Cigarette smoke-induced proinflammatory alterations in the endothelial phenotype: role of NAD(P)H oxidase activation

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Cigarette smoking is the leading cause of preventable morbidity and mortality in the United States and constitutes a major risk factor for atherosclerotic vascular disease, including stroke and coronary artery disease. Close to one-quarter of the United States population are current smokers, and a large number of them will likely die as a consequence of atherosclerosis. Yet, the mechanisms by which cigarette smoke promotes the development of a proinflammatory and proatherogenic environment in the vessel wall are not fully understood.

It is generally believed that increased production of reactive oxygen species (ROS) plays a central role in vascular inflammation and atherogenesis (17, 41). Cigarette smoke can be divided into two phases: tar and gas-phase smoke. Both phases contain high concentrations of ROS, nitric oxide (NO), peroxynitrite, and free radicals of organic compounds (30, 34, 35, 54). In addition to these short-lived, highly reactive substances, previous studies have shown that aqueous cigarette tar extracts also contain pro-oxidant substances that have the potential to increase cellular production of ROS (2, 4, 35, 39, 43, 44, 54). Thus we hypothesized that water-soluble components of cigarette smoke that are likely to reach the systemic circulation can directly promote vascular oxidative stress in systemic vascular beds. This hypothesis was supported by clinical and animal studies showing that cigarette smoke produces generalized endothelial dysfunction in virtually every vascular bed (1, 5, 6, 12, 13, 36), which is usually an indicator of an increased oxidative stress.

Importantly, ROS, including O$_2^-$ and hydrogen peroxide (H$_2$O$_2$), have been implicated in proatherogenic vascular phenotypic alterations (16, 17, 21, 33, 41), including induction of proinflammatory gene expression (14, 18, 24, 25, 37, 38, 42). Previous studies by us and others revealed a central role for H$_2$O$_2$-induced NF-kB activation in vascular inflammation (7, 8). Although the effects of cigarette smoke on proinflammatory mechanisms in lung epithelium and circulating immune cells have been extensively studied in the past (50), the possible link between water-soluble components of cigarette smoke, oxidative stress, and expression of proinflammatory cytokines in intact blood vessels has not been well documented.

On the basis of the aforementioned studies, we hypothesized that water-soluble components of cigarette smoke increase ROS generation in endothelial and/or smooth muscle cells, which activate NF-kB and elicit the expression of proinflammatory mediators. To test this hypothesis, we characterized cigarette smoke extract (CSE)-induced alterations in vascular O$_2^-$ and H$_2$O$_2$ production, endothelial NF-kB activation, and expression of proinflammatory cytokines.

MATERIALS AND METHODS

Animals and vessel isolation. Fourteen- to sixteen-week-old male Wistar rats (n = 20) were used. Protocols were approved by the Institutional Animal Care and Use Committee of New York Medical College and conformed to the current guidelines of the National Institutes of Health and the American Physiological Society for the use and care of laboratory animals. Animals were euthanized by a

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lethal injection of pentobarbital sodium, and the carotid arteries were isolated and cleaned from the surrounding tissue as previously described (45, 49).

**Cigarette smoke exposure.** The experimental group was exposed to the smoke of five commercial cigarettes (11 mg tar and 0.8 mg nicotine per cigarette) each day for a week according to the modified protocols of Meshi et al. (28), whereas the control group was not exposed to cigarette smoke.

**CSE preparation.** CSE (dissolved in DMSO, 40 mg/ml total particular matter, nicotine content of 6%; kept at –80°C) was purchased from Merut Pharmaceuticals (Lexington, KY). From this stock solution, working solutions (from 0.004 to 40 μg/ml final concentration) were prepared immediately before the experiments by dilution with physiological HEPES buffer. With the assumption that cigarette smoke is extracted in the blood and equilibration occurs with the total blood volume, it is likely that plasma levels of water-soluble components of cigarette smoke in smokers overlap with the CSE concentrations used in the present study. Accordingly, the concentrations of nicotine in the CSE solutions used in these studies overlap with plasma levels of nicotine found in smokers.

