Superoxide-mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in vascular endothelium: studies in cultured cells and smokers

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Peluffo G, Calcerrada P, Piacenza L, Pizzano N, Radi R. Superoxide-mediated inactivation of nitric oxide and peroxynitrite formation by tobacco smoke in vascular endothelium: studies in cultured cells and smokers. Am J Physiol Heart Circ Physiol 296: H1781–H1792, 2009. First published April 10, 2009; doi:10.1152/ajpheart.00930.2008.—Tobacco smoke is known to cause nitric oxide (NO) inactivation and endothelial dysfunction. In this work we evaluated the interplay between NO and superoxide (O2·−) radicals and the consequent impact on NO bioavailability and nitrosative stress in bovine aortic endothelial cells exposed to cigarette smoke extract (CSE) and in smokers. Bovine aortic endothelial cells in the presence of CSE triggered O2·− production as indicated by spin-trapping electron paramagnetic resonance experiments. O2·− was produced both extracellularly (3.4 vs. 1.0 nmol·h−1·mg−1; CSE vs. control; cytochrome c′+ reduction assay) and intracellularly (40% inhibition of cytosolic aconitase). CSE also led to the production of peroxynitrite as evaluated by dihydrorhodamine oxidation and protein tyrosine nitration on cells. O2·− and peroxynitrite formation were decreased by ascorbate and α-tocopherol. Additionally, CSE led to the oxidation of endothelial nitric oxide synthase increasing the monomeric inactive form of endothelial nitric oxide synthase. Smokers and age-matched healthy volunteers were supplemented orally with 500 mg ascorbate plus 400 IU all-rac-α-tocopherol every 12 h for 165 days. Smokers had endothelial dysfunction compared with control subjects (95% confidence interval: 2.5, 8.3 vs. 10.6, 14.2; P < 0.05) as assessed by flow-mediated dilation of the brachial artery, and plasma levels of protein 3-nitrotyrosine were 1.4-fold higher. The loss of flow-mediated dilation in smokers reverted after a long-term antioxidant supplementation (95% confidence interval: 13.9, 19.9; P < 0.05), reaching values comparable with the control population. Our data indicate that elements on tobacco smoke revert after a long-term antioxidant supplementation (95% confidence interval: 13.9, 19.9;). In this context, tobacco smoking produces ED as revealed by a decrease in flow-mediated dilation (FMD) (Ref. 7). The measurement of FMD has been successfully applied to monitor the improvement in vascular function caused by a number of drugs (e.g., statins) and antioxidants (ascorbate and α-tocopherol) in smokers (1, 5, 32, 55).

In endothelial cells, NO is synthesized by the constitutively expressed dimeric isoform of nitric oxide synthase (eNOS), which is tightly regulated by Ca2+/calmodulin. Mechanistic forces like shear stress produced by laminar blood flow stimulate eNOS phosphorylation and are responsible for the normal NO tone produced by the endothelium. Tetrahydrobiopterin, a readily oxidizable redox cofactor, is essential for eNOS activity. On pathological conditions that have ED, NO bioavailability is jeopardized either by a decrease on eNOS activity or by an oxidative inactivation of NO (38).

A relevant pathway that decreases NO levels is its diffusion-controlled reaction with superoxide radicals (O2·−) (k ≈ 1 × 1010 M−1·s−1) that gives rise to peroxynitrite (15a). Cardiomyocytes and endothelial and smooth muscle cells are major sites of O2·− production in the cardiovascular system (25). Potential sources of O2·− in the vasculature include the activation of NAD(P)H oxidases (NOX) present in endothelial and smooth muscle cells (9), dysfunctional mitochondria, uncoupled eNOS (59), and xanthine oxidase associated to the endothelium glycocalyx (53, 60). Therefore, O2·− formation in endothelial cells is intimately connected to NO bioavailability governing its output from the endothelium and, as a consequence, its biological actions (35, 38, 44). Additionally, peroxynitrite is a potent oxidizing and nitrating (15a) short-lived species (ca. 10 ms in vivo) (13), which directly reacts with several molecules such as thiols and metal centers (45) and can also yield secondary radicals, including hydroxyl radical (·OH), nitrogen dioxide (NO2), and carbonate radical (CO3·−) (47). When O2·− levels increase, NO is consumed and peroxynitrite is formed despite the superoxide dismutases (SOD)-catalyzed dismutation of O2·− (k = 1 × 108 M−1·s−1) (12, 21, 43) hampering the diffusion of NO. Therefore, not only the beneficial protective actions of NO are lost after its reaction with O2·−, but also a potent oxidant such as peroxynitrite is formed, all of which may shift the biological actions of NO from signal transduction to oxidative pathophysiology.

