without CSE were placed in the upper chamber of a 48-well Boyden chamber. The lower chamber contained the same media as the upper chamber without PAEC. The Boyden chamber was incubated at 37°C in a CO2 incubator. To avoid the influence of cell proliferation and an excess number of cells migrating across the filter, we allowed PAEC to migrate across the filter for only 4 h. The chambers were then disassembled, and the cells on the upper side of the filter membranes were removed. The cells that migrated to the lower side of the filter membranes were fixed and subjected to hematoxylin and eosin staining. The results are expressed as the number of migrated cells per squared millimeter.

PAEC proliferation was assayed with a kit from Roche (Indianapolis, IN) that monitors incorporation of 5-bromo-2’-deoxy-uridine (BrdU) into newly synthesized DNA. PAEC were seeded into 96-well plates (5 × 10^3 cells/well) coated with 1.5% gelatin in 4% FBS. After 24 h, PAEC were incubated with CSE for 24 h. We then labeled the newly synthesized DNA by adding 3 ml of DMEM to this mixture. After the cells reached 70% confluence, the medium was changed to transfection medium. After incubation for 24 h, CSE or control medium was added to the dish. After a 24-h incubation, the cells were subjected to measurements of endothelial monolayer wound repair, tube formation, cell migration, and proliferation.

**Calpain activity assay.** We assayed calpain activities by detecting calpain-mediated cleavage of resorufin-labeled casein in vitro (28) and of natural substrate spectrin in intact cells (22). To detect calpain-mediated cleavage of resorufin-labeled casein in vitro, we scrapped and sonicated the cells in buffer A (0.2 M Tris-HCl, pH 7.5, 1 mM dithiothreitol). Then 100-μl samples were mixed with 50 μl of resorufin-labeled casein substrate solutions (0.4%) and 50 μl of buffer B (buffer A plus 10 mM CaCl2). CaCl2 was substituted by 5 mM EDTA in blanks. After incubation for 30 min at 37°C, we terminated the reactions by adding 480 μl of 5% trichloroacetic acid. The mixtures were incubated again at 37°C for 10 min. After centrifugation, 400-μl supernatants were mixed with 600 μl of 0.5 M Tris-HCl (pH 8.8). The fluorescence was measured by spectrophotometry (excitation, 574 nm; emission, 584 nm). We calculated calpain activity by subtracting the fluorescence units of samples with EDTA from those with CaCl2. We also measured calpain activity in intact cells by detecting calpain-specific spectrin breakdown products as reported by Newcomb et al. (22). Briefly, after treatments the cells were lysed by Western blot sample buffer and subjected to immunoblot with antibody against α-II-spectrin (catalog no. FG 6090, clone AA6; AFFINITI Research Products, Mamhead Castle, UK). This antibody recognizes 280-kDa α-II-spectrin and a 150-kDa calpain-specific fragment. The calpain activity is expressed as the spectrin proteolytic ratio (150 kDa/280 kDa).

**Western blot analysis.** After treatments, PAEC were washed with phosphate-buffered saline and lysed in boiling sample buffer (0.06 M Tris-HCl, 2% SDS, and 5% glycerol, pH 6.8). The lysates were boiled for 5 min. The lysate proteins (15–20 μg) were separated on a 7.5% or 12% SDS-PAGE and electrophotographically transferred onto nitrocellulose membranes. The membranes were incubated in blocking solution overnight at 4°C and then hybridized with primary antibody against spectrin and calpastatin at room temperature for 1–2 h. The bands were detected by an immunochromiluminescence method.

**Statistical analysis.** In each experiment, experimental and control endothelial cells were matched for cell line, age, seeding density, number of passages, and number of days postconfluence to avoid variation in tissue culture factors that can influence measurements of angiogenesis and calpain activity. Results are shown as means ± SE for n experiments. Student’s paired t-test is used to determine the significance of differences between the means of experimental and control cells. A value of P < 0.05 was taken as significant.