**Vessel culture and functional studies.** Isolated carotid arteries were maintained in a stainless steel vessel culture chamber (Danish Myo Technology) under sterile conditions in F-12 medium (GIBCO-BRL) containing antibiotics (100 UI/ml penicillin, 100 μg/ml streptomycin) and supplemented with 5% FCS (GIBCO/Invitrogen), as previously described (11, 46–48). Arteries were treated with CSE (0.004 to 40 μg/ml) or vehicle for 6 or 24 h in the absence or presence of inhibitors of signaling pathways, depending on the protocol. After the incubation period, arterial segments were used for ROS measurements or were snap frozen in liquid nitrogen for molecular biological processing. In other experiments, endothelial function was assessed as previously described (19). In brief, cultured arteries were cut into ring segments 2 mm in length and mounted on 40-μm stainless steel wires in the myographs chambers (Danish Myo Technology, Atlanta, GA) containing Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.1 mM CaCl2, 25 mM NaHCO3, 1.1 mM MgSO4, 1.2 mM KH2PO4, and 5.6 mM glucose; at 37°C; gassed with 95% air-5% CO2) for processing. In other experiments, endothelial function was assessed as previously described (11, 46–48). Arteries were treated with CSE (0.004 to 40 μg/ml) or vehicle for 6 or 24 h in the absence or presence of inhibitors of signaling pathways, depending on the protocol. After the incubation period, arterial segments were used for ROS measurements or were snap frozen in liquid nitrogen for molecular biological processing. In other experiments, endothelial function was assessed as previously described (19). In brief, cultured arteries were cut into ring segments 2 mm in length and mounted on 40-μm stainless steel wires in the myographs chambers (Danish Myo Technology, Atlanta, GA) containing Krebs buffer solution (118 mM NaCl, 4.7 mM KCl, 1.1 mM CaCl2, 25 mM NaHCO3, 1.1 mM MgSO4, 1.2 mM KH2PO4, and 5.6 mM glucose; at 37°C; gassed with 95% air-5% CO2) for measurement of isometric tension. After an equilibration period of 1 h, during which an optimal passive tension of 0.5 g was applied to the rings (as determined from the vascular length-tension relationship), the vessels were contracted by phenylephrine (10−6 mol/l) and relaxations to acetylcholine (from 10−9 to 10−3 mol/l) were obtained. In separate experiments, vessels of control rats were incubated with the serum of cigarette smoke-exposed rats (for 6 h) or with nicotine (from 2.4 to 240 ng/ml, to match the nicotine concentration in the CSE used), and vascular ROS production was determined.

**Measurement of vascular O2− production.** O2− production was assessed from vascular samples by the lucigenin chemiluminescence (5 mol/l) method as we previously described (7, 9, 48). In separate experiments, O2− production in CSE-treated vessels according to the modified protocols of Miura et al. (29). C−H2DCFDA is a 2′,7′-dichlorodihydrofluorescein diacetate-ester (ester) (Invitrogen, Carlsbad, CA) was used to assess H2O2 production in CSE-treated vessels according to the modified protocols of Miura et al. (29). C−H2DCFDA is a 2′,7′-dichlorodihydrofluorescein derivative that has longer retention within the cells. In brief, vessel segments were preincubated with CSE (0.04 to 40 μg/ml) and then treated with C−H2DCFDA (10−5 mol/l; at 37°C for 60 min). In separate experiments, vessels of control rats were incubated with the serum of cigarette smoke-exposed rats or with nicotine. In all experiments, untreated arteries were used as controls. The arteries were then washed three times. The endothelial layer of en face preparations was imaged as described above. Each experiment was performed in quadruplicate. Ten to fifteen entire fields per treatment group were analyzed with one image per field. The mean fluorescence intensities of each ethidium bromide (EB)-stained nuclei were measured in each view field.