Tobacco smoke can be divided into a gas phase and particulate matter, both containing oxidants and free radicals such as O2·−, H2O2; nitrogen oxides including NO, NO2, and peroxynitrite; reactive aldehydes; redox active quinones; and carbon monoxide (41), which are either present in cigarette smoke or generated by it (i.e., redox cycling of hydroquinones in the tar phase). Additionally, cigarette smoking causes an inflammatory response of the endothelium, characterized by the
activation, adhesion, and accumulation of leukocytes in vivo that release proinflammatory cytokines, stimulating secondary tissue production of reactive species (3). Exposure to reactive oxygen and nitrogen species during cigarette smoking can be a direct result of oxidants and free radicals generated during the organic combustion or following the activation of inflammatory processes triggered by smoke components that are solubilized in the pulmonary lining fluid; in this way, tobacco smoke components access the rest of the vasculature producing nitrooxidative effects away from the primary entrance point, which might be modulated by pharmacological intervention with natural or synthetic antioxidants.

In this work, we evaluated the hypothesis that the cigarette smoke extract (CSE)-driven \( \text{O}_2^- \) production on endothelial cells stimulated to produce \(^{-}\)NO will lead to a decreased \(^{-}\)NO bioavailability and peroxynitrite formation in vitro and in vivo. To test the hypothesis we developed two experimental strategies. For in vitro experiments, we explored oxidant formation in cultured vascular endothelial cells exposed to a preparation of CSE using a combination of sensitive and specific assays for the different reactive species taking place in the process. For the in vivo measurements, we conducted a small-scale clinical trial for the evaluation of \(^{-}\)NO-dependent FMD of the brachial artery, basal levels of nitrated plasma proteins, and the long-term effect of oral supplementation with a combination of dietary antioxidants ascorbate and \( \alpha \)-tocopherol (57) in smokers and controls.

**MATERIALS AND METHODS**

**Materials**

Culture medium 199 (M199) and fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Grand Island, NY). Iron-supplemented bovine calf serum was obtained from Hyclone (Logan UT). Phenol red-free M199 was obtained from Sigma (St. Louis, MO). 1,1-Dioctadecyl-3,3,3,3-tetramethyl- in-docarbocyanine perchlorate-acylated low-density lipoprotein (DiI-Ca-LDL), dihydrorhodamine chrome, Na\(_2\)HPO\(_4\) (pH 7.4), centrifuged at 800 \( \times \) 10\(^3\) for 15 min at 4°C, and resuspended in 100 \( \mu \)l of fractionation buffer (FB) containing 0.25 M sucrose (Sigma, St. Louis, MO), 5.5-Dimethyl-1-pyrroline-N-oxide (DMPO) (Sigma) was kindly provided by Dr. Ronald P. Mason (National Institute of Environmental Health Sciences). 2,3-Dimethoxy-1-naphthoquinone (DMNO), 1,3-morpholinosydnonimine (SIN-1), and all other reagents were from Sigma.

