Involvement of Calpain-Calpastatin in Cigarette Smoke-Induced Inhibition of Lung Endothelial Nitric Oxide Synthase

Zhaoqiang Cui*, Zhaosheng Han*, Zhaozhong Li, Hanbo Hu, Jawaharlal M. Patel, Veena Antony, Edward R. Block, and Yunchao Su

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

We reported that cigarette smoke extract (CSE) causes decreases in the activity and expression of endothelial nitric oxide synthase (eNOS) and calpain activity in pulmonary artery endothelial cells (PAECs). Calpains are a family of calcium-dependent endopeptidases, and their specific endogenous inhibitor is calpastatin. In this study, we evaluated the role of calpain-calpastatin in CSE-induced decrease in eNOS gene expression. PAEC were incubated with 5–10% CSE for 2–24 h. eNOS gene transcription rate, eNOS messenger ribonucleic acid (mRNA) half-life, and the activity and protein contents of calpain and calpastatin were measured. Incubation of PAEC with CSE caused significant decreases in eNOS gene transcription and calpain activity and an increase in calpastatin protein content. eNOS mRNA half-life was not significantly altered by CSE. To investigate whether CSE-induced inhibition of eNOS gene expression is caused by decreased calpain activity due to an increase in calpastatin protein content, we cloned calpastatin gene from PAEC and constructed adenovirus vectors containing calpastatin. Overexpression of calpastatin mimics the inhibitory effects of CSE on calpain activity and on the activity, protein, and mRNA of eNOS. The cell-permeable calpain inhibitor, calpastatin peptide, inhibits acetylcholine-induced endothelium-dependent relaxation of the pulmonary artery. Incubation of PAEC with an antisense oligodeoxyribonucleotide of calpastatin prevented CSE-induced increases in calpastatin protein and CSE-induced decreases in calpain activity, eNOS gene transcription, activity and protein content of eNOS, and NO release. These results indicate that CSE-induced inhibition of eNOS expression in PAEC is caused by calpain inhibition due to an increase in calpastatin protein content.

Keywords: endothelium; nitric oxide; lung; tobacco

Cigarette smoke has been implicated as a major risk factor in chronic obstructive pulmonary disease (COPD) (1–3). It has been reported that endothelium-dependent vasorelaxation is diminished in animals exposed to cigarette smoke (4) and in human cigarette smokers (5). Cigarette smoking also reduces exhaled nitric oxide (NO), suggesting that cigarette smoke inhibits NO production (6, 7). NO is an important endogenous vasodilator that contributes to the low vascular resistance in the pulmonary circulation and to the counteraction of hypoxic pulmonary vaso-

constriction (8). NO is also an important mediator in angiogenesis (9, 10). A reduction in NO production by cigarette smoke might be responsible, at least in part, for the increased risk of systemic and pulmonary vascular disease and dysfunction in cigarette smokers.

The mechanism for cigarette smoke-induced decrease in NO production is not completely understood. It might be related to decreased endothelial nitric oxide synthase (eNOS) protein contents. Barbera and colleagues (11) reported that the expression of eNOS is reduced in the pulmonary arteries of smokers. We have previously reported that cigarette smoke extract (CSE) causes inhibition of eNOS activity and decreases in eNOS protein and messenger ribonucleic acid (mRNA) contents in pulmonary artery endothelial cells (PAEC) (12). We have also reported that CSE inhibits calpain activity (13) and that the regulation of eNOS activity in hypoxic PAEC involves calpain (14). Calpain is a family of calcium-activated, nonlysosomal neutral cysteine endopeptidases. There are at least 15 isozymes in the family (15, 16). μ -Calpain (calpain I) and m-calpain (calpain II) are major typical calpain isoforms and are responsible for calpain activity in endothelial cells (15, 17, 18). μ -Calpain and m-calpain isoforms consist of a distinct larger catalytic subunit (~ 80 kD) and a common smaller subunit (~ 30 kD) that helps regulate catalytic activity (17, 19). Calpastatin functions as the major specific endogenous inhibitor for μ -calpain and m-calpain (20–22). The calpain-calpastatin system has been found to have an important role in transmembrane signaling, cell differentiation, transcriptional regulation, cytokine processing, and apoptosis (17, 23). In the present study, we examined the role of the calpain-calpastatin system in CSE-induced inhibition of eNOS activity in PAEC. Our results indicate that CSE-induced inhibition of eNOS expression and activity in PAEC is caused by an inhibition of calpain activity due to an increase in calpastatin protein content.

