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ORIGINAL RESEARCH COMMUNICATION

Cigarette Smoke–induced Oxidative/Nitrosative Stress Impairs VEGF- and Fluid Shear Stress–Mediated Signaling in Endothelial Cells

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Abstract

VEGF receptor 2 (VEGFR2), a tyrosine kinase receptor, is activated by VEGF and fluid shear stress (FSS), and its downstream signaling is important in the regulation of endothelial functions, such as cell migration, endothelium-dependent relaxation, and angiogenesis. Cigarette smoke (CS) is known to cause oxidative/nitrosative stress, leading to modifications of tyrosine kinase receptors and impaired downstream signaling. We hypothesized that CS-induced oxidative/nitrosative stress impairs VEGF- and FSS-mediated VEGFR2 activation, leading to endothelial dysfunction. Human lung microvascular endothelial cells and human umbilical vein endothelial cells were treated with different concentrations of cigarette smoke extract (CSE) to investigate the VEGF- or FSS-mediated VEGFR2 phosphorylation and its downstream signaling involved in endothelial function. CSE treatment impaired both VEGF- and FSS-mediated VEGFR2 phosphorylation, resulting in impaired endothelial nitric oxide synthase (eNOS) phosphorylation by Akt. CS-derived reactive oxygen/nitrogen species react with VEGFR2, rendering VEGFR2 inactive for its downstream signaling. Pretreatment with nitric oxide scavenger (PTIO), reactive oxygen species scavengers (combination of SOD with catalase), and *N*-acetyl-L-cysteine, significantly attenuated the CSE-induced impairment of VEGF-mediated Akt and eNOS phosphorylation. These findings suggest that CSE-induced oxidative/nitrosative stress impairs VEGF- and FSS-mediated endothelial cell function and has important implications in the pathogenesis of CS-induced pulmonary and cardiovascular diseases associated with endothelial dysfunction. *Antioxid. Redox Signal.* 12, 1355–1369.

Introduction

THE VASCULAR ENDOTHELIUM is a vital homeostatic cell layer responsible for a variety of functions, such as thromboresistance, control of vascular tone, and vascular growth (9, 46). Cigarette smoking is the most important risk factor for the development of chronic obstructive pulmonary disease (COPD) and has been shown to induce significant pulmonary vascular changes characterized by endothelial dysfunction and vascular remodeling involved in pulmonary hypertension (3, 33, 58, 59). It is known that cigarette smoke (CS)-induced emphysematous alveolar septa are remarkably thin and almost avascular (34, 60). Furthermore, it is well established that cigarette smoking is one of the major risk factors for atherosclerosis and is associated with premature coronary and peripheral vascular dysfunction (1, 36, 47). However, the underlying mechanisms involved in the pathophysiology of endothelial dysfunction in response to cigarette smoking remain to be understood fully.

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR2) play an important role in endothelial cell functions, such as cell migration, endothelium-dependent relaxation, and angiogenesis (16). VEGFR2 is activated by VEGF and fluid shear stress (FSS), and its downstream signaling activates endothelial nitric oxide synthase (eNOS) through phosphorylation of Akt (25, 26). Vascular endothelial cells modulate their structure and function in response to changes in FSS generated by blood flowing over the endothelium. Laminar blood flow–generated fluid shear stress, frictional dragging force (per unit area) acting on the endothelium, is the most potent physiologic stimulus for nitric oxide (NO) production by activating eNOS in endothelial cells (6, 12, 15, 20). FSS-stimulated VEGFR2 recruits phosphoinositide 3 (PI3)-kinase (PI3K) and hence leads to the activation of Akt and eNOS (26).

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It has been shown that physiologic FSS plays an important role in protecting the development of atherosclerosis, mainly through an NO-dependent mechanism (4). Endothelial dysfunction, manifested mainly by impaired flow-dependent NO production and vasodilation in human coronary circulation, has been shown to predict long-term atherosclerotic disease progression and high cardiovascular event rate, particularly in smokers (24, 39, 42, 63).

An alternative mechanism also has been suggested for VEGFR2-independent activation of eNOS. Shear stress stimulates PI3K, which in turn activates protein kinase A (PKA) through phosphoinositide-dependent protein kinase-1 (PDK1). Activated PKA then either directly or indirectly phosphorylates eNOS (5). Furthermore, shear stress–induced tyrosine phosphorylation of platelet endothelial cell adhesion molecule-1 also modulates the activation of Akt and eNOS (18).

CS contains reactive oxygen/nitrogen species that cause oxidative/nitrosative stress in endothelial cells (45). It has been shown that oxidative/nitrosative stress impairs the cellular signaling pathway, possibly through posttranslational modification of various receptors (14, 41). In our previous study, we demonstrated that CS-induced oxidative stress impaired VEGF-mediated VEGFR2 phosphorylation and VEGFR2 expression in human lung microvascular endothelial cells and in mouse lungs, leading to endothelial dysfunction, as assessed by decreased levels of eNOS, cell migration, and angiogenesis (16). However, the mechanism underlying CS-induced impairment of VEGF- and FSS-mediated VEGFR2 signaling leading to endothelial dysfunction has not been studied.

We hypothesized that CS-induced oxidative/nitrosative stress impairs VEGF- and FSS-mediated VEGFR2 activation and its downstream signaling, leading to endothelial dysfunction. Our study for first time demonstrates the effect of CS on VEGF- and FSS-mediated endothelial signaling, along with redox modulation of VEGFR2 and its downstream signaling, which may have implications in pathogenesis of COPD and its comorbid conditions.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals used were of analytic grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO).

In vitro studies using human lung microvascular endothelial cells and human umbilical vein endothelial cells

Human lung microvascular endothelial cells (HMVEC-Ls) were purchased from Lonza (Walkersville, MD; previously known as Cambrex). Human umbilical vein endothelial cell (HUVECs) cultures were established as described previously by using umbilical cords collected within 48 h of delivery (19). HMVEC-Ls and HUVECs were used in the experiments described for VEGF and FSS studies and were used between three and six passages. However, the experiment described for pretreatment of NO and reactive oxygen species (ROS) scavengers in FSS studies were performed only in HUVECs. Endothelial cells were grown in EGM-2 Lonza media containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown in 75-mm flasks coated with 0.1% gelatin, and treatments were performed in 0.1% gelatin-coated six-well plates. FSS experiments were performed in cells grown in 60-mm culture plates, as described previously (25). Cigarette smoke extract (CSE) treatments were performed after cells were starved for 6 h with EGM-2 media containing 0.1% FBS.

CSE preparation

Research grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky, Lexington, KY. The composition of 1R3F research-grade cigarettes was as follows: total particulate matter, 17.1 mg/cigarette; tar, 15 mg/cigarette; and nicotine, 1.16 mg/cigarette.

CSE (10%) was prepared by bubbling smoke from one cigarette into 10 ml of EGM-2 culture media without FBS at a rate of one cigarette/2 min, as described previously (38, 61), by using a modification of the method described by Carp and Janoff (8). The pH of the CSE was adjusted to 7.4 and was sterile filtered through a 0.45-μm filter (Acrodisc; Pall Corporation, Ann Arbor, MI). CSE preparation was standardized by measuring the absorbance (OD, 0.86 ± 0.05) at a wavelength of 320 nm. The pattern of absorbance (spectrogram) observed at λ_{320} showed insignificant variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture media supplemented with 0.1% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml of culture media without FBS; pH was adjusted to 7.4, and sterile filtered as described earlier.

Immunoblotting

Endothelial cells were lysed in ice-cold radioimmunoprecipitation assay buffer (RIPA) lysis buffer (50 m*M* Tris-HCl, 150 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid, 0.25% deoxycholate, 1 m*M* Na_3VO_4, 1 m*M* NaF, 1 μg of leupeptin/ml, 1 μg of aprotinin/ml, and 1 m*M* phenylmethyl sulfonyl fluoride). The cell lysates were kept on ice for 45 min and then centrifuged at 10,000 *g* for 15 min at 4°C, and the supernatants were separated.

Protein levels were measured by using bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). Protein (30 μg) was electrophoresed on 4 to 15% gradient PAGE gel and transblotted on nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% (wt/vol) nonfat milk in phosphate buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 and incubated with relevant primary antibody (1:1,000 dilution).

After washing, bound antibody was detected by using anti-rabbit/mouse antibody (1:20,000 dilution) linked to horseradish peroxidase, and bound complexes were detected by using enhanced chemiluminescence (Perkin Elmer, Waltham, MA).