**Cell Culture**

Bovine aortic endothelial cells (BAECs) were extracted from fresh bovine aorta obtained at a local slaughterhouse (Frigoricof Carlos Schneck) as previously described (42, 51). Bovine aortas were opened sagittally under the laminar flow hood to expose the endothelium. A scalpel blade was gently scraped along the aorta surface only once, and the cellular debris accumulated in the blade, which contains endothelial cells, were resuspended in 10 ml of M199 supplemented with 5% (vol/vol) FBS, 5% (vol/vol) iron-supplemented calf serum, 10 \( \mu \)M thymidine, 100 U/ml penicillin G, and 100 \( \mu \)g/ml streptomycin sulfate. Cells were collected by centrifugation at 800 \( g \) for 15 min, resuspended in 10 ml of prewarmed (37°C) M199, plated in 80-mm culture dishes and incubated at 37°C in a 5% CO\(_2\)-95% room air atmosphere. BAEC colonies were sealed using sterile 8-mm clamping rings and expanded on 50-ml cell culture flasks in M199. Culture purity was >99% as evaluated by immunocytochemistry using a human antibody against von Willebrand factor (Sigma) and by the uptake of the fluorescently labeled acetylated LDL (DiI-Ca-LDL). All experiments were performed on BAEC cultures at 95% confluence from passages 4 to 14. For all the experiments involving BAECs, fetal bovine serum was lowered to 0.5% (%vol/vol) 24 h before treatment with the different experimental conditions.

**Cigarette Smoke Extract**

CSE was prepared as previously described (54). Briefly, three cigarettes were combusted (commercially available 80-mm filtered cigarettes; Fiesta, Philip Morris) in 30 ml of M199 without phenol red and serum using a Venturi-driven vacuum aspirator. The appropriate vacuum was applied to allow the combustion to last 5 min for each cigarette. The smoke was bubbled through the media under stirring, sterilized by filtration through 0.22 \( \mu \)m and aliquot frozen at \(-20^\circ\)C until use. All experiments were performed with a CSE concentration of 10% (%vol/vol), a nonlethal concentration to BAECs under our experimental conditions as evaluated by the trypan blue exclusion assay (not shown).

**Electron Paramagnetic Resonance Studies**

The detection of \( \text{O}_2^- \) radicals was evaluated by electron paramagnetic resonance (EPR) studies using the spin-trap DMPO (50). BAECs were grown on 6-well culture plates and incubated with 100 mM DMPO for 1 h at room temperature in the different experimental conditions. Following incubation, the cells were scraped and resuspended in 200 \( \mu \)l of PBS. Superoxide \( \text{O}_2^- \) formation was determined at room temperature in an EPR spectrophotometer using flat cell. The spectrum was recorded at a central field of 3,480 G with a sweep width of 100 G, sweep time of 80 s, and microwave frequency of 9.769 GHz, power of 20 mW, and receiver gain of 5 \( \times \) 10\(^5\). Four scans were accumulated for each condition.

**Extracellular \( \text{O}_2^- \) Detection**

\( \text{O}_2^- \) production by BAECs in the presence of CSE was measured by the cytochrome \( \text{c}^3^- \) reduction assay as described previously (26, 27). BAECs grown at 95% confluence in 24-well culture plates were preincubated for 30 min with 110 \( \mu \)M ascorbate, 35 \( \mu \)M \( \alpha \)-tocopherol, or both. After incubation, 0.5 ml of fresh M199 without phenol red and serum was added, containing 40 \( \mu \)M cytochrome \( \text{c}^3^- \) in the presence or absence of Cu-Zn SOD (100 IU/ml). CSE was added to the cell culture, and the incubation proceeded for 6 h at 37°C in a 5% CO\(_2\)-95% room air atmosphere. \( \text{O}_2^- \) was determined in the culture supernatant by measuring the SOD-inhibitable reduction of cytochrome \( \text{c}^3^- \) (ES\(_50\) = 21,000 M\(^{-1}\)-cm\(^{-1}\)). The results are expressed as nanomoles of \( \text{O}_2^- \) per hour per milligram and represent the mean of three independent determinations.

