Stable Compounds of Cigarette Smoke Induce Endothelial Superoxide Anion Oxidase Activation

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Objective—Endothelial dysfunction is an early manifestation of cigarette smoke (CS) toxicity. We have previously demonstrated that CS impairs nitric oxide (NO)-mediated endothelial function via increased generation of superoxide anion ($O_2^-$). In these studies, we investigated whether stable compounds present in CS activate specific pathways responsible for the increased endothelial $O_2^-$ production.

Methods and Results—Short exposure of bovine pulmonary artery endothelial cells (BPAECs), human pulmonary artery endothelial cells, and rat pulmonary arteries to CS extracts (CSEs) resulted in a large increase in $O_2^-$ production (20-fold, 3-fold, and 2-fold increase, respectively; P<0.05 versus control), which was inhibited by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitors diphenyleneiodinium, apocynin, and gp91 docking sequence-tat peptide but not by oxypurinol, the NO synthase inhibitor N$^\omega$-nitro-$\epsilon$-arginine methyl ester, or the mitochondrial respiration inhibitor rotenone. Exposure of BPAECs to acrolein, a stable thiol-reactive agent found in CS, increased $O_2^-$ production 5-fold, which was prevented by prior inhibition of NADPH oxidase.

Conclusions—These studies demonstrate that thiol-reactive stable compounds in CS can activate NADPH oxidase and increase endothelial $O_2^-$ production, thereby reducing NO bioactivity and resulting in endothelial dysfunction. Clinically, these studies may contribute to the development of agents able to mitigate CS-mediated vascular toxicity. (Arterioscler Thromb Vasc Biol. 2004;24:1031-1036.)

Key Words: nitric oxide ■ superoxide anion ■ cigarette smoke ■ NADPH oxidase ■ endothelium

Cigarette smoking is the major cause of preventable morbidity and mortality in the United States and constitutes a major risk factor for vascular disease, including atherosclerosis and pulmonary hypertension. Clinical and animal studies have demonstrated that cigarette smoke (CS) produces endothelial dysfunction in the systemic and pulmonary circulation. However, the mechanisms by which CS impairs nitric oxide (NO)-mediated endothelial function have not been fully elucidated, although substantial evidence is accumulating suggesting that increased superoxide anion ($O_2^-$) generation may play a critical role.

Two potential inducible intracellular sources of reactive oxygen species (ROS) are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a flavin-containing enzyme present in polymorphonuclear cells and in the systemic and pulmonary vasculature, and xanthine oxidase, a modified form of the ubiquitous enzyme xanthine dehydrogenase. In resting cells, NADPH oxidase has a low level of activity, and its protein components are segregated into cytoplasmic and plasma membrane compartments. Once activated, NADPH oxidase catalyzes the transfer of electrons from NADPH to molecular oxygen with subsequent production of $O_2^-$. Cytokines such as tumor necrosis factor-$\alpha$ and interleukin-1, and vasoactive agents such as Angiotensin II increase the expression and activity of this enzyme in several cell types, including endothelial cells, vascular smooth muscle cells, as well as glomerular mesangial cells, resulting in the production of $O_2^-$ for prolonged periods.

CS can be divided into 2 phases: tar and gas-phase smoke. Both phases contain high concentrations of ROS, NO, peroxynitrite, peroxynitrate, and free radicals of organic compounds. In addition to these short-lived, highly reactive substances, the gas phase of CS contains varying amounts of more stable substances that also have the potential to increase intracellular production of ROS. These include a series of $\alpha,\beta$-unsaturated aldehydes, such as acrolein and crotonaldehyde, $\alpha,\beta$-unsaturated ketones, and a number of saturated aldehydes. These stable compounds have been shown to react nonenzymatically with thiol groups that may be involved in the regulation of enzymes, including NADPH oxidase and xanthine oxidase. Furthermore, because of their stability, these compounds could induce the production of $O_2^-$.
of ROS in vascular fields remote from the primary site of exposure.\(^1\)

During smoking, the pulmonary circulation is the first site exposed to the gas phase of CS. In these studies, we tested the hypothesis that the thiol-reactive substances in CS induce the synthesis of ROS using both intact pulmonary arteries and pulmonary artery endothelial cells. This study was designed to show that: (1) production of \(O_2^-\) by intracellular sources of ROS is increased after exposure of pulmonary arteries or isolated cells to CS, (2) NADPH oxidase or xanthine oxidase mediates the CS-induced generation of intracellular \(O_2^-\) production, and (3) acrolein and related thiol-reactive substances, known to be present in CS, mimic the actions of CS.