Effect of CSE on VEGF-mediated VEGFR2 phosphorylation and its downstream signaling

HMVEC-Ls and HUVECs were grown (90% confluent) in six-well culture plates and starved for 6 h in 0.1% serum and

VEGF-free media. Cells were then treated with CSE (0.1–0.5%) or VEGFR2 inhibitor (NVP-AAD777; Novartis Pharma AG, Basel, Switzerland, 1.0 μM) for 2 h (16). At the end of the 2 h period, cells were washed twice with PBS and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h in a fresh media, so as to remove the direct effect of CSE on Akt and eNOS phosphorylation. Thereafter, VEGF (50 ng/ml, Cell Signaling Technology, Danvers, MA) was added and incubated for 10 min at 37°C in a humidified atmosphere containing 5% CO_2.

Parallel control experiments were carried out by adding PBS instead of VEGF. The reaction was stopped immediately by adding ice-cold PBS, and the cells were washed twice with ice-cold PBS and then lysed by using RIPA buffer containing protease inhibitor cocktail. Finally, cell lysates were sonicated for 10 s and centrifuged at 10,000 *g* for 15 min, and supernatant was separated and analyzed for protein content by using the BCA kit (Pierce). The levels of phosphorylated VGFR2 (rabbit anti-phospho-VEGFR2 (Tyr 1175); Cell Signaling), total VEGFR2 (rabbit anti-VEGFR2; Cell Signaling), phosphorylated Akt [rabbit anti-phospho-Akt (Ser 473), Cell Signaling], total Akt (rabbit anti-Akt; Cell Signaling), phosphorylated eNOS [rabbit anti-phospho-eNOS (Ser-1177); Cell Signaling], and total eNOS (rabbit anti-eNOS; Cell Signaling) were analyzed by immunoblotting, as described earlier.

Effect of CSE on fluid shear stress–mediated VEGFR2 phosphorylation and its downstream signaling

Confluent endothelial cells (90%) cultured in 60-mm dishes were serum starved for 6 h. Cells were then treated with CSE (0.1 to 0.5%) for 2 h. At the end of 2 h, cells were rinsed with PBS and replaced with HEPES-buffered saline solution (HBSS; 130 m*M* NaCl, 5 m*M* KCl, 1.5 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 20 m*M* HEPES, pH 7.4). These cells either were maintained in static conditions or were exposed to FSS (shear stress, 12 dyn/cm^2 for 10 min) in a cone-and-plate viscometer at 37°C, as described previously (43). Cells were lysed in RIPA buffer, and the levels of phosphorylated (mouse anti-phosphotyrosine, clone 4G10; Millipore, MA) and total VEGFR2, Akt, and eNOS were analyzed with immunoblotting, as described earlier.

Effect of CSE on nitration of tyrosine residue on VEGFR2

Endothelial cell lysates were prepared in RIPA buffer, as described earlier, and VEGFR2 protein was immunoprecipitated by using anti-VEGFR2 antibody (1:100 dilution; rabbit anti-VEGFR2; Cell Signaling), which was added to 200 μg of protein in a final volume of 400 μl and incubated for 1 h. Protein-A/G agarose beads (20 μl) (Santa Cruz Biotechnology, Santa Cruz, CA) were added to each sample and left overnight at 4°C on a rocker. The samples were then centrifuged at 10,000 *g* at 4°C for 5 min. The supernatant was discarded, and the beads were washed 3 times and then resuspended in 100 μl of RIPA buffer. The samples were then mixed with 5× SDS sample buffer, boiled, and the proteins were resolved with SDS-PAGE.

Immunoblotting was performed for nitrated tyrosine (rabbit anti-nitro tyrosine; Cell Signaling Technology) and VEGFR2 (rabbit anti-VEGFR2; Cell Signaling Technology).

Effect of NO and ROS scavengers on CSE-induced impairment of VEGF- and FSS-mediated VEGFR2 phosphorylation and its downstream signaling

Confluent endothelial cells (90%) were serum-starved for 6 h. Cells were then treated with either NO scavenger [PTIO; (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; EMD Chemicals, Inc., Gibbstown, NJ), 100 μM] or ROS scavengers (combination of polyethylene glycol–superoxide dismutase (PEG-SOD) and polyethylene glycol-catalase (PEG-CAT), 400 U/ml of each) for 30 min before CSE treatments. PTIO or PEG-SOD plus PEG-CAT remained in the media throughout the CSE treatments to cells. At the end of a 2 h period, cells were washed twice with PBS and incubated in 37°C in a humidified atmosphere containing 5% CO_2 for another 2 h in fresh medium to remove the direct effect of CSE on Akt and eNOS phosphorylation. VEGF- and FSS-mediated experiments were carried out as described in Materials and Methods.

Effect of NAC on CSE-induced impairment of VEGF- and FSS-mediated VEGFR2 phosphorylation and its downstream signaling

Confluent endothelial cells (90%) were serum starved for 6 h. Cells were then treated with NAC (1 m*M*) for 1 h before the CSE treatments. NAC remained in the media throughout the CSE treatments. At the end of the 2 h period, cells were washed and maintained in fresh media, as mentioned earlier. VEGF- and FSS-mediated experiments were carried out as described in Materials and Methods.

Statistical analysis

The Sigma Stat 3.0 statistical program was used to analyze the data. The results are shown as the mean ± SEM of at least three experiments. All pair-wise multiple comparisons were performed by using the ANOVA; values of $p < 0.05$*, $p < 0.01$**, and $p < 0.001$*** were considered significant.

Results

CSE impaired VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells

VEGFR2 is an essential mediator for endothelial function. The effect of CSE on VEGF-induced VEGFR2 phosphorylation and its downstream signaling was assessed in HMVEC-Ls and HUVECs. Our previous data showed that at 2 h of CSE treatments, VEGFR2 protein was neither downregulated nor phosphorylated, but at 12 h of CSE treatment, VEGFR2 levels were decreased (16). Therefore, to determine whether CSE had any effect on VEGF-mediated VEGFR2 phosphorylation, the 2 h time point was chosen to rule out the downregulation and phosphorylation effect of CSE on VEGFR2. To assess the effect of CSE on VEGFR2 phosphorylation and its downstream signaling, cells pretreated with CSE for 2 h were washed twice with PBS and incubated again with VEGF (50 ng/ml) for 10 min. The levels of total and phosphorylated VEGFR2 (Tyr 1175), Akt (Ser 473), and eNOS (Ser 1177) were measured by immunoblotting after the cells were treated with VEGF. VEGF-mediated phosphorylation of VEGFR2, Akt, and eNOS phosphorylation were significantly decreased in