**Aconitase activity**. Cells were grown on 140-mm\(^2\) culture dishes in M199 and in the presence of CSE for 24 h in M199 without phenol red and serum supplementation at 37°C in a 5% CO\(_2\)-95% room air atmosphere. After incubation, the cells were scraped and resuspended in 5 ml of ice-cold PBS containing (in mM) 140 NaCl, 4 KCl, and 10 Na\(_2\)HPO\(_4\) (pH 7.4), centrifuged at 800 \( g \) for 15 min at 4°C, and resuspended in 1 ml of fractionation buffer (FB) containing 0.25 M Tris-HCl (pH 7.4), 0.1 mM EDTA, 2 mM sodium citrate, and 1 mM sodium succinate. Cells from three plates were pooled and homogenized on a Potter-Elvehjem homogenizer at 4°C, by 10 strokes at 800 rpm and centrifuged at 1,500 \( g \) for 10 min at 4°C. Supernatants containing the cytosolic and mitochondrial fraction were further centrifuged at 13,000 \( g \) for 15 min at 4°C. The mitochondrial-containing pellet was resuspended in 200 \( \mu \)l of FB, centrifuged at for 13,000 \( g \) for 15 min at 4°C, and resuspended in 100 \( \mu \)l of FB. The mitochondrial fraction was sonicated at maximum power four times with 1-s pulses on an ice bath. Aconitase activity was immediately measured in cytosolic and mitochondrial extracts using the coupled assay with porcine isocitrate dehydrogenase as previously reported (17, 42) by following the
increase in absorbance due to NADPH formation at 340 nm. Enzyme activity is expressed as nanomoles of NADPH per minute per milligram.

Mito-Hydroethidine Oxidation

BAECs were incubated in the presence of 10% CSE for 6 and 18 h. As a positive control for mitochondrial O$_2^-$ production, cells were incubated with the respiratory chain inhibitor antimycin A (1 μM) for 30 min. BAECs were loaded with 1 μM of Mito-hydroethidine (MitoSox red) for 20 min, washed with phenol red-free M199, and analyzed on a Nikon Eclipse TE200 inverted epifluorescence microscope using a filter cube with an excitation filter of (wavelength/band pass) 540/25 nm, emission filter of 605/55 nm, and beam splitter at 546 nm. Photographs were taken at a fixed exposure time and gain settings (29, 42).

DHR Oxidation

DHR oxidation was used to evaluate peroxynitrite production of BAECs exposed to CSE as previously described (47). Cells were grown in 24-well culture plates, and M199 was replaced with 0.5 ml of Dulbecco’s phosphate-buffered solution (dPBS) consisting of (in mM) 137 NaCl, 8.1 Na$_2$HPO$_4$, 0.9 CaCl$_2$, 0.5 MgCl$_2$, 2.7 KCl, and 1.45 KH$_2$PO$_4$ (pH 7.4), supplemented with 5.6 glucose and 1 L-/H9262 reactor as described previously (46). Peroxynitrite concentration was 137 NaCl, 8.1 Na$_2$HPO$_4$, 0.9 CaCl$_2$, 0.5 MgCl$_2$, 2.7 KCl, and 1.45 KH$_2$PO$_4$ (pH 7.4), supplemented with 5.6 glucose and 1 L-

Chemical Nitration of Cellular Proteins

Peroxynitrite was synthesized in our quenched flow reactor as described previously (46). Peroxynitrite concentration was determined spectrophotometrically at 302 nm (ε$_{302}$ = 1,670 M/cm). Alternatively, the peroxynitrite donor SIN-1 was added to the monolayer at a concentration of 500 μM that releases 1.3 μM/min of peroxynitrite. Media containing freshly added SIN-1 was added every 60 min to avoid oxygen depletion by SIN-1. Peroxynitrite release form SIN-1 was determined by measuring DHR oxidation (ε$_{500}$ = 78,800 M$^{-1}$·cm$^{-1}$) as described previously (47).