**Materials and Methods**

**Preparation of CS Extracts**

CS extracts (CSEs) were prepared as described in our previous studies.\(^3\) Briefly, CS from one research filtered cigarette (Tobacco-Health Research, University of Kentucky) was combusted for 5 minutes using a constant negative pressure machine that simulates the act of smoking. The CS was bubbled through 5.0 mL of Krebs buffer at room temperature. Once this process was completed, the CSE was collected and diluted with untreated Krebs buffer as indicated in Results.

**Pulmonary Arteries Protocol**

Fresh rat pulmonary arteries were cut in 3- to 4-mm segments and exposed to CSE (25%) for 30 minutes. At the end of the exposure period, vessel segments were washed and \(O_2^-\) production was measured by chemiluminescence of lucigenin as described.\(^4\) Briefly, pulmonary artery segments were added to a vial containing dark-adapted lucigenin (5 \(\mu\)mol/L) in 1 mL Krebs buffer (pH 7.4). Photon emission was measured every minute for 10 minutes in a Berthold FB12 luminometer. A buffer blank was subtracted from each reading and \(O_2^-\) production was adjusted for dry weight and expressed as cpm/mg dry weight.

**Cell Culture**

All cells were grown in a humidified cell incubator 95\% \(O_2\)/5\% \(CO_2\), and passed when confluent by trypsinization. Bovine pulmonary artery endothelial cells (BPAECs) were purchased from Cell Applications, Inc. and grown in DME (GIBCO) supplemented with 10\% FCS (Hyclone, Logan, UT). Human pulmonary artery endothelial cells (HPAEcs) were purchased from Cascade Biologicals (Portland, OR) and grown in medium 200 supplemented with low serum supplement (Cascade).

**Cell Suspension Protocol**

All cell types used in these experiments were grown in their respective cell culture media in 75-cm\(^2\) flasks. When confluent, cells were made quiescent in cell culture media supplemented with 0.5\% FCS for 72 hours. To determine a dose response to CSE, BPAECs were exposed to CSE in various concentrations (6\% to 25\%) for 30 minutes. Subsequently, all cell suspension experiments, including those with BPAECs and HPAECs, were performed using 25\% CSE with and without the following compounds: (1) the NADPH oxidase inhibitors diphenyleneiodinium (DPI; 10 \(\mu\)mol/L), apocynin (100 \(\mu\)mol/L), or gp91 docking sequence-tat peptide (gp91ds-tat; 50 \(\mu\)mol/L), kindly provided by Patrick J Pagano, PhD, Henry Ford Hospital, Detroit, Mich.; (2) the mitochondrial respiration inhibitor rotenone (1 \(\mu\)mol/L); (3) the xanthine oxidase inhibitor oxypurinol (Oxy; 100 \(\mu\)mol/L); or (4) the NO synthase inhibitor \(\text{N}^\text{G}\)-nitro-L-arginine methyl ester (L-NAME 2 \(\text{mmol/L}\)). In separate experiments, instead of CSE, BPAECs were exposed for 30 minutes to Krebs containing the thiol-reactive \(\alpha\),\(\beta\)-unsaturated aldehyde acrolein (10 to 300 \(\mu\)mol/L), the 3-carbon-saturated aldehyde propionaldehyde (300 \(\mu\)mol/L), or the thiol reagent \(N\)-ethylmaleimide (NEM 40 \(\mu\)mol/L) with and without DPI (10 \(\mu\)mol/L) in the medium.