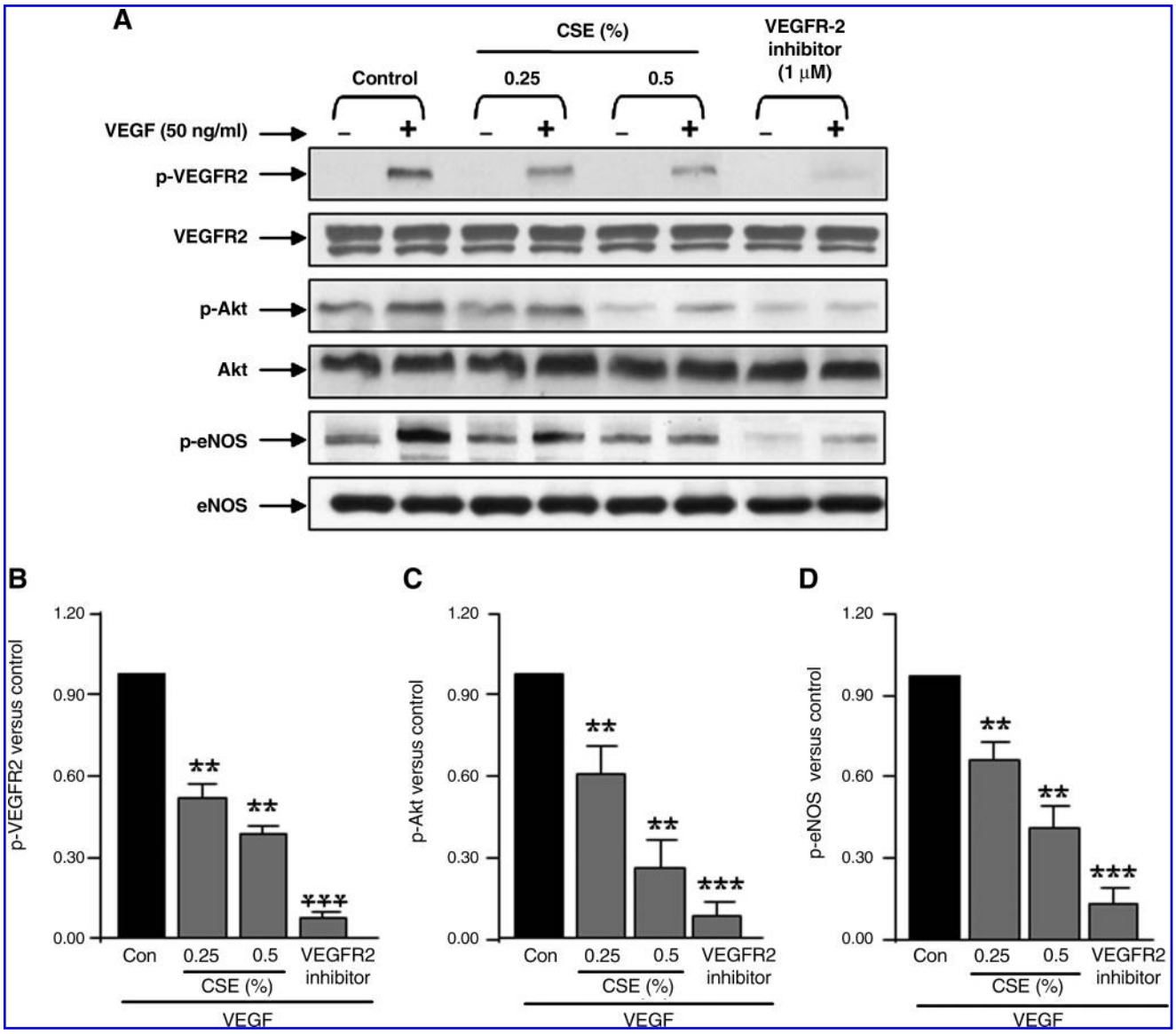

FIG. 1. CSE impaired VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells. (**A**) CSE- or VEGFR2-inhibitor–treated cells (2 h) were incubated with VEGF, and the levels of phosphorylated and total VEGFR2, Akt, and eNOS were measured with immunoblotting. The VEGFR2 blot showed two bands, a 230-kDa, fully glycosylated functional receptor, and a 200-kDa, semiglycosylated nonfunctional receptor. Phosphorylation was seen only in the fully glycosylated functional receptor. CSE treatments downregulated VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in a concentration-dependent manner. Pretreatment of cells with NVP-AAD777 (1 μM) abolished the VEGF-mediated VEGFR2 phosphorylation. Histograms represent mean ± SEM of the percentage of VEGFR2 (**B**), Akt (**C**), and eNOS (**D**) phosphorylation compared with respective control experiments ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group. p-VEGFR2 = phosphorylated VEGFR2 (Tyr 1175); p-Akt = phosphorylated Akt (Ser 473); p-eNOS = phosphorylated eNOS (Ser 1177).

CSE-treated cells in a concentration-dependent manner when compared with control treatments ($p < 0.01$; Fig. 1). Furthermore, VEGF-mediated VEGFR2 phosphorylation was completely inhibited by the VEGFR2 inhibitor NVP-AAD777 at a concentration of 1.0 μM in endothelial cells. These data revealed that CSE dose-dependently downregulated VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells.

CSE impaired FSS-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells

It is known that FSS activates VEGFR2 phosphorylation and induces its downstream signaling (26). Therefore, the effect of CSE on FSS-mediated VEGFR2, Akt, and eNOS phosphorylation was assessed with immunoblotting. We found that FSS-

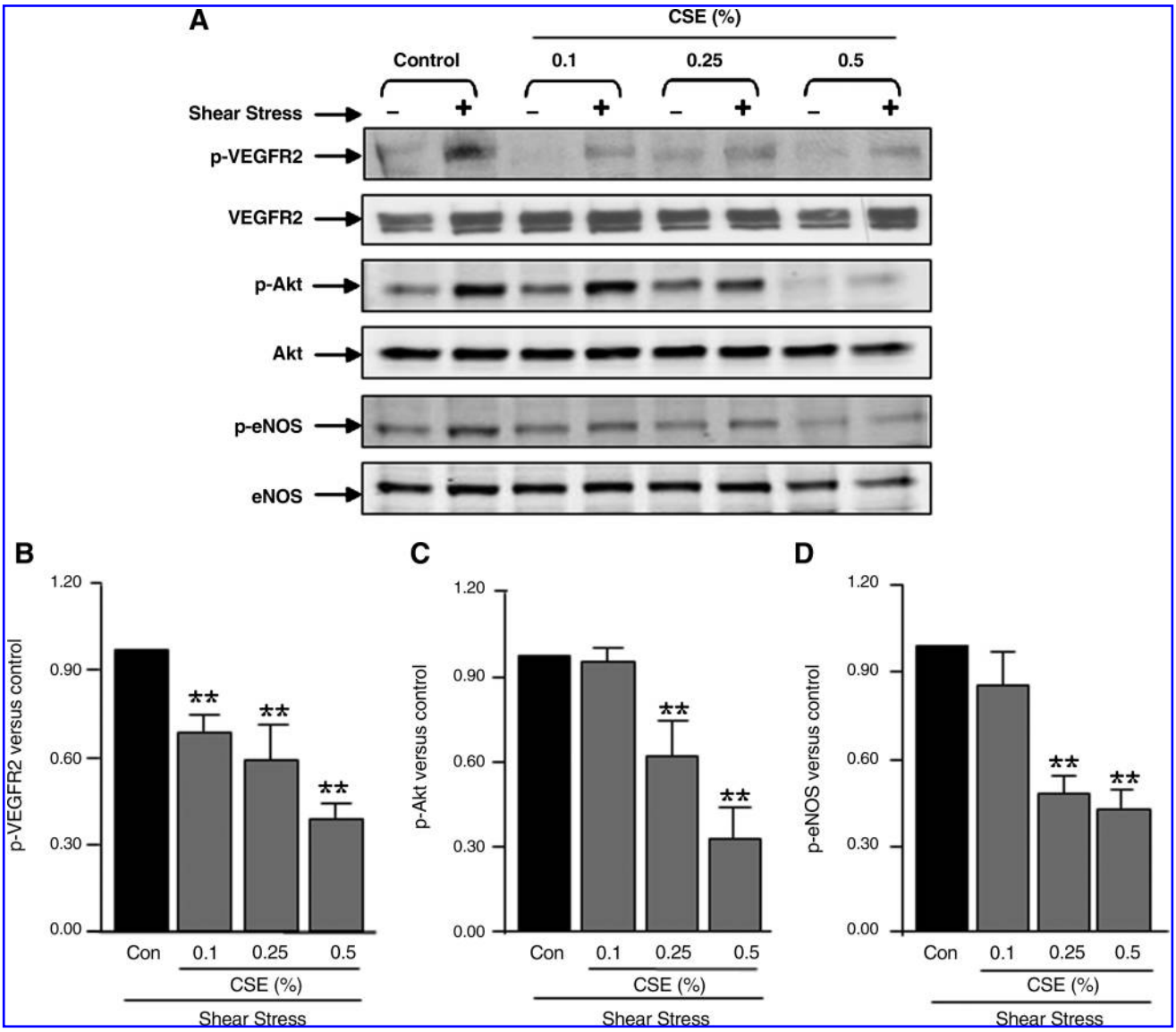

FIG. 2. CSE-impaired fluid shear stress–mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells. (A) Fluid shear-stress force (12 dyn/cm^2) was applied to CSE-treated HMVEC-Ls (2 h), as described in Materials and Methods. The levels of phosphorylated and total VEGFR2, Akt, and eNOS were measured with immunoblotting. The VEGFR2 blot showed two bands: a 230-kDa, fully glycosylated functional receptor, and a 200-kDa, semiglycosylated nonfunctional receptor. Phosphorylation was seen only in the fully glycosylated functional receptor. CSE treatment downregulated the shear stress–mediated VEGFR2 phosphorylation and its downstream signaling in a concentration-dependent manner. Histograms represent the mean ± SEM of the percentage of VEGFR2 (**B**), Akt (**C**), and eNOS (**D**) phosphorylation compared with the respective control experiments ($n = 3$). **$p < 0.01$ *vs.* control group. p-VEGFR2 = phosphorylated VEGFR2 (Tyr 1175); p-Akt = phosphorylated Akt (Ser 473); p-eNOS = phosphorylated eNOS (Ser 1177).

mediated phosphorylation of VEGFR2, Akt, and eNOS were significantly decreased in CSE-treated cells in a concentration-dependent manner when compared with control treatments ($p < 0.01$; Fig. 2). This indicates that CSE treatment impaired FSS-mediated VEGFR2 activation and its downstream signaling. However, FSS-mediated Akt and eNOS phosphorylation were not inhibited by the VEGFR2 inhibitor (NVP-AAD777) in endothelial cells (data not shown), possibly because of VEGFR2-independent pathways (5, 18).