Protein 3-Nitrotyrosine Determination

Levels of nitrated plasma proteins were determined in smokers ($n$ = 5) and nonsmokers ($n$ = 5) at baseline and after 3 mo of oral supplementation with antioxidants. Dot blot analysis of the samples with an anti-3-nitrotyrosine antibody (6) was performed by seeding 100 μg of proteins in 2 μl onto nitrocellulose membrane. Western blot analysis was performed on total cell extracts. Proteins (30 μg) were resolved by 12% SDS-PAGE. Membranes from Western blot and dot blot were blocked overnight in Tris-buffered saline (TBS) containing (in mM) 25 Tris (pH 7.4), 140 NaCl, and 3 KCl, supplemented with 0.6% vol/vol Tween-20 and 5% wt/vol BSA. The membrane was probed with a polyclonal rabbit anti-3-nitrotyrosine antibody (diluted 1:2,000 in TBS 0.1% wt/vol BSA and 0.6% vol/vol Tween-20) produced in our laboratory (6). Immunoreactive proteins were detected using the Immun-Star Chemiluminescence Kit (Bio-Rad). The specificity of the signal was verified by a pretreatment of the blotted membrane with 100 mM sodium hydrosulfitte in 100 mM sodium borate (pH 9.0), which reduces 3-nitrotyrosine to 3-aminotyrosine and precludes the immunoreactivity (62). A semiquantitative determination of nitrated proteins was performed by densitometric analysis using the J-Image freeware (National Institutes of Health) by measuring the ratio between de bands and total protein load per lane as developed by Ponceau-S staining of the nitrocellulose membrane ($n$ = 5 experiments). Figure 6 shows an illustrative experiment. The results are expressed as means and 95% confidence intervals. A $P < 0.05$ was considered significant. For immunocytochemistry studies, the cells were fixed with 4% paraformaldehyde in buffer for 15 min at 4°C. The cells were permeabilized with 0.1% Triton X (30 min) and blocked overnight with 0.1% Triton, 2% BSA, and 0.5% goat serum. The cells were incubated with the polyclonal anti-3-nitrotyrosine antibody (1:100), diluted in the presence of 0.1% Triton for 2 h at 37°C, and developed using an Alexa Fluor 546-streptavidin-conjugated antibody. Stain cells were visualized on a Nikon Eclipse TE epifluorescence inverted microscope, and photographs were taken at a fixed exposure time and gain settings.

Determination of eNOS Monomerization

BAECs were cultured in six-well plates as above in dPBS supplemented with 5 mM l-arginine and exposed to CSE in the presence or absence of 5 mM t-NAME, 1 mM NOC-18, 30 μM of the redox cycling compound DMNQ, and 5 μM ionomycin during 4 h at 37°C in a 5% CO$_2$-95% room air atmosphere. After incubation, the cells were harvested in 500 μl ice-cold dPBS and collected by centrifugation for 5 min at 4°C at 800 g. Cells were lysed in 100 μl potassium phosphate buffer (50 mM, pH 7.4), supplemented with 0.1% Triton X-100 and 10 mM EDTA, and sonicated at maximum power four times with 1-s pulses on an ice bath. Samples were supplemented with 100 μl of loading buffer containing 30 mM Tris·HCl (pH 6.6), 1% (wt/vol) SDS, 2.5% (vol/vol) 2-mercaptoethanol, and 5% (vol/vol) glycerol. Proteins (100 μg) were resolved by 6% SDS-PAGE at 4°C, followed by Western blotting onto nitrocellulose membranes (18 h, 100 mA at 4°C). After protein transfer, the membranes were stained with Ponceau-S solution (Applichem) to confirm equal protein loading. Membranes were incubated (1 h) with polyclonal anti-eNOS antibody (Sigma), diluted 1:10,000 in TBS 0.1% Tween-20. Immunoreactive proteins were detected using the Immun-Star Chemiluminescence Kit (Bio-Rad). Densitometry analysis was performed with the J-Image freeware. The results are expressed as percentages of eNOS monomer respect to the total eNOS detected (monomer + dimer) on each condition and reported as fold increases of control.