At the end of the incubation period, cells were washed, trypsinized, and resuspended in 1 mL Krebs buffer and kept on ice until use. Cell suspensions were added to a cuvette containing 1 mL of phosphate buffer (pH 7.4) and lucigenin (5 \(\mu\)mol/L).\(^4\) \(O_2^-\) production was measured as described above, adjusted for protein content, and expressed as cpm/mg protein.

In addition, we measured the production of ROS by using a dichlorofluorescein diacetate (DCFDA) assay, as described by Norenberg et al.\(^2\) On the day of the experiment, the cells were washed and incubated for 30 minutes at 37\°C in 2 mL of DMEM containing 10 \(\mu\)mol/L DCFDA. After loading with DFCDA, the cells were washed with 25\% CSE with and without DPI (10 \(\mu\)mol/L) for 30 minutes at 37\°C. After incubation, the cells were washed and resuspended in 500 \(\mu\)L 0.2\% Triton X-100, centrifuged, and transferred to a 96-well microtiter plate. Fluorescence was measured at an excitation wavelength of 485 nm and emission light of 535 nm. Protein was measured by Bio-Rad, and results were expressed as relative light units (RLU)/mg protein.

**NADPH Oxidase Activity**

Cells exposed to different experimental conditions were washed 3 times with Hanks' balanced salt solution, trypsinized, resuspended in 300 \(\mu\)L homogenization buffer (phosphate buffer 50 \(\mu\)mol/L, EDTA 0.01 \(\mu\)mol/L, leupeptin 2 \(\mu\)mol/L, pepstatin A 2 \(\mu\)mol/L, phenylmethylsulfonyl fluoride or phenylmethanesulfonyl fluoride 1 \(\mu\)mol/L, pH 7.4), and homogenized by sonication (15 s pulses \times 3). NADPH oxidase activity was measured by using a chemiluminescence assay in a 50 \(\mu\)mol/L phosphate buffer pH 7.4, containing 1.0 \(\mu\)mol/L EGTA, 5 \(\mu\)mol/L lucigenin, and 100 \(\mu\)mol/L NADPH as substrate. The assay was initiated by addition of 20 \(\mu\)L homogenate. In some experiments, 10 \(\mu\)mol/L DPI was added before the readings, and in others the homogenates were denatured by boiling for 10 minutes before performing the assay. \(O_2^-\) production was measured by chemiluminescence of lucigenin as described above.\(^4\) In some experiments, cell homogenates were fractionated in cytosolic and a membranous fraction. Briefly, after lysis cell homogenates were centrifuged at 13 000 \(\times g\) for 20 minutes at 4\°C. The supernatant (cytosolic fraction) was removed and the pellet-containing plasma and mitochondrial membranes were resuspended in 300 \(\mu\)L lysis buffer. Protein content in homogenates and in cell fractions was measured by using the Bio-Rad method. \(O_2^-\) production was adjusted for protein content and expressed as cpm/\mu g protein.

**Statistical Analysis**

Data are expressed as mean±SEM. For statistical comparison involving 2 groups, an unpaired Student \(t\) test was used, whereas for comparison involving >2 groups, ANOVA using the Statview 512 statistical program was used (BrainPower, Inc.). Significance was considered present when \(P<0.05\).

**Results**

**CS Induces \(O_2^-\) Production in Pulmonary Artery Endothelial Cells**

The role of the endothelium as one of the sites of CS-induced \(O_2^-\) production was studied by exposing BPAECs to a range of CSE concentrations (Figure 1). BPAECs were used because they are readily available and are known to express NADPH
oxidase.22,23 As shown in Figure 1, CSE significantly increased $O_2^\cdot_-$ production in BPAECs in a concentration-dependent manner. The production of $O_2^\cdot_-$ by 25% CSE-treated cells was 20-fold greater than control cells and nearly 2-fold greater than control cells treated with 12% CSE. Based on these results, 25% CSE was selected for use in all subsequent experiments. Importantly, this concentration of CSE did not result in increased cell lethality as assessed by trypan blue exclusion.