**RESULTS**

**Effects of CSE on endothelial angiogenesis as measured by endothelial monolayer wound repair, endothelial cell tube formation, migration, and proliferation in PAEC.** To investigate the effects of CSE on endothelial angiogenesis, we measured endothelial monolayer wound repair, endothelial cell tube formation, migration, and proliferation in control PAEC and CSE-exposed PAEC. As shown in Fig. 1, incubation of PAEC with CSE for 16 h resulted in a dose-dependent decrease in monolayer wound repair. Incubation of PAEC with CSE (7.5%) also resulted in a significant decrease in tube formation (28). As shown in Fig. 3, incubation of PAEC with 7.5% CSE caused a decrease in cell migration and proliferation. Incubation of PAEC for 2–24 h with 2.5–10% CSE, which corresponds approximately to exposures associated with smoking slightly less than 0.5 pack per day to slightly less than 2
packs per day of cigarettes, had no effect on total cellular protein contents, lactate dehydrogenase (LDH) release, or reduced glutathione contents and did not cause cell death or apoptosis as assessed by trypan blue exclusion, cell morphology, and DNA fragmentation, suggesting that the effects of CSE on endothelial angiogenesis did not arise from a generalized toxic effect of CSE on these cells.

Effects of CSE on calpain activity in vitro. To investigate whether CSE affects calpain activity, we measured calpain activities in CSE-exposed PAEC. As shown in Fig. 4, incubation of PAEC with CSE (10%) resulted in a time-dependent decrease in calpain activity.
Effects of calpain inhibitor-1 on the CSE-induced inhibition of monolayer wound repair, tube formation, migration, and proliferation and on calpain activity in intact cells. Calpain plays an important role in the formation of actin stress fibers and in the regulation of endothelial angiogenesis. To investigate whether calpain is involved in CSE-induced inhibition of endothelial angiogenesis, we incubated PAEC with CSE in the presence and absence of calpain inhibitor-1, after which monolayer wound repair, tube formation, migration, and proliferation were evaluated. As shown in Fig. 5, incubation of PAEC with 10 μM calpain inhibitor-1 resulted in significant decreases in monolayer wound repair, tube formation, migration, and proliferation. Calpain inhibitor-1 also potentiated the inhibitory effects of CSE on monolayer wound repair, tube formation, migration, and proliferation in PAEC.

To confirm that the effect of calpain inhibitor-1 and CSE on endothelial angiogenesis is achieved through inhibiting calpain activity, we measured calpain activities by detecting its specific spectrin breakdown products. As shown in Fig. 6, both calpain inhibitor-1 and CSE inhibit calpain activity. Corresponding to the changes in angiogenesis, calpain inhibitor-1 potentiated the inhibitory effects of CSE on calpain activity, suggesting that CSE-induced inhibition of endothelial angiogenesis might involve calpain.

Effects of antisense ODN of calpastatin on the CSE-induced decreases in monolayer wound repair, tube formation, migration, and proliferation in PAEC. To further confirm whether CSE-induced inhibition of calpain is responsible for decreased endothelial angiogenesis in CSE-exposed PAEC, we manipulated calpain activity using antisense ODN of calpastatin. The effects of CSE on angiogenesis in PAEC transfected with scrambled and antisense ODN of calpastatin were evaluated, respectively. Transfection of PAEC with the antisense ODN of calpastatin for 48 h did not reduce calpastatin protein content under basal conditions, but it prevented the CSE-induced increase in calpastatin protein content and the CSE-induced decrease in calpain activity, suggesting that antisense ODN of calpastatin blocks new synthesis of calpastatin protein induced by CSE, which is responsible for CSE-induced inhibition of calpain activity (Fig. 7). Incubation of PAEC transfected with scrambled ODN with 7.5% CSE exhibited decreases in monolayer wound repair, tube formation, migration, and proliferation, respectively (Fig. 8). Moreover, incubation of PAEC transfected with antisense ODN of calpastatin with 7.5% CSE attenuated the decreases in monolayer wound repair, tube formation, and migration caused by CSE but did not affect the CSE-induced decrease in proliferation (Fig. 8).

Fig. 5. Effect of calpain inhibitor-1 (Calp-inh) on the inhibitory effect of CSE on monolayer wound repair, tube formation, migration and proliferation in PAEC. Monolayer wound repair (A), tube formation (B), migration (C), and proliferation (D) were evaluated in control PAEC and PAEC exposed to CSE (7.5%) for 16, 8, 4 and 24 h, respectively, in the absence and presence of Calp-inh (10 μM). Results are expressed as means ± SE; n = 5 experiments. *P < 0.01 vs. control, #P < 0.05 vs. CSE.