CSE-induced oxidative/nitrosative stress caused the nitration of tyrosine residues in VEGFR2 in endothelial cells

CS contains reactive nitrogen species, which can nitrate proteins on tyrosine residues. Therefore, we hypothesized that VEGFR2 is nitrated on tyrosine residues after CS exposure of endothelial cells. Immunoprecipitated VEGFR2 was used to determine the nitrated tyrosine residue of VEGFR2 by

immunoblotting. CSE treatments significantly increased the levels of nitrated tyrosine residues on VEGFR2 in a concentration-dependent manner (Fig. 3). Pretreatment of endothelial cells with NO donor (Deta-NONOate) also increased the level of nitrated tyrosine residues on VEGFR2. Pretreatment of endothelial cells with NO scavenger (PTIO) and NAC significantly ($p < 0.001$) attenuated the CSE-induced nitration of tyrosine on VEGFR2, suggesting the involvement of CSE-induced oxidative/nitrosative stress in impaired VEGF- and FSS-mediated phosphorylation of tyrosine residues in VEGFR2 and its downstream signaling in endothelial cells.

NO and ROS scavengers inhibited the CSE-induced impairment of VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells

CS is known to induce oxidative/nitrosative stress in endothelial cells, leading to endothelial dysfunction (16). Therefore, we determined whether NO (PTIO) and ROS (SOD and catalase) scavengers can inhibit the CSE-induced impairment of VEGF-mediated VEGFR2 phosphorylation and its downstream signaling. We found that pretreatment with PTIO (100 μM) and the combination of PEG-SOD plus PEG-catalase (400 U/ml each) attenuated the CSE-impaired VEGF-mediated VEGFR2, Akt, and eNOS phosphorylation (Figs. 4 and 5). These data show that CSE-induced impairment of VEGF-mediated phosphorylation of VEGFR2 and its downstream signaling were significantly inhibited by NO and ROS scavengers. These data further support the notion that CSE-induced nitrosative/oxidative stress impairs the VEGF/VEGFR2 signaling pathway in endothelial cells.

Impact of NAC on CSE-induced impairment of VEGF-mediated phosphorylation of VEGFR2 and its downstream signaling in endothelial cells

We further investigated whether NAC, a thiol antioxidant and precursor of glutathione, attenuated the CS-induced impaired VEGFR2 phosphorylation and its downstream signaling. CSE-impaired phosphorylation of VEGFR2 and its downstream signaling were significantly attenuated by NAC (1 mM) pretreatment (Fig. 6). These data indicated the involvement of CSE-induced nitrosative/oxidative stress in the impairment of the VEGF/VEGFR2 signaling pathway in endothelial cells. However, post-treatment with NAC was unable to rescue the CSE-induced impaired phosphorylation of VEGFR2, suggesting that the observed effects were due to irreversible covalent modifications of VEGFR2 (data not shown).

Effect of NO and ROS scavengers on CSE-induced impairment of FSS-mediated signaling in endothelial cells

CSE-derived oxidants are known to cause endothelial dysfunction, whereas FSS is required for normal homeostasis of endothelial function. Furthermore, reactive oxygen and nitrogen species interfere with FSS-mediated endothelial function (2). We therefore determined whether NO scavenger (PTIO) and ROS scavengers (PEG-SOD and PEG-CAT) inhibited the CSE-induced endothelial dysfunction in an FSS model. As shown in Fig. 7, pretreatment with NO scavenger (PTIO, 100 μM) significantly inhibited the deleterious effects of CSE on FSS-mediated Akt and eNOS phosphorylation. However, CSE-induced impairment of FSS-mediated Akt and eNOS phophorylation was not inhibited by ROS scavengers. FSS is known to generate low levels of ROS (55), which was confirmed by diminished Akt and eNOS phosphorylation in ROS scavengers pretreatment alone and with CSE (Fig. 7).

NAC inhibited the CSE-induced impairment of FSS-mediated signaling in endothelial cells

Further to determine whether NAC inhibited the CSE-induced covalent modifications, we pretreated the cells with NAC, and phosphorylated and total protein levels of Akt and eNOS were measured with immunoblotting. Pretreatment with NAC (1 mM) significantly inhibited CSE-induced impairment of FSS-mediated phosphorylation of Akt and eNOS (Fig. 8). Taken together, these data indicated the CSE-impaired FSS-mediated Akt and eNOS phosphorylation were dependent on oxidative/nitrosative stress.

Discussion

Posttranslational modifications of receptor proteins induced by oxidative/nitrosative stress are known to impair cellular signaling (14, 41). CS contains reactive oxygen/nitrogen species that causes oxidative/nitrosative stress in endothelial cells (45). In our previous study, we demonstrated that CS-induced oxidative stress impaired VEGF-mediated endothelial function in HMVEC-Ls and in mouse lung (16). Furthermore, FSS-mediated endothelial function was impaired by CS exposure (51). However, the mechanism of CS-induced impaired VEGF- and FSS-mediated endothelial function was not studied. It has been shown that FSS-mediated endothelial function is associated with VEGFR2, PI3K, and eNOS phosphorylation (26). Hence, we studied the effect of CSE on VEGF- and FSS-mediated endothelial function by monitoring these signaling events. We demonstrated that CSE impaired VEGF- and FSS-mediated VEGFR2 phosphorylation, leading to decreased activation of Akt and eNOS. Tyrosine residues in VEGFR2 were nitrated in response to CSE treatment, which was attenuated by pretreatment of endothelial cells with the NO scavenger (PTIO) and NAC, confirming the involvement of CSE-induced oxidative/nitrosative stress in VEGFR2 modifications. Furthermore, our data suggest that VEGF-mediated decreased phosphorylation of VEGFR2 (Tyr 1175) caused by CSE was associated with increased nitration of tyrosine residue. Therefore, CSE-induced oxidative/nitrosative stress may modify the key phosphorylation sites in VEGFR2 and render it inactive for VEGF- and FSS-mediated signaling. VEGF- and FSS-mediated VEGFR2 activation and its downstream signaling are important in regulating endothelial function, including cell survival, proliferation, and angiogenesis (25, 29, 31). Complex mixtures of chemical species from CS potentially affect different events of angiogenesis, vessel development, vessel migration, and cell proliferation (17). These data are in accordance with our previous findings that showed that the CSE-induced impaired VEGF-mediated cell migration and angiogenesis are significantly attenuated by pretreatment with NAC in endothelial cells (16).