**Measurement of vascular H2O2 production.** The cell-permeant oxidative fluorescent indicator dye C−H2DCFDA (5 and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate-ester (ester) (Invitrogen, Carlsbad, CA) was used to assess H2O2 production in CSE-treated vessels according to the modified protocols of Miura et al. (29). C−H2DCFDA is a 2′,7′-dichlorodihydrofluorescein derivative that has longer retention within the cells. In brief, vessel segments were preincubated with CSE (0.04 to 40 μg/ml) and then treated with C−H2DCFDA (10−5 mol/l; at 37°C for 60 min). In separate experiments, vessels of control rats were incubated with the serum of cigarette smoke-exposed rats or with nicotine. In all experiments, untreated arteries were used as controls. The arteries were then washed three times. The endothelial layer of en face preparations was imaged as described above. Each experiment was performed in quadruplicate. Ten to fifteen entire fields per treatment group were analyzed with one image per field. The background-corrected mean fluorescence intensities of each image were averaged. In some experiments, vessels co incubated with catalase were used as positive controls.

In addition, H2O2 production was also measured using the modified methods of Werner (53), after CSE treatment. Vessels were incubated with an assay mix consisting of homovanillic acid (HVA; 100 μmol/l) and horseradish peroxidase (5 U/ml) in HEPES-buffered salt solution (pH 7.5) at 37°C for 1 h. The reaction was stopped with 80 μmol glycine solution (0.1 mol/l, pH 10, 0°C). H2O2-induced fluorescent product was assessed using a fluorometer (excitation 321 nm, emission 421 nm), and the background-corrected fluorescent signal was normalized to tissue weight. Calibration curve was constructed using 0.01–100 μM H2O2 standards in assay mix (1 h at 37°C) with or without catalase (200 U/ml).

### Table 1. Oligonucleotides for real-time RT-PCR

<table>
<thead>
<tr>
<th>mRNA Targets</th>
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<th>Antisense</th>
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<tbody>
<tr>
<td>Rat TNFα</td>
<td>TCGTACGAAACCACCAAG</td>
<td>CTGACGGTGTGGTGTGA</td>
</tr>
<tr>
<td>Rat IL-1β</td>
<td>CAGCAATGTCGGGACCC</td>
<td>ATAGGTAAGTGTGTCCT</td>
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<tr>
<td>Rat IL-6</td>
<td>TACCACAACCTCAATGG</td>
<td>GATACCCATCGACAGGAT</td>
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<td>GAGGTAGCCGTTGTCG</td>
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<tr>
<td>Rat ICAM-1</td>
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<td>CCCCCCTTAAGTGGTGGA</td>
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<td>Rat gp91imm</td>
<td>GGAATGATTCATCGAGGCCAA</td>
<td>TTACGCAAGGCTCCCG</td>
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<tr>
<td>Rat β-actin</td>
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Real-time quantitative PCR. Quantitative RT-PCR (QRT-PCR) was used to elucidate the effect of smoking on the expression of inflammatory master cytokines (TNF-α, IL-1β, and IL-6) and inducible NOS (iNOS) in coronary arteries. These factors were shown to be associated with oxidative stress-related vascular inflammation and are considered to be early indicators of a proatherogenic microenvironment in the vascular wall. To elucidate the role of ROS and NAD(P)H oxidase in vascular inflammation, carotid arteries were treated with cigarette smoke extract (CSE) in organoid culture with or without pretreatment with polyethylene glycol (PEG)-catalase (200 U/ml), PEG-SOD (200 U/ml), apocynin [3 × 10−4 mol/l, which inhibits NAD(P)H oxidases; Refs. 47, 48], or DPI. In separate experiments, mRNA expression of the NAD(P)H oxidase catalytic subunit gp91phox was assessed in CSE-treated arteries. Total RNA from the arteries was isolated with MiniRNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript II RT (Invitrogen) as described previously (9, 10). Real-time RT-PCR technique was used to analyze mRNA expression using the Stratagen MX3000, as previously re-