The enzymes xanthine oxidase and NADPH oxidase were considered the 2 most likely sources of intracellular CS-induced $O_2^\cdot_-$ production. Their functional participation in this reaction was assessed through the use of metabolic inhibitors. DPI, an inhibitor of NADPH oxidase, almost completely blocked the increase in $O_2^\cdot_-$ production in 25% CSE-treated BPAECs, whereas Oxy, the xanthine oxidase inhibitor, showed only minimal inhibition (Figure 2). However, in addition to inhibiting NADPH oxidase, DPI can also inhibit other flavin-containing enzymes such as NO synthase (NOS) and $O_2^\cdot_-$ superoxide production catalyzed by the mitochondrial respiratory chain. We therefore performed separate experiments using apocynin and gp91ds-tat, 2 specific NADPH oxidase inhibitors. As control for gp91ds-tat, cells were also exposed to an inactive form of this inhibitor, gp91ds-tat scramble (gp91Scramble). Pretreatment with apocynin or gp91ds-tat but not with gp91Scramble resulted in a significant reduction in $O_2^\cdot_-$ production in BPAECs exposed to CSE: control 1092±78 cpm/μg protein, CSE 7134±896 cpm/μg protein, CSE+apocynin 2719±133 cpm/μg protein, CSE+gp91ds-tat 1679±0.3 cpm/μg protein, CSE+gp91Scramble 6914±920 cpm/μg protein (n=6, *$P<0.05$ versus CSE), suggesting that NADPH oxidase is the main source of $O_2^\cdot_-$ in the endothelium after CSE exposure. In contrast, pretreatment with rotenone, an inhibitor of the mitochondrial respiratory chain, did not inhibit $O_2^\cdot_-$ formation, suggesting a nonsignificant role for the mitochondrial respiratory chain, which did not inhibit $O_2^\cdot_-$ production induced by CSE in BPAECs is inhibited

The role of NADPH oxidase in CS-induced $O_2^\cdot_-$ production was further corroborated by measuring the activity of this enzyme in cell homogenates prepared from control and CSE-treated BPAECs. Significantly more $O_2^\cdot_-$ production was observed in homogenates prepared from CSE-treated BPAECs than in homogenates prepared from control BPAECs (Figure 3A). No $O_2^\cdot_-$ production was observed in homogenates prepared from control BPAECs (Figure 3A). No $O_2^\cdot_-$ production was detected when homogenates were denatured by boiling before performing the assay (not shown). $O_2^\cdot_-$ production in homogenates from both control and CSE-pretreated cells was inhibited by DPI, indicating that the source of $O_2^\cdot_-$ was NADPH oxidase. No $O_2^\cdot_-$ production was observed in the absence of added NADPH, thereby establishing the dependency of the reaction on this reduced cofactor (not shown).

Membrane and cytoplasm fractions prepared by homogenation followed by differential centrifugation from control and CSE-pretreated BPAECs were assayed for $O_2^\cdot_-$-producing activity. Nearly all of the $O_2^\cdot_-$ production in the CS-exposed cells as well as in the control cells was associated with the membrane fraction, which is consistent with the localization of activated NADPH oxidase7 (Figure 3B).
Thiol-Reactive Substances Mimic CS in the Induction of $O_2^\cdot$ Production in BPAECs

Acrolein, a thiol-reactive α,β-unsaturated aldehyde, is the most abundant α,β-unsaturated aldehyde present in the gas phase of CS. This relatively stable but reactive aldehyde could potentially be responsible for the activation of endothelial NADPH oxidase after CS exposure. We therefore measured $O_2^\cdot$ production in BPAECs exposed to authentic acrolein over a range of concentrations similar to those found in CSE. As with CSE (Figure 1), acrolein increased $O_2^\cdot$ production in a dose-dependent manner (Figure 4A). This increase in $O_2^\cdot$ generation was inhibited by DPI (Figure 4B). In contrast, the less reactive 3-carbon-saturated aldehyde propionaldehyde (300 μmol/L) did not induce $O_2^\cdot$ production in BPAECs (not shown), thereby demonstrating that the thiol-reactive properties of acrolein are responsible for the increase in NADPH oxidase activity.