MATERIALS AND METHODS

Materials

Mouse monoclonal antibodies directed against eNOS and calpastatin were obtained from BD Biosciences (Lexington, KY) and Biomol (Plymouth Meeting, PA), respectively. L-[3 H]-arginine was obtained from Amersham (Arlington Heights, IL). Reagents for the cDNA cloning and expressional vector construction of porcine calpastatin were obtained from Invitrogen (Carlsbad, CA), Clontech (Palo Alto, CA), and ATCC (Manassas, VA), respectively. Oligodeoxyribonucleotides (ODN) were synthesized by Invitrogen. Effectene was obtained from Qiagen (Valencia, CA). rATP, rUTP, rGTP, and rCTP were obtained from Promega (Madison, WI).

Cell Culture

Endothelial cells were obtained from the main pulmonary artery of 6- to 7-mo-old pigs and were cultured and characterized as previously reported (12). Third- to sixth-passage cells in monolayer culture in 100-mm dishes were used. PAEC were maintained in RPMI 1640 medium

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Correspondence and requests for reprints should be addressed to Yunchao Su, M.D., Ph.D., Department of Medicine, MSB M452, Box 100225, University of Florida, 1600 S.W. Archer Road, Gainesville, FL 32610. E-mail: ysu@ufl.edu

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plus 4% fetal bovine serum and 100 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin, and 2 µg/ml amphotericin B.

Preparation of CSE

CSE was prepared as described previously (12). It has been used to study the effects of cigarette smoke on isolated vessels (24) and cultured cells (25–28) and is a good model for defining the effects of cigarette smoke on cell and tissue function (28, 29). Commercial cigarettes (Marlboro brand) were smoked, and the mainstream smoke was passed through 30 ml of RPMI 1640 medium, which was pre-warmed 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 medium. Final concentrations are expressed as %vol/vol. The control medium was prepared with the same protocol except that the cigarettes were unlit.

Calpain Activity Assay

Calpain activity was measured as reported previously with modification (30). After treatments, the cells were scraped and sonicated in buffer A (0.2 M Tris-HCl, pH 7.5, 1 mM dithiothreitol). Then 100-µl samples were mixed with 50 µl resorufin-labeled casein substrate solutions (0.4%) and 50 µl buffer B (buffer A plus 10 mM CaCl₂). CaCl₂ was substituted by 5 mM ethylenediaminetetraacetic acid (EDTA) in blanks. After incubation for 30 min at 37°C, the reactions were terminated by adding 480 µl of 5% trichloroacetic acid. The mixtures were incubated again at 37°C for 10 min. After centrifugation, 400 µl of the supernatant was mixed with 600 µl 0.5 M Tris-HCl (pH 8.8). The fluorescence was measured by spectrophotometry (Excitation 574 nm, Emission 584 nm). Calpain activity was calculated by subtracting the fluorescence units of samples with EDTA from those with CaCl₂.