Fig. 1. Relaxations to acetylcholine (A) and S-nitrosopenicillamine (SNAP; B) in ring preparations of carotid arteries of control rats and rats exposed to cigarette smoke (“smoking”; see MATERIALS AND METHODS). The effect of preincubation with the NAD(P)H oxidase inhibitor apocynin (3 × 10−4 mol/l) is also shown. Data are means ± SE (n = 4–6 for each group). *P < 0.05. #P < 0.05 vs. cigarette smoke exposure only. C: O2−• generation in vessels of control rats and rats exposed to cigarette smoke. O2−• generation was determined by the lucigenin (5 μmol/l) chemiluminescence (CL) method in the absence and presence of the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI; 10−5 mol/l) or SOD (200 U/ml). Data are normalized to the mean value of the untreated control group. *P < 0.05 (n = 5–6 in each group). D: O2−• generation in rat carotid arterial segments treated with various concentrations of cigarette smoke extract (CSE). *P < 0.05 (n = 5–6 in each group). E: O2−• generation in rat carotid arteries treated with CSE (4 μg/ml, for 6 h) in the absence or presence of DPI (10−5 mol/l), SOD (200 U/ml), indomethacin (Indo, 10−5 mol/l) and N- nitro-L-arginine methyl-ester (l-NAME; 10−4 mol/l). Data are normalized to the mean value of the control group. *P < 0.05; #P < 0.05 vs. CSE exposure only (n = 8). F: NAD(P)H (10−4 mol/l)-driven lucigenin CL in homogenates of vessels incubated overnight in the presence or absence of CSE (4 μg/ml). *P < 0.05. G: mRNA expression of gp91phox in vessels incubated overnight in the presence or absence of CSE (4 μg/ml). *P < 0.05.
ported (7, 9–11, 46). Samples were run in triplicate. Efficiency of the PCR reaction was determined using dilution series of a standard vascular sample. Quantification was performed using the ΔΔCT method. The housekeeping gene β-actin was used for internal normalization. Oligonucleotides used for real-time QRT-PCR are listed in Table 1. The fidelity of the PCR reaction was determined by melting temperature analysis and visualization of product on a 2% agarose gel.

Transient transfection and luciferase assays. The effect of CSE on NF-κB activity in primary rat coronary arterial endothelial cells (CAECs) was tested by a reporter gene assay. CAECs were cultured as previously reported (7). We used a NF-κB reporter composed of a NF-κB response element upstream of firefly luciferase (NF-κB-Luc, Stratagene) and a renilla luciferase plasmid under the control of the cytomegalovirus promoter (as an internal control). All transfections were performed with Novafector (Venn Nova LLC, Pompano Beach, FL) following manufacturer protocols. Firefly and renilla luciferase activities were assessed after 42 h using the Dual Luciferase Reporter Assay Kit (Promega) and a luminometer. Pyrrolidine dithiocarbamate (10−5 mol/l), an inhibitor of NF-κB activation, was used as control.

Monocyte adhesion assay. We measured adhesion of fluorescently labeled human monocytic (THP-1) cells to confluent monolayers of CAECs using a microplate-based assay as previously reported (7). In brief, CAECs were grown to confluence in 96-well plates and were treated with increasing concentrations of CSE (incubation time of 6 h at 37°C) in the absence or presence (60-min preincubation) of apocynin, DPI, or catalase. H2O2 and TNF-α were used as positive controls. THP-1 cells were labeled with the fluorescent dye BCECF (5 × 10−6 mol/l final concentration; Molecular Probes, Eugene, OR; in serum-free RPMI medium for 45 min at 37°C). Cells were then washed twice with prewarmed (37°C) RPMI. Phorbol myristate acetate (PMA; 10−6 mol/l)-pretreated fluorescently labeled THP-1 cells (5 × 105/well) were added to the microplate wells containing confluent CAECs