Trial Design

Subjects. The present study was performed with the approval by the Institutional Review Board and the Bioethics Committee of the Hospital de Clínicas, Facultad de Medicina, Universidad de la República, and in accordance to the Helsinki declaration regarding ethical principles of medical investigations on human subjects. All participants signed a statement of informed, written consent for the participation in the intervention trial. Inclusion criteria included young (age range, 24–40 yr old) nonsmokers and heavy smokers (>20 cigarettes per day). Exclusion criteria included any known illness state, current antioxidant supplementation, or drug intake. All volunteers were screened for hypertension, hyperglycemia, hypercholesterolemia, renal function, and hyperuricemia. Subjects where divided in two groups: nonsmokers ($n$ = 20) and smokers ($n$ = 5). Table 1 shows the clinical characteristics and biochemical parameters of both populations performed at baseline by routine clinical laboratory determinations at the Laboratorio de Análisis Clinicos (Montevideo, Uruguay).

Experimental trial. The trial design consisted of a longitudinal pharmacological intervention study to assess the effect of long-term (165 days) oral supplementation with ascorbate and α-tocopherol on the endothelial function of smokers compared with an age-matched healthy population. The study was designed to detect a change on

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endothelial function of 40% of the control population with α set at 0.05 and with a power of 85%.

All volunteers received a single pill (Gestativa, Gramon-Bagé, Uruguay) of ascorbate (500 mg) plus all-rac-α-tocopherol (400 IU) every 12 h during 165 days. It is important to note that 400 IU of all-rac-synthetic α-tocopherol is bioequivalent to 200 α tocopherol (22). Blood samples were drawn at days 0, 10, 30, 103, 165, 175, and 195, and the levels of ascorbate and α-tocopherol were determined. The endothelial function, the main physiological outcome of the study, was evaluated in both groups at baseline and after reaching the plateau phase (103–165 days) in blood levels for both ascrobate and α-tocopherol (Fig. 1).

Endothelium-Dependent FMD of the Brachial Artery

Endothelial function was evaluated by measuring the FMD of the brachial artery by high-resolution ultrasonography (7, 10) before and during the antioxidant supplementation (Fig. 1) as previously described. Briefly, fasting volunteers (8 h) were asked to lie in a supine position for 10 min in a thermostatized, quieted, and dim room before performing the measurements. Resting longitudinal images of the brachial artery of the nondominant arm were acquired using a Hewlett-Packard Sonos 2000 ultrasound system equipped with a 7.5-MHz linear-array transducer in B-mode. Images were recorded in S-VHS, including a continuous ECG to align the images with the cardiac cycle for off-line analysis. Typically, optimal imaging of the brachial artery was found to be 2 to 3 cm above the antecubital fossa where the characteristic m-line (media adventitia interface) was identified. After recording the basal parameters (arterial diameter, heart rate, and the velocity time integral), a blood pressure cuff positioned on the antecubital fossa was inflated at 50 mmHg above the systolic pressure during 5 min to induce a transient distal ischemia that generates a distal dilation of the vasculature. Images of the brachial artery were acquired for 90 s, starting 30 s before the cuff release. Maximum FMD was achieved at 45 s after the cuff release.

The brachial artery diameter measurements reported here represent the average value observed for three cardiac cycles; blood flow was calculated by multiplying the velocity time integral of the Doppler signal by heart rate and the artery cross-sectional area.

Ascrobate and α-Tocopherol Blood Level Determinations

Ascrobate was determined within 20 min after blood sampling (to avoid its further oxidation) by ion-pairing reverse-phase HPLC with electrochemical detection as previously described (37). Fresh plasma from heparinized tubes was deproteinized by 10 min incubation with ice-cold methanol-1 mM diethylenetriaminepentaacetic acid (1.9 vol/ vol) and centrifuged at 13,000 g for 5 min at 25°C to remove precipitated proteins before injection. The supernatant (15 μl) was injected onto a Spherisorb ODS-2 column (250 x 4.6 mm); prequillibrated for 12 h with the mobile phase consisting of (in mM) 40 potassium acetate, 0.45 EDTA, and 1.5 dodecyltrimethyl-ammonium bromide as the counter-ion (pH 4.75); and equipped with an appropriate guard column. The mobile phase was delivered isocratically at a flow rate of 1 ml/min. Ascrobate detection was achieved using a Gilson model 142 amperometric electrochemical detector set at +0.6 V and the sensitivity to 20 nA/V. For the determination of α-tocopherol, an aliquot (10 μl) of the deproteinized supernatant was injected into a reverse-phase column (C-18 small pore from Vydac), eluted isocratically with methanol at 1 ml/min, and detected using a fluorescence detector with excitation at 295 nm and emission at 330 nm. Plasma ascrobate and α-tocopherol concentrations were calculated using a freshly prepared external standard calibration curve for both antioxidants (56).