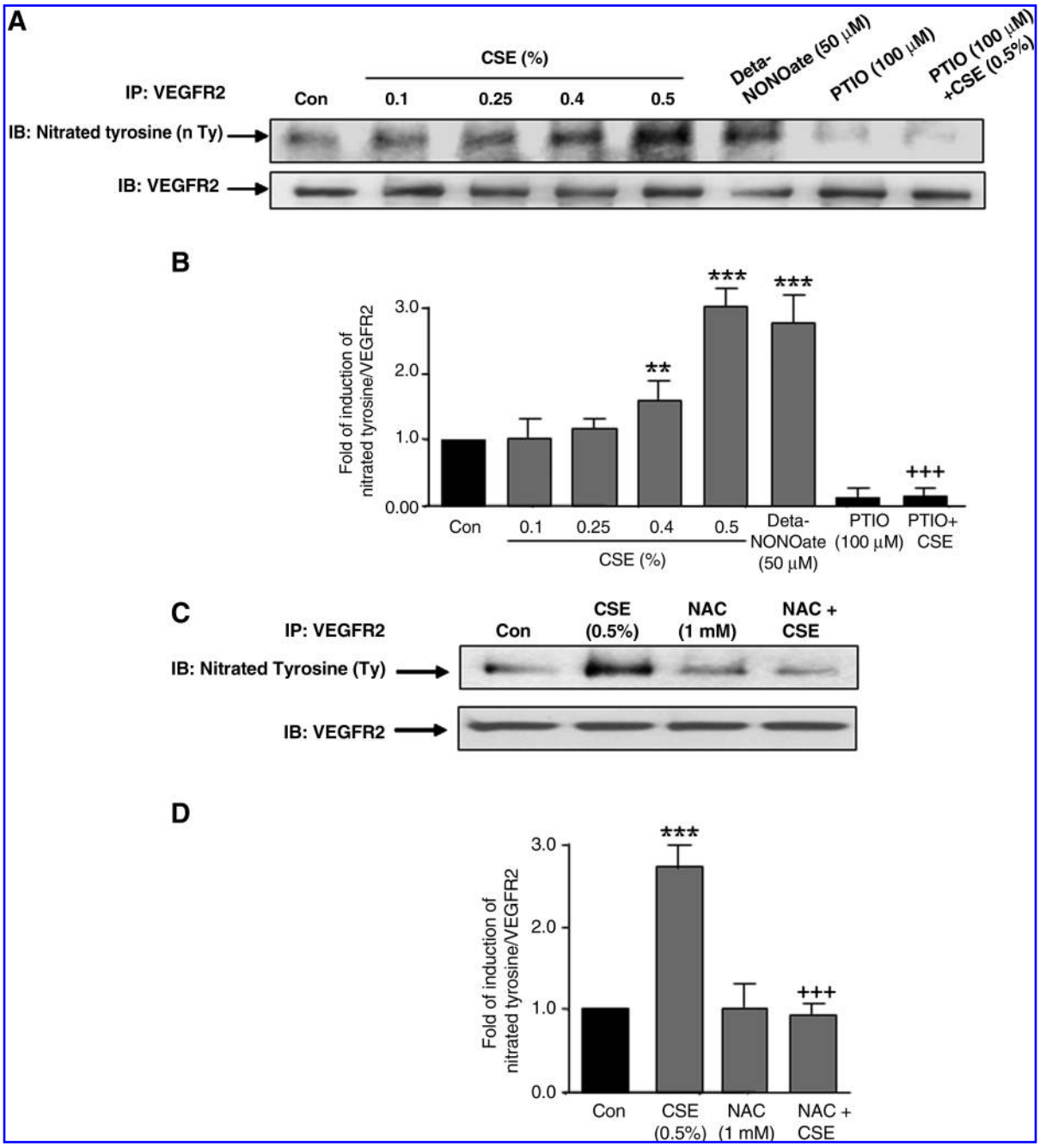

FIG. 3. CSE caused nitration of tyrosine residue in VEGFR2 in endothelial cells. (**A**) HMVEC-Ls were treated with CSE (0.1–0.5%), NO donor (Deta-NONOate, 50 μM), NO scavenger (PTIO, 100 μM), or a combination of CSE with NO scavenger. VEGFR2 was immunoprecipitated, and immunoblot was performed to determine the nitration of tyrosine residues of VEGFR2. CSE treatment increased the level of nitrated tyrosine residues on VEGFR2 in a concentration-dependent manner. Pretreatment with NO scavenger (PTIO) attenuated the CSE-induced nitration of tyrosine on VEGFR2. (**B**) Histograms represent the mean ± SEM of the percentage of nitrated tyrosine residues in VEGFR2 compared with those in the control experiments ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group. $^{+++}p < 0.001$ *vs.* CSE-alone–treated group. (**C**) HMVEC-Ls were treated with CSE (0.5%), NAC (1 mM), or a combination of CSE with NAC. VEGFR2 was immunoprecipitated, and immunoblot was performed to determine the nitration of tyrosine residues of VEGFR2. CSE increased the level of nitrated tyrosine residues on VEGFR2. Pretreatment with NAC attenuated the CSE-induced nitration of tyrosine on VEGFR2. (**D**) Histograms represent the mean ± SEM of the percentage of nitrated tyrosine residues in VEGFR2 compared with those in the control experiments ($n = 3$). $^{***}p < 0.001$ *vs.* control group. $^{+++}p < 0.001$ *vs.* CSE-alone–treated group.

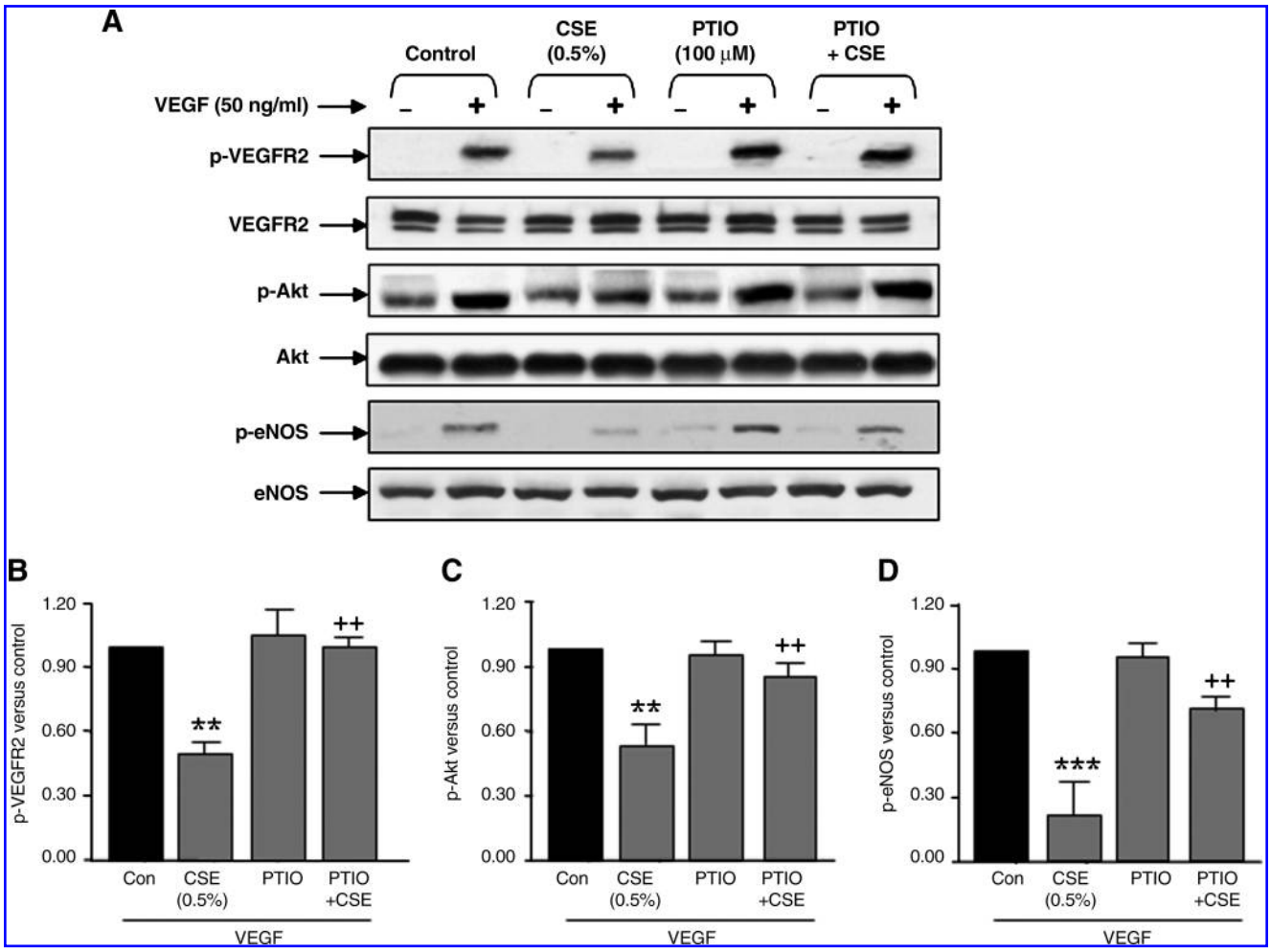

FIG. 4. Nitric oxide scavenger (PTIO) attenuated the CSE-induced impairment of VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells. (**A**) HMVEC-Ls were treated with CSE (0.5%) and incubated with VEGF in the presence or absence of the NO scavenger (PTIO, 100 μM), and the levels of phosphorylated and total VEGFR2, Akt, and eNOS were measured with immunoblotting. CSE-induced impairment of VEGF/VEGFR2 signaling was attenuated in presence of the NO scavenger (PTIO). Histograms represent the mean ± SEM of the percentage of VEGFR2 (**B**), Akt (**C**), and eNOS (**D**) phosphorylation compared with the respective control experiments ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group; $^{++}p < 0.01$ *vs.* CSE-alone–treated group. p-VEGFR2 = phosphorylated VEGFR2 (Tyr 1175); p-Akt = phosphorylated Akt (Ser 473); p-eNOS = phosphorylated eNOS (Ser 1177).

CS-induced oxidative stress is involved in the development of vascular dysfunctions due to loss in the vascular bed, particularly in emphysema (34, 60). However, the mechanism of impaired endothelial functions caused by CS is not known. In the present study, we demonstrated that CSE induced impaired VEGF- and FSS-mediated VEGFR2 activation and its downstream signaling in endothelial cells. This is supported by other studies indicating that long-term CS exposure downregulated VEGFR2 and VEGF expressions/levels in human and rodent lungs (16, 27, 35, 50, 53). Furthermore, VEGFR2 blockade caused endothelial cell apoptosis and transdifferentiation to smooth muscle-like and neuron-like cells (49). A recent report showed that neutrophil elastase treatment cleaves VEGF, thus influencing VEGF activity, leading to the recruitment of inflammatory cells, compared with intact VEGF (32). CS also impairs angiogenesis and cell migration in endothelial cells (16, 17, 37). Hence, our results support the concept that CS causes disruption of VEGFR-mediated survival signals in pulmonary capillary endothelial cells, resulting in avascular alveolar septa and emphysematous lungs (27, 28, 54).