![Fig. 2. A: nuclear ethidium bromide (EB) fluorescence in endothelial and smooth muscle cells in sections of carotid arteries of control rats and rats exposed to cigarette smoke (“smoking”: for the cigarette smoke exposure protocol, see MATERIALS AND METHODS). Vessels were incubated with the dye dihydroethidium, which produces a red nuclear fluorescence when oxidized to EB by O2•−. B: representative fluorescent photomicrographs of en face preparations of rat carotid arteries incubated with CSE (4 μg/ml, for 6 h; A) and untreated controls (C). On these compressed images, the EB-stained elongated nuclei of vascular smooth muscle cells and the round nuclei of endothelial cells are visualized. Identical results were observed in 4 separate experiments. Scale bars, 10 μm. D: summary graph showing the averaged fluorescence intensities of EB-stained nuclei of endothelial and smooth muscle cells (n = 4 vessels in each group). Data are means ± SE. *P < 0.05.](http://ajpheart.physiology.org/)

![Fig. 3. A: CSE (4 μg/ml) significantly increases H2O2 production in en face preparations of rat carotid arteries, as shown by the dichlorofluorescein (DCF) fluorescence method. Data are means ± SE; FLU, fluorescent units. *P < 0.05. B: demonstration of increased H2O2 production in rat carotid arteries exposed to CSE (4 μg/ml) using a homovanillic acid (HVA)/horseradish peroxidase method. Results are expressed as arbitrary FLU (means ± SE; n = 3 for each group). *P < 0.05 vs. untreated control.](http://ajpheart.physiology.org/)
Smoking-induced endothelial dysfunction. In vivo exposure of rats to cigarette smoke elicited impaired vascular relaxations to acetylcholine (Fig. 1A) and to SNAP (Fig. 1B), which were improved by apocynin treatment. In vitro CSE treatment tended to reduce NO-dependent relaxation of rat carotid arteries to acetylcholine (EC$_{50}$; control, 3.0 ± 0.8 × 10$^{-6}$ mol/l; CSE, 5.0 ± 1.3 × 10$^{-6}$ mol/l; not significant) and SNAP (EC$_{50}$; control, 2.5 ± 0.4 × 10$^{-7}$ mol/l; 10.0 ± 4.3 × 10$^{-7}$ mol/l; not significant); however, the differences did not reach statistical significance.

Smoking and in vitro CSE exposure increase vascular O$_2$$^{•-}$ and H$_2$O$_2$ production. In carotid arteries of cigarette smoke-exposed rats, there was an increased SOD- and DPI-inhibitable lucigenin chemiluminescent signal, indicating an increased NAD(P)H oxidase-dependent O$_2$$^{•-}$ generation (Fig. 1C). Also, in a dose-dependent manner, CSE significantly increased O$_2$$^{•-}$ production in the carotid arteries (Fig. 1D) and aortas (not shown). O$_2$$^{•-}$ production in CSE-treated vessels was significantly decreased by administration of DPI, Tiron, or SOD, whereas it was unaffected by indomethacin or l-NAME (Fig. 1E). In CSE-treated vessels, there was an increased NAD(P)H-driven lucigenin chemiluminescence (Fig. 1F) and an upregulation of gp91$^{phox}$ mRNA (Fig. 1G).

Using the EB staining method, we found that, in cross sections of carotid arteries of smoke-exposed rats, the mean fluorescence intensity of endothelial and smooth muscle cell nuclei was significantly greater than that of control rats (Fig. 2A). Representative fluorescent photomicrographs of EB-stained untreated and CSE-treated carotid arteries (en face preparations) are shown in Figs. 2, B and C. In CSE-treated vessels, there was an intensive nuclear ethidium bromide staining, localized to the endothelial and smooth muscle cells, indicating that CSE promotes O$_2$$^{•-}$ generation in both cell types (Fig. 2D).

Exposure to CSE elicited substantial increases in vascular H$_2$O$_2$ generation as measured by both the DCF fluorescence (Fig. 3A) and HVA fluorescence (Fig. 3B) methods. With the use of exogenous H$_2$O$_2$, a calibration curve was constructed for HVA fluorescence, which was linear in the 10$^{-7}$ to 10$^{-4}$ mol/l range. DCF and HVA fluorescence could be inhibited by catalase, showing the specificity of the signal (not shown).