Real-Time Reverse-Transcription Polymerase Chain Reaction for eNOS mRNA

Total ribonucleic acid (RNA) was extracted using the kit from Promega, and real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using ABI 5,700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA). The primers used for eNOS amplification were as follows: forward primer: 5'-CGATCCTCACCGCTACAACA-3', reverse primer: 5'-CTCGTTCTCCAGGTGCTTCA-3'. GAPDH or 18S RNA were included as an internal control, and for GAPDH the forward primer was 5'-CCACCCACGGCAAGTTCCACGGCA-3', and the reverse primer was 5'-TCCAGGCGGCAGGTCAGATCCACA-3'. For 18S RNA the forward primer was 5'-TGGTTGCAAAGCTGAAACT-3', and the reverse primer was 5'-GGTGAGGTTTCCCGTGTT-3'. Of each primer, 60 nM was used in each 50 µl reaction volume. The reaction was performed in the following conditions: 50°C for 30 min for reverse transcription, 95°C for 10 min, 95°C for 30 s, 65°C for 1 min, 72°C for 30 s, total 40 cycles. SYBR green reagent (Stratagene, La Jolla, CA) was used for detection of RT-PCR products. Two standard curves were obtained for eNOS and GAPDH or 18S RNA, respectively. For both mRNA, linear inverse correlation was observed between C_T values (cycles at threshold lines) and the amount of applied cellular RNA. The input amount (TGia) was determined by the following formula: TGia = (C_T value-b)/m, where b is y-intercept and m is the slope of the standard curve. eNOS mRNA amount was normalized by that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

eNOS mRNA Half-Life Assay

The cells were incubated with actinomycin D (2.5 µg/ml) for 2–24 h in the presence and absence of CSE. Total RNA was extracted, and real-time RT-PCR was performed as described previously, after which an eNOS mRNA decay curve was constructed. The half-life of eNOS mRNA was equal to the time for one half of the amount of mRNA to decay.

Nuclear Run-Off Assay

To measure the eNOS gene transcription rate, polymerase chain reaction (PCR)-based nuclear runoff assay was performed as described by Rolfe and Sewell (31) with modification. After treatments, nuclei of PAECs were isolated and divided into two equal groups. The two

groups with equal numbers of nuclei were incubated for 30 min at 30°C in the presence and absence of rATP, rUTP, rGTP, and rCTP, respectively, and RNA was extracted from the nuclei. Real-time RT-PCR for RNA transcripts of eNOS and GAPDH was performed as described previously, and the differences in the ratios of nascent eNOS mRNA to nascent GAPDH mRNA with and without nucleotides were used to determine the eNOS gene transcription rate.

Western Blot Analysis

After treatments, PAEC were washed with phosphate-buffered saline and lysed in boiled sample buffer (0.06 M Tris-HCl, 2% sodium dodecyl sulfate, 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% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred onto nitrocellulose membranes. The membranes were incubated in blocking solution overnight at 4°C and hybridized with primary antibody against eNOS, calpastatin, calpain-1, calpain-2, and GAPDH at room temperature for 1–2 h. The bands were detected using an immunochemiluminescence method.

cDNA Cloning and Sequence Analysis of Porcine Calpastatin

Total RNA (1 µg) isolated from porcine PAEC was used to perform RT-PCR using one-step RT-PCR amplification (PowerScript reverse transcriptase and Advantage 2 PCR enzyme system; BD Biosciences, Clontech). The PCR primers (forward 5'-ATTAATGAGCTCAAATGAATCCACAGAAACCAA-3', reverse 5'-ATTTAGAATTCTTA ACTTGTA CTCTTCCATCAGATTT-3') were designed on the basis of published calpastatin cDNA sequences in GenBank from *Sus scrofa* (accession no. M20160) (32). RT-PCR conditions were: 42°C 45 min, 94°C 10 min, followed by 35 cycles of amplification (94°C 30 s, 60°C 30 s, 68°C 3 min). The resulting PCR products were sequenced using a Perkin Elmer/Applied Biosystems automated DNA Sequencer (DNA Sequencing Laboratory, ICBR, University of Florida). The sequence analyses were performed using NTI Vector 7.1.

Construction and Transient Expression of Adenoviral Vector Containing Porcine Calpastatin in PAEC

The recombinant adenovirus-encoding porcine calpastatin-cyan fluorescence protein (CFP) fusion protein was constructed using the BD Adeno-X Tet-off expression system (Clontech). Briefly, after *Sac* I/*Eco*RI digestion and gel-purification, the PCR products were inserted in the *Sac* I/*Eco*RI sites of pEGFP-C1. The resulting plasmid was named calpastatin-CFP. The inserts excised from *Nhe* I/*Sal* I sites of calpastatin-CFP were used to construct recombinant adenoviruses containing tetracycline (Tet)-regulated gene inserts according to the manufacturer's instructions. After package, propagation, purification, and titration, the resulting adenoviruses and Adeno-X Tet-Off regulatory virus were used to coinfect PAEC.