FSS force can be atheroprotective or atherogenic, depending on the fluid pattern (steady laminar *vs.* oscillatory) (4, 13). Atheroprotective FSS is known to activate Nrf2 through the PI3K/Akt-dependent pathway and regulates antioxidant-responsive genes that maintain the endothelial intracellular redox state, thereby protecting against oxidative stress challenge (13). However, the direct relation between FSS, VEGFR2, and Nrf2 is not known. Disruption of the *Nrf2* gene in mice leads to early onset of CS-induced emphysema (48). It is known that Nrf2, a redox-sensitive transcription factor, is prevented from translocating into the nucleus from the cytosol of macrophages, alveolar, and airway epithelial cells because of CSE-induced posttranslational modifications, such as aldehyde/carbonyl adduct formation and nitration, and thereby failed to induce antioxidant-responsive genes (30).

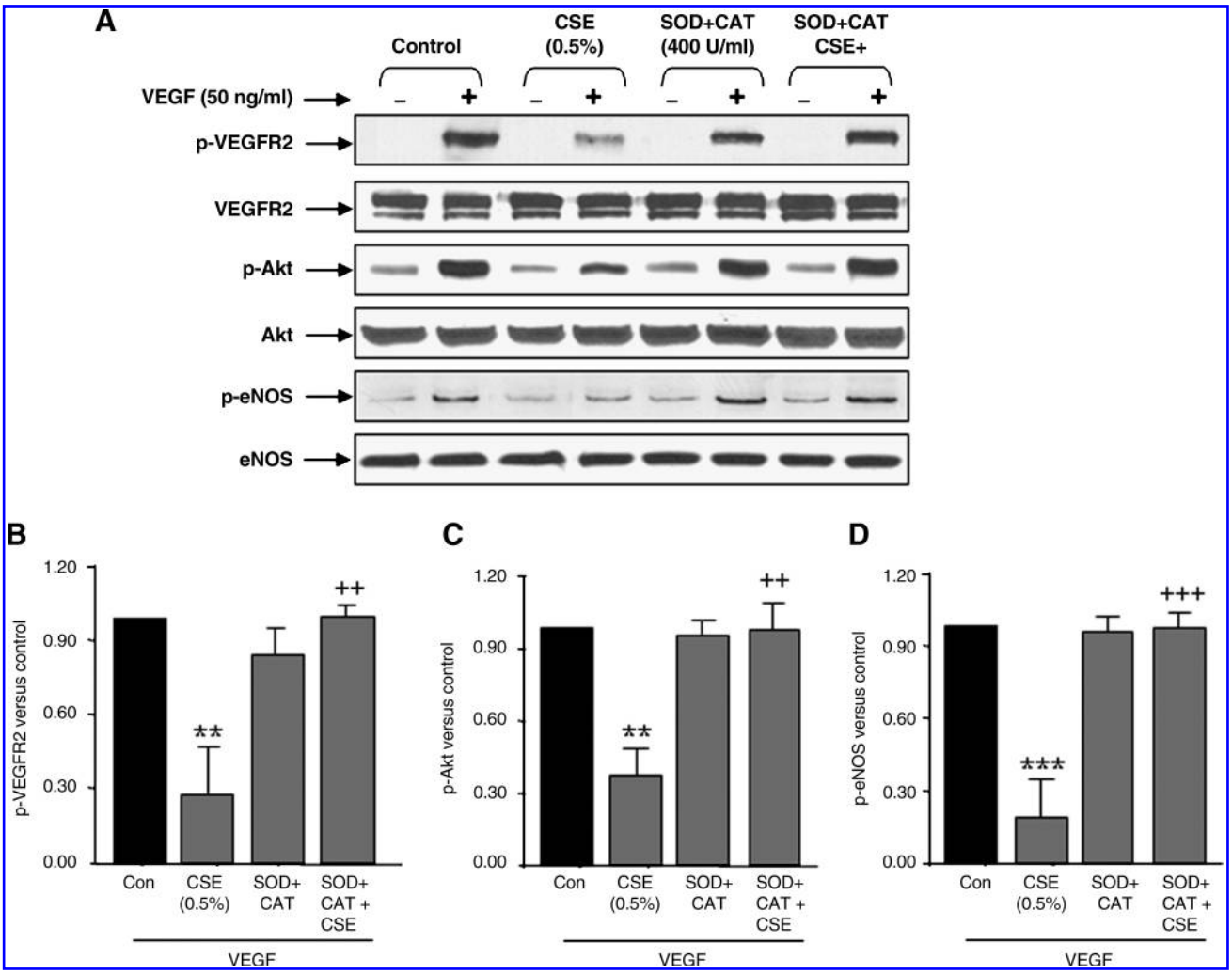

FIG. 5. ROS scavengers (SOD and catalase) attenuated the CSE-induced impairment of VEGF-mediated VEGFR2 phosphorylation and its downstream signaling in endothelial cells. (**A**) HMVEC-Ls were treated with CSE (0.5%) in the presence or absence ROS scavengers (SOD and catalase, 400 U/ml each), and incubated with VEGF, and the levels of phosphorylated and total VEGFR2, Akt, and eNOS were measured with immunoblotting. CSE-induced impairment of VEGF/VEGFR2 signaling and its downstream signaling were attenuated by ROS scavengers. Histograms represent the mean ± SEM of the percentage of VEGFR2 (**B**), Akt (**C**), and eNOS (**D**) phosphorylation compared with the respective control experiments ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$ *vs.* control group; $^{++}p < 0.01$; $^{+++}p < 0.001$ *vs.* the CSE-alone–treated group. p-VEGFR2 = phosphorylated VEGFR2 (Tyr 1175); p-Akt = phosphorylated Akt (Ser 473); p-eNOS = phosphorylated eNOS (Ser1177).

Hence we tested the hypothesis that CSE-induced impaired endothelial function is mediated by an oxidant/antioxidant imbalance in endothelial cells. Our data showed that antioxidants can inhibit the CSE-induced impairment of VEGF- and FSS-mediated cellular signaling. Therefore, it is possible that CSE-induced impairment of VEGF- and FSS-mediated Akt activation leads to endothelial dysfunction through a deficient/inactive Nrf2-dependent mechanism. However, further studies are required to confirm this possibility.

Endothelial dysfunction is an early event in atherosclerosis and is known to occur in smokers (1, 22, 47, 57). However, the mechanism for increased risk of impaired endothelial function in response to CS is not well understood. It is presumed to be the consequence of oxidative and nitrosative constituents present in CS. eNOS is exclusively expressed in endothelial cells and plays a vital role in regulating endothelial function (7). eNOS can be activated by chemical stimuli such as acetylcholine and VEGF or by a mechanical stimulus such as FSS in the blood vessels (27). As a result of eNOS activation, NO is released and causes vasodilatation (7). In the present investigation, we showed that CSE induced impaired VEGF- and FSS-mediated eNOS activation through a VEGFR2/Akt-dependent mechanism. This is consistent with previous studies showing that CSE-induced irreversible inhibition of eNOS activity is due to impaired kinase signaling (52, 56). However, NO is converted into peroxynitrite in the presence of reactive oxygen species derived from CSE (21, 40). This not only results in nitration of tyrosine residues on VEGFR2, thereby inhibiting its downstream signaling, as shown in our study, but also can lead to impairment of endothelial functions such as cell migration, angiogenesis, and endothelium-dependent relaxation.