Fig. 6. Effect of CSE and Calp-inh on in vivo calpain activity. PAEC were incubated with CSE (7.5%) and Calp-inh (10 μM) for 24 h, after which calpain activity was measured by detecting calpain-specific spectrin breakdown products. A: representative immunoblot against 150-/280-kDa fragments of spectrin. B: bar graph depicting the changes of spectrin 150/280 ratio. Results are expressed as means ± SE; n = 5 experiments. *P < 0.01 vs. control, #P < 0.05 vs. CSE.
Angiogenesis is a complex process involving endothelial cell migration, proliferation, and differentiation, as well as tube formation. In the present study, angiogenesis was evaluated by measuring endothelial monolayer wound repair, tube formation, cell migration, and proliferation in PAEC. Each measurement assesses a different aspect of angiogenesis. Monolayer wound repair reflects the integration of endothelial migration, proliferation, and differentiation. The cell migration assay assesses endothelial mobility, which is more closely related to cytoskeletal reorganization. The tube formation assay measures endothelial differentiation, which is associated with cytoskeletal reorganization and intracellular organelle trafficking. The proliferation assay assesses cell cycle changes and DNA synthesis. Our results have shown that incubation of PAEC with CSE resulted in significant decreases in monolayer wound repair, tube formation, cell migration, and proliferation, indicating that CSE affects a number of the processes involved in endothelial angiogenesis.

The decreases in monolayer wound repair, tube formation, cell migration, and proliferation in PAEC exposed to CSE do not arise from a generalized toxic effect of cigarette smoke on these cells, because PAEC exposed to 2.5–10% CSE for 2–24 h do not manifest changes in LDH release, cell protein, GSH content, or DNA fragmentation. However, our data suggest that CSE-induced inhibition of endothelial angiogenesis is due, at least in part, to calpain inhibition. Exposure of PAEC to CSE causes an inhibition of calpain activity in PAEC (8, 30). We also found that calpain inhibitor-1, a specific inhibitor of calpain, inhibits angiogenesis and potentiates the inhibitory effects of CSE on angiogenesis in PAEC, which is accompanied by corresponding additive changes in calpain activity. Moreover, an antisense ODN of calpastatin prevented CSE-induced inhibi-
bition of calpain activity and of endothelial monolayer wound repair, cell migration, and tube formation but not proliferation. Together, these results indicate that CSE-induced inhibition of endothelial monolayer wound repair, tube formation, and migration are mediated by calpain inhibition.

Transfection of PAEC with an antisense ODN of calpastatin for 48 h did not significantly decrease calpastatin protein content under basal conditions. The failure to detect a decrease in protein content is most likely due to the long half-life of calpastatin protein [at least 5 days as reported by Zhang et al. (34)] and the fact that our measurements were made at 48 h. However, anti-sense ODN of calpastatin was able to prevent the CSE-induced increase in calpastatin protein and the attendant CSE-induced decrease in calpain activity in PAEC, suggesting that it effectively blocks calpastatin expression. This is supported by preliminary data (not shown) demonstrating a 20–30% decrease in calpastatin mRNA content in ODN-treated cells.

Cigarette smoke has been implicated as a major risk factor in the pathogenesis of COPD. Cigarette smoke not only causes direct injury to lung tissue but also inhibits compensatory angiogenesis and thus impairs repair and adaptive mechanisms in a smoker’s lung. Hypoxia has been shown to induce angiogenesis in the pulmonary circulation (12, 13, 18). Hypoxic pulmonary angiogenesis plays a compensatory role in the hypoxic lung to improve total lung diffusion capacity and to attenuate the development of hypoxic pulmonary hypertension. The present study indicates that calpain activity is required for lung angiogenesis. We have previously reported that hypoxia increases calpain activity (28, 29). Therefore, we speculate that calpain may play an important role in hypoxic pulmonary angiogenesis. Inhibition of calpain and angiogenesis by cigarette smoke may damage the compensatory angiogenic response in the pulmonary circulation to hypoxia and thus contribute to the pathogenesis of hypoxemia and pulmonary hypertension in COPD patients.

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