Transfection of Antisense ODN for Calpastatin

We took advantage of an antisense ODN against porcine calpastatin, which has been previously reported to prevent the CSE-induced increase in calpastatin expression in PAEC (13). Antisense ODN and scrambled ODN (2 µg) were mixed with 300 µl of buffer followed by 16 µl of enhancer. Then, 60 µl of the transfection reagent effectene was added to the mixture. The transfection medium was prepared by adding 3 ml Dulbecco's modified Eagle's medium into the mixture. After the cells reached ~70% confluence as assessed by inverted light microscopy, the medium was changed to transfection medium. After incubation for 24 h, CSE was added to the dish. After 24 h, calpain and eNOS activities, calpastatin and eNOS protein contents, and eNOS gene transcription rate were measured.

eNOS Activity Assay

eNOS activities were measured as reported previously (12). Briefly, PAEC were scraped and homogenized in buffer A (50 mM Tris-HCl, pH 7.4, containing 0.1 mM each EDTA and ethyleneglycol-bis-[β-aminoethylether]-N,N'-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1.0 µg/ml leupeptin, and 10 µM calpain inhibitor-1). The homogenates were centrifuged at 100,000 × g for 60 min at 4°C, and the total membrane pellet was resuspended in buffer B (buffer A + 2.5 mM CaCl₂).

The resulting suspension was used for determination of eNOS activity by monitoring the formation of L-[³H]-citrulline from L-[³H]-arginine. The total membrane fractions (100 µg of protein) were incubated (total volume 0.4 ml) in buffer B containing 1 mM NADPH, 100 nM calmodulin, 10 µM tetrahydrobiopterin, and 5 µM combined L-arginine and purified L-[³H]-arginine for 30 min at 37°C. Purification of L-[³H]-arginine and measurement of L-[³H]-citrulline formation were performed as reported previously (12). The specific activity of eNOS is expressed as pmol L-citrulline min⁻¹ mg protein⁻¹. Protein contents in the total membrane fractions were determined by the method of Lowry and colleagues (33). We found that CSE did not induce type II NO synthase expression in PAEC. Thus, the conversion of L-[³H]-arginine to L-[³H]-citrulline reflects eNOS activity.

Measurement of NO Production

NO production was measured by detecting nitrite formation in the culture medium. Briefly, after treatment, the PAEC cell monolayer was rinsed twice. The cells were incubated with fresh RPMI 1640 medium for 10 min, 100 µl of medium were collected, and the nitrite in the medium was reduced to NO using potassium iodide. NO was measured using an inNOII Nitric Oxide Measuring System (Innovative Instruments, Inc., Tampa, FL).

Measurement of Endothelium-Dependent Vasorelaxation

Pulmonary artery segments (1.5–2.0 mm in diameter, 3–4 mm in length) were isolated from the lungs of 6- to 7-mo-old pigs. Pulmonary artery segments were incubated with calpastatin peptide (80 µM) (Calbiochem) or control peptide in RPMI 1640 medium at 37°C and 5% CO₂ for 24 h. The vessels were suspended in individual organ chambers (Radnoti Four-Unit Tissue Bath System; Radnoti Glass Technology, Inc., Monrovia, CA) with 5 ml of Krebs buffer and oxygenated with 95% O₂ and 5% CO₂ at 37°C. After equilibration at a resting force of 1.5 × g, the vessels were contracted using 0.5 µM U-46619 (a thromboxane A₂ mimetic). After stable contraction, acetylcholine (5.0 µM)-induced relaxations were recorded. An isometric force transducer (Harvard Apparatus, Holliston, MA) and PO-NE-MAH PTP-Tissue Bath System software (Gould Instrument System, Valley View, OH) were used in vessel tension recording. The endothelium-dependent vasorelaxations were expressed as a percentage of U46619 contraction.