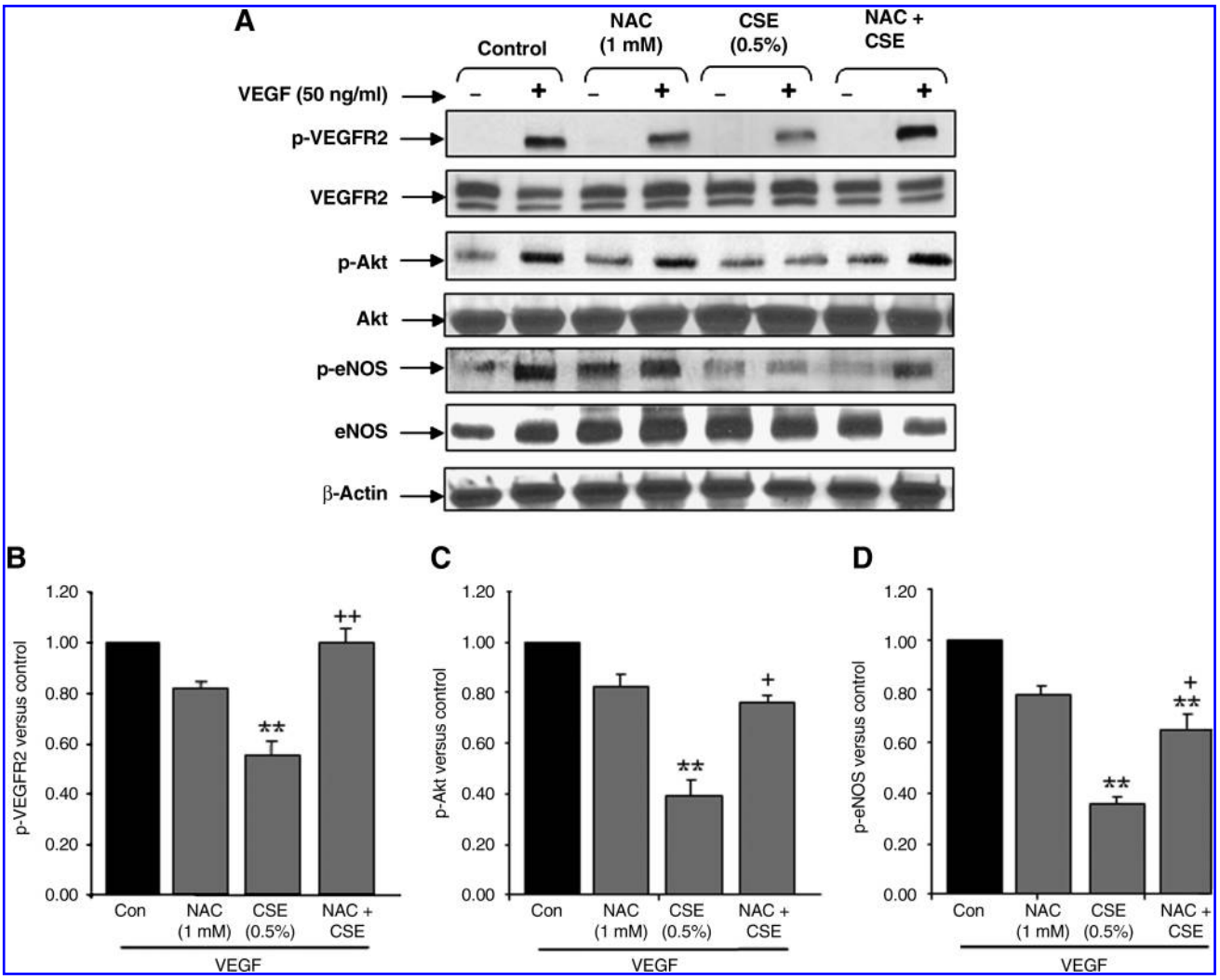

FIG. 6. Effect of *N*-acetyl-L-cysteine on CSE-induced impairment of VEGF-mediated phosphorylation of VEGFR2 and its downstream signaling in endothelial cells. (**A**) HMVEC-Ls were treated with CSE (0.5%) in the presence or absence of NAC (1 m*M*), and incubated with VEGF, as described in Materials and Methods. The levels of phosphorylated and total VEGFR2, Akt, and eNOS were measured with immunoblotting. CSE-induced impairment of VEGF/VEGFR2 and its downstream signaling were attenuated in the presence of NAC. Histograms represent the mean ± SEM of the percentage of VEGFR2 (**B**), Akt (**C**), and eNOS (**D**) phosphorylation compared with respective control experiments ($n = 3$). $^{**}p < 0.01$ *vs.* control group; $^{+}p < 0.05$; $^{++}p < 0.01$ *vs.* CSE-alone–treated group. p-VEGFR2 = phosphorylated VEGFR2 (Tyr 1175); p-Akt = phosphorylated Akt (Ser 473); p-eNOS = phosphorylated eNOS (Ser 1177).

Our finding is corroborated by a recent study that showed that CSE treatment increased the peroxynitrite production and monomeric inactive form of eNOS, thus leading to decreased NO bioavailability in bovine aortic endothelial cells (44). Nevertheless, our data provide evidence that CSE-induced impaired VEGF- and FSS-mediated endothelial function was inhibited by NO and ROS scavengers.

Our data further revealed the inhibition of CSE-induced impairment of VEGF- and FSS-mediated downstream signaling, as evidenced by pretreatment of cells with NO and ROS scavengers. ROS can cause phosphorylation/dephosphorylation of Akt, which has been shown to be essential for VEGFR2 downstream signaling and endothelial function (10, 23, 62). Here, we showed that pretreatment of cells with NO and ROS scavengers significantly inhibited the impairment of VEGF-mediated Akt and eNOS phosphorylation in response to CSE. Consistent with our data, previous studies also showed that pretreatment of cells with antioxidants inhibited the impairment of VEGF-induced phosphorylation of Akt and eNOS in response to CSE treatment (37). However, ROS scavengers were unable to inhibit the CSE-induced impairment of FSS-mediated phosphorylation of Akt and eNOS. FSS signals are known to be mediated by ROS-dependent mechanisms (55). Therefore, it is possible that ROS scavengers, along with the complex chemicals present in CSE (tar and electrophilic compounds), can potentially impair the Akt-eNOS signaling pathway. This contention is further validated by the finding that pretreatment of cells with the nucleophilic thiol agent NAC (because of its reducing property) significantly inhibited the impairment of VEGF- and FSS-mediated Akt and eNOS phosphorylation in response to CSE. NAC, a key thiol antioxidant (precursor of glutathione), has the