Incubation of vessels from control rats with the serum of cigarette smoke-exposed rats (for 6 h) significantly increased endothelial DCF fluorescence (Fig. 4A) and nuclear EB staining (not shown). Administration of nicotine did not result in significant increases in endothelial DCF fluorescence (Fig. 4B, C and D) and nuclear EB staining (not shown).

Smoking and in vitro CSE exposure upregulate vascular expression of inflammatory markers. In coronary arteries of cigarette smoke-exposed rats, mRNA expression of iNOS, TNF-α, IL-1β, and IL-6 (Fig. 5, A–D) and ICAM (not shown) significantly increased. Exposure of rat carotid arteries to increasing concentrations of CSE in vitro also elicited upregulation of iNOS, TNF-α, IL-1β, and IL-6 (Fig. 6, A–D). The

Data analysis. Data were normalized to the respective control mean values. Data are means ± SE. Statistical analyses of data were performed by Student’s t-test or by two-way ANOVA followed by the Tukey post hoc test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

**Fig. 4.** A: incubation with serum from rats with cigarette smoke exposure (for 6 h) increases H$_2$O$_2$ production in en face preparations of rat carotid arteries, as shown by the DCF fluorescence method. Data are means ± SE. *$P < 0.05$. B: H$_2$O$_2$ production in arteries treated with increasing doses of nicotine (DCF fluorescence method). Data are means ± SE. *$P < 0.05$. C: Results from plate reader-based measurements of DCF fluorescence in primary coronary arterial endothelial cells treated with increasing doses of nicotine. Data are means ± SE ($n = 8$ for each group).
effect of CSE on ICAM-1 expression did not reach statistical significance (not shown). Expression of iNOS, TNF-α, IL-1β, and IL-6 in CSE-treated vessels was significantly reduced by apocynin and PEG-catalase (Fig. 6, A–D). Similar results were obtained also with DPI.

Demonstration of CSE induced activation of NF-κB in endothelial cells. We demonstrated that CSE, in a concentration-dependent manner, significantly enhanced the transcriptional activity of NF-κB in CAECs (as indicated by an increase in the luciferase activity; Fig. 7A). Importantly, CSE-induced NF-κB activity could be inhibited by catalase, DPI, and apocynin (Fig. 7B), suggesting that NAD(P)H oxidase-derived H₂O₂ production plays a key role in CSE-induced NF-κB activation in CAECs.

Smoking and in vitro CSE exposure enhance monocyte adhesion to the endothelium. Adhesiveness of activated THP-1 monocyte cells to the endothelial surface of carotid arteries of smoke-exposed rats was significantly increased (Fig. 8A). In
vitro exposure of cultured carotid arteries and aortas to CSE also increased monocyte adhesiveness to the endothelium (Fig. 8B). Incubation of CAECs with CSE also resulted in dose-dependent increases in the adhesion of THP-1 cells (Fig. 8C), which could be inhibited by apocynin, DPI, or catalase (Fig. 8C). Increased monocyte adhesiveness induced by H2O2 and TNF-α is shown as positive controls in Fig. 8C.