Statistics

In each experiment, control and experimental endothelial cells were matched for cell line, age, seeding density, number of passages, and confluence state to avoid variation in tissue culture factors, which can influence the measurement of eNOS activity and eNOS protein analysis. Results are shown as mean ± SE for *n* experiments. Student's paired *t* test was used to determine the significance of differences between the means of treated and control groups, and a *P* value of < 0.05 was taken as significant.

RESULTS

Effect of CSE on eNOS Gene Transcription Rate and eNOS mRNA Half-Life

We have previously reported that CSE causes decreases in the activities, protein contents, and mRNA contents of eNOS in PAEC (12). To investigate whether CSE-induced decreases in eNOS mRNA content are caused by decreased gene transcription rate and/or mRNA stability, we measured eNOS gene transcription rate and eNOS mRNA half-life in CSE-exposed PAEC. Incubation of PAEC with CSE resulted in a time- and dose-dependent inhibition of eNOS gene transcription (Figure 1). The inhibition was observed after 4-h incubation, and more prolonged exposures to CSE resulted in greater inhibition of eNOS gene transcription. However, CSE did not influence eNOS mRNA half-life (20.5 ± 2.1 h versus 20.1 ± 1.8 h). Incubation of PAEC with CSE solutions (2.5–10%) for 2–24 h had no effect on total cellular protein contents or lactate dehydrogenase release, suggesting that the effects of CSE on eNOS gene transcription are not accompanied by cytotoxicity.

Figure 1. Effect of CSE on eNOS gene transcription rate. (A) PAEC were incubated without CSE (Control) or with CSE (5–10%) for 24 h, after which eNOS gene transcription rate was measured as described in MATERIALS AND METHODS. (B) PAEC were incubated with 7.5% CSE for 4–24 h, after which eNOS gene transcription rate was measured as described in MATERIALS AND METHODS. Results are expressed as means ± SE; *n* = 4 experiments. **P* < 0.05 versus control.

Effect of CSE on Calpastatin Protein Content and Calpain Activity

To investigate whether CSE-induced inhibition of calpain activity is caused by increased protein expression of calpastatin, an endogenous calpain inhibitor, we measured calpastatin protein content and calpain activities in CSE-exposed PAEC. Incubation of PAEC with CSE (7.5%) resulted in a time-dependent increase in calpastatin (125 kD) protein content and a time-dependent decrease in calpain activity (Figure 2). The protein contents of the large subunits of μ -calpain and m-calpain were not changed (data not shown). These results suggest that CSE-induced inhibition of calpain activity might be caused by increased protein expression of calpastatin.

Molecular Cloning and Characterization of Porcine Calpastatin

We obtained two calpastatin cDNA fragments using RT-PCR and primers that were designed on the basis of published calpastatin sequences from pig heart. The first is a shorter cDNA fragment (accession no. AY904066), which is an alternative splicing transcript variant of calpastatin with exon 3 deletion. It encodes calpastatin protein containing domain-L with 22 amino acid deletion and all four repeats of calpain inhibitory domain (domains 1–4). The second is a longer calpastatin cDNA fragment (GeneBank no. AY372988) containing an open reading frame of 2,142 bp that encodes a protein of 714 amino acid

Figure 2. Effect of CSE on calpain activity and calpastatin protein contents in PAEC. Cells were incubated with control medium or CSE (7.5%) for 4–24 h, after which calpain activity and calpastatin protein contents were measured as described in MATERIALS AND METHODS. (A) Changes in calpain activity. Results are expressed as mean $\pm$ SE; $n = 4$ experiments. $*P < 0.05$ versus control (0 h). (B) Immunoblots from three experiments using antibodies directed against calpastatin and GADPH. (C) Bar graph depicting the change in density of calpastatin protein in immunoblots. Results are expressed as means $\pm$ SE; $n = 4$ experiments. $*P < 0.05$ versus control (0 h).

residues. Its putative amino acids share a 100% sequence similarity with a published calpastatin gene from pig heart (accession no. M20160) (32). It contains a full-length domain-L and domains 1–4.