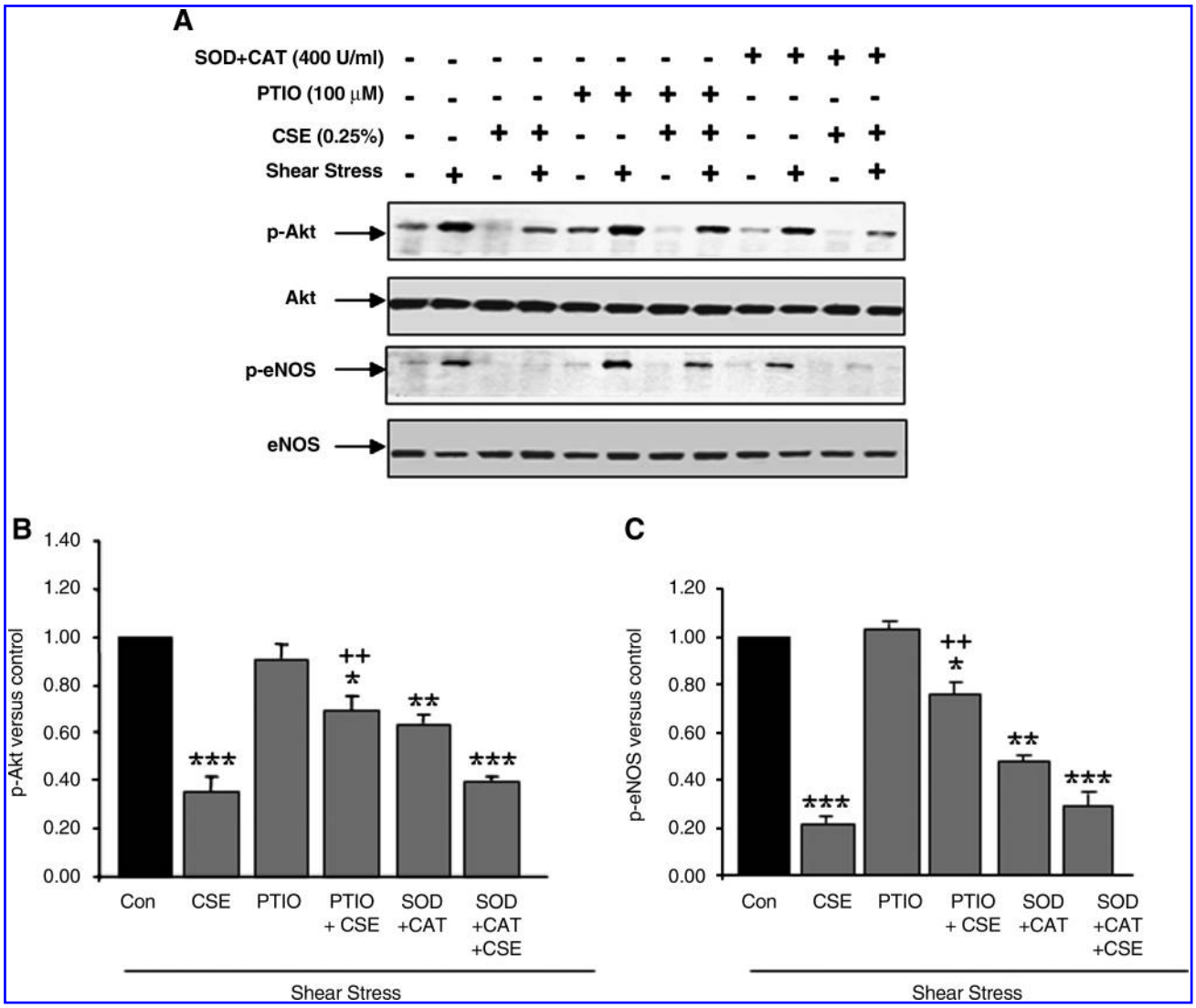

FIG. 7. Effect of nitric oxide scavenger (PTIO) and ROS scavengers (SOD + catalase) on CSE-induced impairment of FSS-mediated phosphorylation of Akt and eNOS in endothelial cells. (**A**) HUVECs were treated with CSE (0.25%) in the presence or absence of NO scavenger (PTIO, 100 μM) and ROS scavengers (combination of SOD, 400 U/ml, and catalase, 400 U/ml); thereafter, fluid shear-stress force (12 dyn/cm^2) was applied, as mentioned in Materials and Methods. The levels of phosphorylated and total Akt and eNOS were measured by using immunoblotting. Histograms represent the mean ± SEM of the percentage of Akt (**B**) and eNOS (**C**) phosphorylation compared with the respective control experiments ($n = 3$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ *vs.* control group.$^{++}p < 0.01$ *vs.* CSE-alone–treated group. p-Akt = phosphorylated Akt (Ser 473); p-eNOS = phosphorylated eNOS (Ser 1177).

capability of maintaining the reducing environment at a physiologic level in the cells; hence, NAC treatment alone also showed a small decrease in FSS-mediated phosphorylation of Akt and eNOS. Consistent with our data, a recent study showed that lung and aorta isolated from glutathione-*S*-transferase–null mice were more vulnerable to CS-induced toxic insults than were their wild-type controls (11), which further reveals the protective role of antioxidants in preventing CSE toxicity.

Taken together, our data from HMVEC-Ls and HUVECs support the concept that CSE-induced oxidative/nitrosative stress impairs VEGF- and FSS-mediated VEGFR2 signaling, leading to endothelial dysfunction. We further showed for the first time that CSE causes modifications in VEGFR2 by NO-mediated modifications, perhaps on tyrosine residues, leading to its inactivation. The present study also showed that reactive oxygen/nitrogen scavengers inhibited the CSE-induced impairment of VEGF-mediated VEGFR2, Akt, and eNOS phosphorylation, whereas ROS scavengers had no effect on Akt and eNOS phosphorylation, particularly during the FSS condition. These data suggest that CSE-induced downregulation of Akt and eNOS occurs by covalent modifications under the FSS condition. Our data further suggest that pretreatment with NAC significantly inhibited CSE-induced impairment of VEGF- and FSS-mediated phosphorylation of VEGFR2 and its downstream signaling through its direct antioxidant properties and its indirect role as a glutathione precursor. Our study showed the modulatory effect

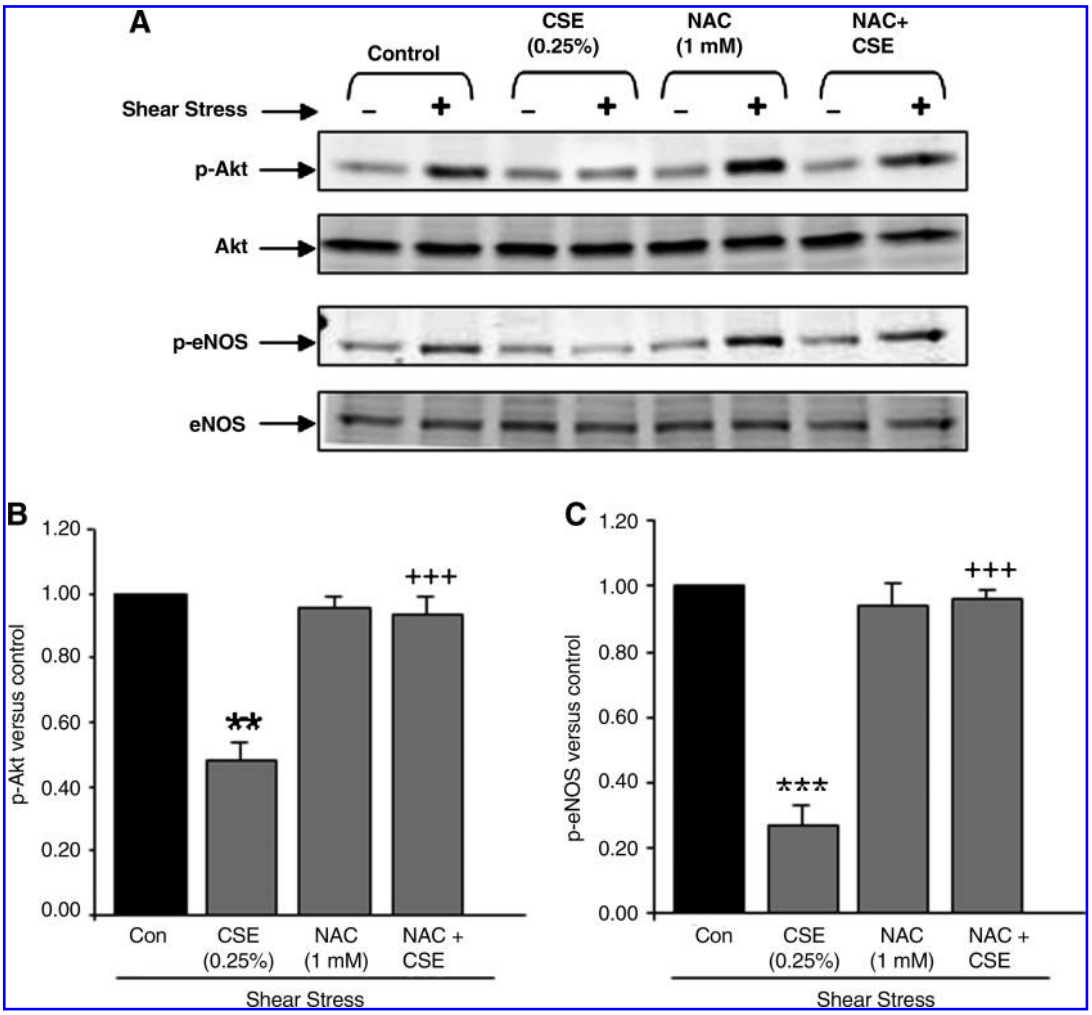

FIG. 8. Effect of *N*-acetyl-L-cysteine on CSE-induced impairment of FSS-mediated phosphorylation of Akt and eNOS in endothelial cells. (**A**) HUVECs were treated with CSE (0.25%) in presence or absence of *N*-acetyl-L-cysteine (1 m*M*); thereafter, fluid shear-stress force (12 dyn/cm^2) was applied, as mentioned in Materials and Methods. Phosphorylated and total protein levels of Akt and eNOS were measured with immunoblotting. CSE-induced impaired Akt and eNOS signaling were attenuated in presence of *N*-acetyl-L-cysteine. Histograms represent the mean ± SEM of the percentage of Akt (**B**) and eNOS (**C**) phosphorylation compared with respective control experiments ($n = 3$). $^{**}p < 0.01$; $^{***}p < 0.001$; *vs.* control group. $^{+++}p < 0.001$ *vs.* CSE-alone–treated group. p-Akt = phosphorylated Akt (Ser 473). p-eNos = phosphorylated eNos (ser1177).

of CS on VEGF- and FSS-mediated downstream signaling, and the potential role of antioxidants in quenching deleterious effect of CS in endothelial cells. These new findings not only define the basic understanding of CS-induced oxidative/nitrosative stress-mediated impairment of endothelial function/signaling in response to VEGF/FSS, but also have broad implications in pathogenesis of various CS-induced pulmonary and cardiovascular diseases associated with endothelial dysfunction.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

COPD = chronic obstructive pulmonary disorder
CS = cigarette smoke
CSE = cigarette smoke extract
EGM-2 = endothelial cell growth medium 2
eNOS = endothelial nitric oxide synthase
FSS = fluid shear stress
HMVEC-Ls = human lung microvascular endothelial cells
HUVECs = human umbilical vein endothelial cells
NAC = *N*-acetyl-L-cysteine
NO = nitric oxide
Nrf2 = nuclear factor erythroid-2–related factor 2
PBS = phosphate-buffered saline
PI3K = phosphoinositide 3-kinase
PTIO = 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
ROS = reactive oxygen species
SOD = superoxide dismutase
VEGF = vascular endothelial growth factor
VEGFR2 = VEGF receptor 2

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