**DISCUSSION**

There are three important findings in this study. First, cigarette smoke exposure elicits significant endothelial dysfunction in rat carotid arteries, which could be reversed by inhibition of the NAD(P)H oxidase (Fig. 1, A and B). This finding accords with the increased NAD(P)H oxidase-dependent O2•− generation in these vessels (Fig. 1C). It is likely that water-soluble components of cigarette smoke are directly responsible for the activation of the vascular NAD(P)H oxidase, because exposure of isolated arteries to CSE in vitro, in the absence of activated leukocytes, elicited significant O2•− production in a concentration-dependent manner (Fig. 1D). The primary source of CSE-induced O2•− generation seems to be the NAD(P)H oxidase (Fig. 1, E and F), supporting the ex vivo observations (Fig. 1, A–C). Accordingly, CSE seems to increase the expression of gp91phox in rat arteries (Fig. 1G), and in human pulmonary artery endothelial cells, gp91 docking sequence-tat peptide (similar to apocynin) was reported to inhibit CSE-induced O2•− generation (22). Dihydroethidium imaging revealed that both endothelial cells and vascular smooth muscle cells exhibit an upregulated O2•− generation in vessels of cigarette smoke-exposed animals (Fig. 2A). Similarly, CSE challenge elicited oxidative stress in both cell types (Fig. 2, B–D), mimicking the effects of in vivo exposure to cigarette smoke. Recent studies support the idea that CSE in vitro may induce NAD(P)H oxidase(s) in other cell types as well (20, 22, 36). Previously, we have shown that NAD(P)H oxidase(s) are abundantly expressed in rat arteries, and an increased NAD(P)H oxidase activity is responsible for enhanced endothelial O2•− production in pathophysiological conditions such as aging (9), hyperhomocysteinemia (46), and hypertension (47, 48). NAD(P)H oxidase represents a common pathway eliciting endothelial dysfunction; thus it is logical to hypothesize that smoking will aggravate vascular injury in these pathophysiological conditions. We have shown previously that PKC-dependent phosphorylation of the p47phox subunit is central to the regulation of vascular NAD(P)H oxidase activity (47). One can hypothesize that cigarette smoke particulate constituents activate protein kinase C (23), which leads to the increased vascular oxidative stress. It should be noted that, in addition to the NAD(P)H oxidase, other cellular sources (such as xanthine oxidase, cytochrome P-450, and mitochondrial sources) can also produce significant amounts of O2•−; however, the role of these enzymes in CSE-induced oxidative stress is not well understood (our data suggest that cyclooxygenase-derived O2•−; however, the role of these enzymes in CSE-induced oxidative stress is not well understood (our data suggest that cyclooxygenase- and endothelial NOS do not play a major role in CSE-induced oxidative stress) (Fig. 1D). We would like to point out that, in the present study, in addition to apocynin [which inhibits the activation of NAD(P)H oxidase by inhibiting the association of the cytoplasmic regulatory subunits and the membrane-bound oxidase subunits], we have also used the nonspecific inhibitor DPI [which inhibits flavin-containing enzymes, including NAD(P)H oxidase and other enzyme systems as well]. Because SOD enzymes catalyze the removal of O2•− with a rate constant of 2 × 10⁹ mol⁻¹l⁻¹s⁻¹, it is likely that a significant portion of O2•− is dismutated, increasing also H2O2 levels. Indeed, in CSE-treated vessels, there was a substantially increased H2O2 production (Fig. 3).

The component(s) of CSE that activate NAD(P)H oxidase at present are unknown. Although nicotine may impair endothelium-mediated vasodilation in microvessels (27), it could not mimic the effect of serum from cigarette smoke-exposed rats (Fig. 4A) or CSE (Fig. 3) on endothelial ROS production in our experiments (Fig. 4, B and C). A recent study suggested that acrolein, a thiol-reactive α,β-unsaturated aldehyde that is abundantly present in cigarette smoke, is a potent inducer of NAD(P)H oxidase-derived O2•− generation in pulmonary arterial endothelial cells (22). Other components of cigarette tar extracts that may promote ROS generation include semiquinones, hydroquinones, and quinones (35, 54); acrolein-related α,β-unsaturated aldehydes, such as crotonaldehyde, α,β-unsaturated ketones; and a number of saturated aldehydes (4, 39, 44). Because of their stability and water solubility, acrolein and other related compounds are likely to reach vascular beds remote from the primary site of exposure and, possibly, induce the production of ROS.
The second significant finding in this study was that in vivo exposure to cigarette smoke provokes an increase in the expression of proinflammatory cytokines (including IL-6, TNF-α, and IL-1β) and cytokine-sensitive inflammatory mediators (iNOS) in the vascular wall (Fig. 5). Importantly, these proinflammatory phenotypic alterations could also be mimicked by in vitro CSE challenge (Fig. 6). Recent studies suggest that exposure of cultured human endothelial cells to CSE or serum from smokers also results in proinflammatory gene expression (1, 31, 32, 40, 51). Atherosclerosis is a chronic inflammatory disease, and pathological and epidemiological evidence suggest that proinflammatory cytokines play a central role orchestrating the pathological processes underlying the development of the atherosclerotic plaque. Our findings are of great significance, showing that cigarette smoke components are able to elicit a proatherogenic microenvironment in the vascular wall in the absence of circulating factors and immune cells. Previously, we have shown that vascular expression of proinflammatory cytokines and iNOS is frequently upregulated in conditions associated with increased H₂O₂ production, such as hyperhomocysteinemia (46), aging (10), and hypertension (8).