Effect of Calpastatin Overexpression on Calpain Activity and eNOS Protein Content

Infection of PAEC with sham-adenovirus did not affect calpain activity (Figure 3). Infection of PAEC with adenovirus containing the first porcine calpastatin (exon 3-deleted [$\Delta 3$]) cDNA resulted in dramatic decrease in calpain activity. However, the protein contents of calpain-1 or calpain-2 did not change in calpastatin-overexpressed cells, suggesting that the decrease in calpain activity is caused by the inhibitory effect of calpastatin on calpain. Corresponding to the changes in calpain activity, infection of PAEC with adenovirus containing the first porcine

Figure 3. Effect of calpastatin overexpression on calpain proteins and activity. PAEC were infected with or without a sham adenovirus or adenovirus containing $\Delta 3$ -calpastatin gene. After 48 h, calpain proteins and activity were measured as described in MATERIALS AND METHODS. (A) Representative immunoblot of proteins of calpastatin, calpain-1, calpain-2, and GADPH from four experiments. (B) Bar graph depicting the changes in calpain activity. Results are expressed as means $\pm$ SE; $n = 4$ experiments. $*P < 0.05$ versus control.

calpastatin (exon 3-deleted [$\Delta 3$]) cDNA caused a decrease in the activity, protein content, and mRNA of eNOS (Figure 4). Identical results were obtained in PAEC infected with adenovirus containing the second (longer) transcript variant of calpastatin cDNA (data not shown). These results suggest that overexpression of calpastatin mimics CSE-induced inhibition of calpain activity and eNOS protein expression.

Effect of Calpastatin Peptide on Endothelium-Dependent Relaxation of Pulmonary Artery

To investigate whether inhibition of calpain in pulmonary artery endothelium results in a decrease in endothelium-dependent vasorelaxation, porcine pulmonary artery segments were incubated with calpastatin peptide or control peptide for 24 h, after which endothelium-dependent relaxations were evaluated. Incubation of pulmonary artery segments with a cell-permeable calpastatin peptide for 24 h resulted in a decrease in endothelium-dependent vasorelaxation induced by acetylcholine (Figure 5).

Effect of Antisense ODN of Calpastatin on CSE-Induced Decreases in eNOS Gene Transcription Rate

We have reported that transfection of PAEC with the antisense ODN of calpastatin for 48 h prevented the CSE-induced increase in calpastatin protein content and the CSE-induced decrease in calpain activity in PAEC (13). To investigate whether the CSE-induced decrease in eNOS gene transcription rate is caused by inhibition of calpain activity due to an increase in calpastatin protein content, eNOS gene transcription rates were observed in PAEC transfected with scrambled ODN or antisense ODN of calpastatin in the presence and absence of CSE. Transfection

Figure 4. Effect of calpastatin overexpression on activity, protein content, and mRNA level of eNOS. PAEC were infected with or without a sham adenovirus or adenovirus containing $\Delta 3$ -calpastatin gene. After 48 h, activity, protein content, and mRNA level of eNOS were measured as described in MATERIALS AND METHODS. (A) Bar graph depicting the changes in eNOS activity. (B) Representative immunoblot of eNOS and GADPH proteins from four experiments. (C) Bar graph depicting the changes in eNOS mRNA. Results are expressed as means $\pm$ SE; $n = 4$ experiments. * $P < 0.05$ versus control.

of PAEC with the antisense ODN of calpastatin for 48 h largely prevented the CSE-induced decrease in eNOS gene transcription rate (Figure 6).