We reasoned that if the induction of NADPH oxidase is caused by thiol-reactive substances in CS, other thiol-reactive agents should also induce $O_2^\cdot$ production in these cells. Indeed, when BPAECs were exposed to the classical thiol reagent NEM, increased production of $O_2^\cdot$ was observed (Figure 5). Further, this increase in $O_2^\cdot$ production caused by NEM was twice as great as the $O_2^\cdot$ production observed in cells exposed to an equimolar concentration of acrolein (Figure 4). The higher rate of $O_2^\cdot$ production with NEM-treated cells may be attributed to differences in volatility, reactivity, and metabolism of these 2 thiol-reactive substances. As expected, the NADPH oxidase activities in acrolein- and NEM-induced BPAECs were similarly inhibited by DPI (Figures 4 and 5).

Because $O_2^\cdot$ readily reacts with NO to produce peroxynitrite (ONOO−), formation of ONOO− was assessed in CSE-treated BPAECs. BPAECs were exposed to CSE 25% with and without DPI (10 μmol/L) or the NOS inhibitor L-NAME (2 mmol/L). After 30 minutes of exposure, ONOO− was measured by luminol chemiluminescence. As shown in Figure 6, CSE treatment caused a significant increase in ONOO− generation. This increase in ONOO− generation was inhibited by DPI as well as by L-NAME. To determine the specificity of our measurements, cells were pretreated with ebselen (50 μmol/L) or uric acid (250 mmol/L) to quench ONOO−. Ebselen and uric acid pretreatment resulted in profound inhibition of ONOO− production: CSE 12 476 ± 1 797 RLU/min/mg protein, CSE + ebselen 3 829 ± 1 510* RLU/min/mg protein, CSE + uric acid 1649 ± 264* RLU/min/mg protein (n=3 to 6, *P<0.05 versus CSE). In separate experiments, we also pretreated the cells with superoxide dismutase (SOD, 200 U/mL). SOD pretreatment also resulted in significant inhibition of ONOO− production: CSE...
4280±651 RLU/min/mg protein, CSE+SOD 1264±336 RLU/min/mg protein (n=3, P<0.05). These results show that CSE induces an increase in OONO⁻ formation and suggest that endothelial NOS (eNOS) and NADPH oxidase are the main sources of NO and O₂⁻, respectively, that participate in the generation of ONOO⁻ in response to CSE exposure.

To confirm that our results with BPAECs are not species specific, we measured O₂⁻ in CSE-treated HPAECs. Exposure of HPAECs to 25% CSE resulted in a marked increase in O₂⁻ production, ie, 7525±2021 cpm/µg protein for CSE-treated versus 3122±187 cpm/µg protein for controls (P<0.05, n=6). This increase in O₂⁻ production induced by CSE in HPAECs was also inhibited by DPI, ie, CSE+DPI 3244±120 cpm/µg protein (P<0.05 versus CSE alone, n=6). Similar to our results with BPAECs, Oxy did not inhibit O₂⁻ production in CSE-treated HPAECs: CSE+Oxy 7281±554 cpm/µg protein (P=NS versus CSE, n=6). Collectively, these findings indicate that thiol-reactive compounds present in CS stimulate the production of O₂⁻ in pulmonary artery endothelial cells via activation of NADPH oxidase and that xanthine oxidase is not an important source of O₂⁻ in response to CS exposure in these cells. Moreover, this phenomenon appears to be species independent.

**Discussion**

Clinical and animal studies have demonstrated that active and passive cigarette smoking impair NO-mediated bioactivity. The mechanisms involved are not completely understood; however, clinical studies have shown that smokers have increased synthesis of isoprostanes, which are products of lipid peroxidation, suggesting that increased generation of ROS participates in the development of endothelial dysfunction in smokers. In our previous studies, we have clearly demonstrated that ROS, and in particular O₂⁻, have a critical role in impairing endothelial function in rat aortas exposed to CS; however, the mechanisms involved were not elucidated.