To elucidate the possible link between cigarette smoke, production of ROS, and proinflammatory cytokine expression, we pharmacologically inhibited NAD(P)H oxidase and apocynin or the H₂O₂ scavenger catalase on CSE-induced monocyte adhesion (Fig. 7). The results of pretreatment with the NAD(P)H oxidase inhibitors DPI and apocynin or the H₂O₂ scavenger catalase on CSE-induced monocyte adhesion are shown. TNF-α (10 ng/ml) and H₂O₂ were used as positive control. Data are means ± SE. *P < 0.05 vs. control. C: treatment of primary coronary arterial endothelial cells with increasing concentrations of CSE significantly increased the adhesion of fluorescently labeled, PMA-stimulated monocytes. The effects of pretreatment with the NAD(P)H oxidase inhibitors DPI and apocynin or the H₂O₂ scavenger catalase (200 U/ml) and on CSE-induced monocyte adhesion are shown. TNF-α (10 ng/ml) and H₂O₂ were used as positive control. Data are means ± SE. *P < 0.05 vs. control. *P < 0.05 vs. control.

D: proposed scheme for the mechanisms by which water-soluble components of cigarette smoke promote proinflammatory phenotypic alterations in carotid arteries. The model predicts that CSE induces an increased O₂•⁻ generation by NAD(P)H oxidase, which scavenges vasodilator NO, resulting in endothelial dysfunction. NAD(P)H oxidase-derived H₂O₂ (formed from O₂•⁻ via the action of SOD) activates the redox-sensitive transcription factor NF-κB, upregulating inflammatory gene expression. The resulting proinflammatory phenotype of carotid arteries will likely increase monocyte recruitment to the vascular wall and promote the development of atherosclerosis, especially if other risk factors (e.g., hypertension, hypercholesterolemia, and hyperhomocysteinemia) are also present. CAMs, cellular adhesion molecules.
Previous studies suggested that even moderate cigarette smoking leads to an activation of the circulating monocytes and their increased adhesion to the endothelium (3). We found that both in vivo exposure of rats to cigarette smoke and in vitro incubation of vessels with CSE enhance adhesion of activated monocytes to the endothelial surface (15, 23, 26) (Fig. 8, A and B). The role of water-soluble components of cigarette smoke is supported by the findings that serum collected from smokers increases endothelial expression of adhesion molecules, including ICAM-1 (1, 23). Our results support the view that NAD(P)H oxidase-derived H$_2$O$_2$ plays a central role in endothelial activation by cigarette smoke constituents (Fig. 8C). These findings agree with the results that increasing plasma vitamin C concentrations in smokers by oral supplementation decreased monocyte adhesion to values found in nonsmokers (52).

In conclusion, water-soluble components of cigarette smoke increase NAD(P)H-oxidase derived H$_2$O$_2$ generation in endothelial and smooth muscle cells, which induce a proinflammatory vascular phenotype likely via mechanisms that involve NF-κB activation (Fig. 8D). We propose that cigarette smoking-induced oxidative stress and vascular inflammation will support atherosclerotic plaque formation in the carotid arteries, increasing the morbidity of stroke.

GRANTS
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