Effect of Antisense ODN of Calpastatin on CSE-Induced Decreases in eNOS Protein Contents, eNOS Activity, and NO Release

To investigate whether preventing CSE-induced inhibition of eNOS gene transcription by antisense ODN of calpastatin is accompanied by a prevention of CSE-induced decreases in eNOS protein contents, eNOS activity, and NO release, PAEC transfected with scrambled ODN or antisense ODN of calpastatin were exposed to control medium or medium containing CSE. eNOS protein contents, eNOS activity, and NO release were determined. Transfection of PAEC with antisense ODN of calpastatin prevented the CSE-induced decreases in eNOS protein contents, eNOS activity, and NO release (Figures 7 and 8).

DISCUSSION

We have reported that CSE inhibits eNOS activity and that this inhibition is due to decreased eNOS mRNA and protein contents

Figure 5. Effect of calpastatin peptide on endothelium-dependent relaxation of intact pulmonary artery segments. Porcine pulmonary artery segments were incubated with calpastatin peptide or control peptide for 24 h, after which acetylcholine (5.0 μ M)-induced endothelium-dependent relaxations were evaluated as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ SE; $n = 3$ experiments. * $P < 0.05$ versus control peptide.

in PAEC exposed to CSE (12). Barbera and colleagues (11) also reported that the expression of eNOS mRNA and protein is reduced in the pulmonary artery of cigarette smokers. However, the mechanism responsible for cigarette smoke-induced decreases in eNOS mRNA is not clear. In the present study, we found that exposure of PAEC to CSE resulted in a decrease in eNOS gene transcription rate that is not accompanied by alteration of eNOS mRNA half-life, indicating that the CSE-induced decrease in eNOS mRNA content in PAEC is caused by an inhibition of eNOS gene transcription.

We have found that incubation of PAEC with CSE causes an inhibition of calpain activity and an increase in calpastatin protein content. To study whether increased calpastatin protein content is involved in CSE-induced inhibition of calpain activity and eNOS protein expression, we cloned the gene encoding the functional domains of calpastatin from porcine PAEC. Four types of calpastatin cDNA variants have been reported (21). Types I, II, and III contain variants with or without exon 3 deletion (34, 35). Types I and II contain sequences encoding the XL region, which migrates at 145 kD in SDS-PAGE (36). Type III is identical to that originally isolated from porcine heart and lacks a sequence encoding the XL region (32, 37). Expression of type III calpastatin with and without exon 3 deletion produces

Figure 6. Effect of antisense ODN of calpastatin on the CSE-induced decrease in eNOS gene transcription rate. PAEC were transfected with or without scrambled ODN or antisense ODN of calpastatin and exposed to 7.5% CSE for 24 h, after which eNOS gene transcription rates were measured as described in MATERIALS AND METHODS. Controls were incubated with media alone. Results are expressed as means $\pm$ SE; $n = 3$ experiments. * $P < 0.01$ versus scrambled (scrambled ODN).

Figure 7. Effect of antisense ODN of calpastatin on CSE-induced changes in eNOS protein content. PAEC were transfected with scrambled ODN or antisense ODN of calpastatin and then exposed to 7.5% CSE for 24 h, after which protein contents were measured as described in MATERIALS AND METHODS. (A) Representative immunoblot of proteins of eNOS and GADPH from four experiments. (B) Bar graph depicting the changes in density of eNOS protein in immunoblots. Results are expressed as means $\pm$ SE; $n = 4$. * $P < 0.05$ versus scrambled (scrambled ODN).

a polypeptide that migrates at 125 and 135 kD in SDS-PAGE, respectively (21). Type IV calpastatin expression is limited to the testis. We found that porcine PAEC displayed types I, II, and III variants with or without exon 3 deletion and that type III with exon 3 deletion was a predominant variant (data not shown). Consistent with these data, calpastatin protein expressed in porcine PAEC appeared at 125 kD in the Western blotting analysis of the crude protein extract of PAEC, suggesting that the dominant expression variant of calpastatin in porcine PAEC is type III with exon 3 deletion. By using RT-PCR primers specific for type III calpastatin, we found two variants of the calpastatin gene in porcine PAEC. Both variants contained an L-domain and the calpain-inhibitory domains (domains 1–4), but in the first gene we obtained from porcine PAEC, there was a deletion of exon 3 (22 amino acid residues).