The interaction between NO and O₂⁻ occurs at an almost diffusion-limited rate and about 6 times faster than the rate of O₂⁻ removal by SOD, and results in the concomitant generation of highly reactive ONOO⁻ and loss of NO bioactivity. In addition to sequestering free NO, O₂⁻ oxidizes arachidonic acid to F₂-isoprostanes, which are known vasoconstricting substances that also induce platelet aggregation and adhesion. Indeed, the levels of both free and esterified plasma F₂-isoprostanes are higher in smokers than in nonsmokers. In previous studies, we demonstrated that CS induces endothelial dysfunction by increasing the vascular production of O₂⁻ resulting in NO inactivation. More recently, another mechanism by which ROS can induce endothelial dysfunction has been described that involves the release of zinc by ONOO⁻ from the zinc-thiolate center of eNOS. This results in ENOS uncoupling and the production of O₂⁻ instead of NO. We investigated this pathway and found only a slight reduction in CSE-induced O₂⁻ production in the presence of the NOS inhibitor L-NAME. From these results, we conclude that the contribution of NOS to O₂⁻ generation is minimal in this system. On the other hand, we found that persistent NO synthesis by NOS in the presence of increased O₂⁻ production led to enhanced ONOO⁻ production.

CS contains large amounts of ROS, NO, nitrogen dioxide, peroxynitrite, peroxynitrite, and free radicals of organic compounds. However, these substances are short lived and rapidly decompose to innocuous end products in an aqueous solution, and therefore cannot explain the increased vascular production of ROS after exposure to CSE. In the current studies, we investigated whether chemically stable compounds present in the gas phase of CS are able to activate specific intracellular sources of ROS. Our results clearly demonstrate that CSE and the α,β-unsaturated aldehyde acrolein significantly increased O₂⁻ production in cultured endothelial cells.

Our original expectation was that xanthine oxidase would be the primary source of increased ROS production. However, when Oxy was added to cultured CS-treated BPAECs and HPAECs, CS-induced O₂⁻ production was minimally inhibited by Oxy. In contrast, DPI, an inhibitor of NADPH oxidase, almost totally inhibited O₂⁻ production in these cells, suggesting that this enzyme is the main source of O₂⁻ in endothelial cells exposed to CS. These results were further corroborated with apocynin and gp91ds-tat, 2 specific NADPH oxidase inhibitors that also produced a significant reduction in O₂⁻ production. Furthermore, the mitochondrial respiration inhibitor rotenone did not inhibit endothelial O₂⁻ in response to CSE, suggesting that the mitochondria are not a significant source of O₂⁻ in response to CSE.
production and inhibition of this reaction by DPI in BPAECs (Figure 4). Propionaldehyde, the less reactive, saturated, 3-carbon analog of acrolein, did not enhance O$_2^-$ production in these cells. In contrast, the classical sulfhydryl reagent NEM was even more effective in inducing O$_2^-$ production in these cells than acrolein. NEM-induced O$_2^-$ production was also inhibited by DPI (Figure 5). From these results, we surmise that α,β-unsaturated aldehydes in CS enhances O$_2^-$ production in vivo and that the alkylation of enzyme thiols by these α,β-unsaturated aldehydes is involved in this induction.

The thiol-reactive substances in CS may exert adverse effects throughout the vasculature. Although measurable levels of the free form of the highly reactive α,β-unsaturated aldehydes are not likely to occur in the systemic circulation, the carbonyl group of these substances may react with amines and thiols of blood components to produce carrier forms that release the original substance at a remote site.30–32 We suggest that carrier forms of α,β-unsaturated aldehydes produced in the pulmonary vasculature from CS release these thiol-reactive substances throughout the systemic circulation. Indeed, in normal humans, the levels of these adducts are about 150 μmol/L and are even higher in other conditions associated with increased oxidative stress, such as diabetes and chronic renal failure.33 Once these aldehydes are released, they can activate endothelial NADPH oxidase at sites remote from the lung.

In conclusion, as suggested by our results with DPI and gp91ds-tat, we have demonstrated that by activating a flavoprotein-containing enzyme such as NADPH oxidase, CS and certain thiol-reactive substances found in CS induce O$_2^-$ production in the pulmonary artery endothelium. These findings provide a plausible mechanism by which CS exerts its deleterious effects in the systemic and the pulmonary vasculature and may facilitate the development of novel strategies to reduce the incidence of pulmonary and vascular pathology in smokers unable to quit smoking.

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References


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