In the present study, we found that overexpression of calpastatin mimics CSE-induced inhibition of calpain activity and eNOS protein expression, suggesting that CSE-induced inhibition of eNOS protein expression might be caused by decreased calpain activity due to an increase in calpastatin protein content. Moreover, transfection of PAEC with an antisense ODN of calpastatin not only prevented the CSE-induced increase in calpastatin protein content and the CSE-induced decrease in calpain activity but also blocked the inhibition of eNOS gene transcription in CSE-exposed PAEC. Accordingly, transfection of PAEC with an antisense ODN of calpastatin prevented CSE-induced decreases in the protein contents and activity of eNOS and restored NO release in CSE-treated PAEC. Taken together, these results indicate that CSE-induced inhibition of eNOS gene

Figure 8. Effect of antisense ODN of calpastatin on CSE-induced changes in eNOS activity and NO release. PAEC were transfected with scrambled ODN or antisense ODN of calpastatin and exposed to 7.5% CSE for 24 h, after which eNOS activity (A) and NO release (B) were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ SE; $n = 4$. * $P < 0.05$ versus scrambled (scrambled ODN).

transcription is due to an inhibition of calpain activity caused by increased calpastatin protein.

We found that the eNOS mRNA level was lower in calpastatin-overexpressed PAEC. The half-life of eNOS mRNA was not significantly altered (data not shown). Moreover, the pulmonary artery segments with inhibited calpain activity exhibited reduction in endothelium-dependent vasorelaxation. These results indicate that optimal calpain activity is necessary for eNOS gene expression and NO-mediated vasodilation. Calpain may regulate eNOS gene expression in several ways. For example, a number of transcription factors have been shown to be affected by calpain (38). Han and colleagues (39) reported that cytosolic m-calpain cleaves cytoplasmic nuclear factor- κ B transcription factor inhibitor I κ B α , causing nuclear factor- κ B activation. Also, Mellgren (40) and Buki and colleagues (41) reported that histone H1 kinase and histone H1 protein are substrates of calpain *in vivo* and *in vitro*. Interactions of histone and chromatin are associated with regulation of the activity of transcription factors of genes, including the eNOS gene (42). Therefore, calpain may regulate eNOS gene expression by modifying the interactions of histone and chromatin.

We have previously found that calpain regulates eNOS activity in hypoxic PAEC by affecting the eNOS–heat shock protein 90 interaction at a post-translational level (14). The results of the present study suggest that inhibition of calpain is responsible for CSE-induced inhibition of eNOS gene transcription. The effect of calpain may depend on the subcellular localization of the enzyme. Calpain is located in the cytosol and at the cell membrane. Calpain in the cytosol may cleave cytosolic proteins (e.g., transcription factors) to affect the transcription of genes such as the eNOS gene. Calpain located at the cell membrane may affect membrane proteins (i.e., eNOS) in a post-translational manner during hypoxia. Further studies are needed to clarify this thesis.

There is a strong association between vascular endothelial cell injury and cigarette smoking. Tobacco smoke components diffuse across the alveolar capillary membrane and its lining fluid to enter the bloodstream and interact with endothelial cells and circulating formed elements in the blood (43–46). The pulmonary vasculature is the first vascular bed with which these components can interact. The calpain–calpastatin system is very active in pulmonary endothelial cells (14, 30, 47). Pulmonary endothelial cells are also an important source of NO, a reactive gas that functions as a signaling molecule and plays a major role in the regulation of pulmonary vascular tone, leukocyte and platelet adhesion to endothelium, and angiogenesis. The present study has shown that CSE-induced inhibition of eNOS activity is caused, at least in part, by decreased eNOS gene transcription due to an inhibition of calpain activity in CSE-exposed PAEC. This discovery opens the door to the possibility that manipulation of calpain activity may provide a new avenue for preventing or reversing impaired eNOS activity and NO production in cigarette smokers and patients with chronic obstructive pulmonary disease.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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