Data Analysis

All data are given as means and means ± SD or 95% confidence intervals unless otherwise noted. For comparison between two groups, the Student’s t-test was performed. An analysis of variance was performed for comparison of more than two groups. Post hoc analysis was performed by the least significant difference test. A P value of <0.05 was considered significant. All experiments were typically reproduced a minimum of three times on independent days.

RESULTS

$O_2^{-}$ Production by BAECs Exposed to CSE

BAECs in the presence of 10% CSE were incubated for 1 h in the presence of the spin-trap agent DMPO. The reaction of $O_2^{-}$ with DMPO yields the paramagnetic adduct DMPO-OOH that rapidly decays ($t_{1/2}$ < 1 min) to the more stable DMPO-OH adduct (quartet signal; δN = δH = 14.9 G) (Fig. 2). The formation of DMPO-OH from cells was significantly increased over the basal condition in the presence of CSE (Fig. 2D). The EPR signal was not abolished by either of the NOX inhibitors diphenylene iodonium (Fig. 2E) or apocynin (Fig. 2F). The incubation of CSE with DMPO in the absence of cells did not generate a significant EPR signal (Fig. 2B).

Since DMPO readily diffuses through the plasma membrane, the original site of oxygen radical formation cannot be determined; thus, we designed experiments to evaluate the extracellular and intracellular components of $O_2^{-}$ production. First, the extracellular production of $O_2^{-}$ was explored by the Cu-Zn-SOD inhibitable reduction of cytochrome c$^{3+}$ assay. BAECs in the presence of CSE led to a significant increase on
O$_2^-$ production (3.4 vs. 1.0 nmol·h$^{-1}$·mg protein$^{-1}$ CSE vs. control) (Fig. 3). Ascorbate (110 μM) and α-tocopherol (35 μM) were used to modulate O$_2^-$ production by BAECs at the concentrations reached in the plasma of supplemented volunteers (vide infra). The addition of ascorbate mildly decreased the detectable O$_2^-$ after CSE exposure (3.4 vs. 2.2 nmol·h$^{-1}$·mg protein$^{-1}$ CSE vs. CSE plus ascorbate), whereas α-tocopherol had no effect. The presence of both antioxidants further inhibited O$_2^-$ production to 1.4 nmol·h$^{-1}$·mg protein$^{-1}$, indicating a synergistic effect (Fig. 3). Apocynin did not have any effect on O$_2^-$ production in BAECs (not shown).

To further characterize O$_2^-$ production, we measured aconitase activity from cytosolic and mitochondrial fractions of BAECs, given that this enzyme is readily inactivated by O$_2^-$. and has been validated as a sensitive intracellular redox target (42). After 24 h of incubation in the presence of CSE, we observed that the cytosolic aconitase activity dropped to 60% of control values, whereas no inhibition of the mitochondrial aconitase was evidenced (Fig. 4). Moreover, BAECs incubated and...
in the presence of CSE for 6 and 18 h did not increase the oxidation of the mitochondrial-targeted oxidant-sensor probe (MitoSox) (not shown). Apocynin had no effect on the cytosolic and mitochondrial aconitase inactivation extent. Together, these results support that mild and short-term CSE exposure leads to a NOX and mitochondria-independent production of $O_2^-$, which is detected both extracellularly and in the cytosol.

Peroxynitrite Production of BAECs Exposed to CSE

The fluorescent probe DHR reacts with peroxynitrite-derived free radicals but not with $O_2^-$ or NO directly (47, 61). BAECs in the presence of CSE and ionomycin led to DHR oxidation, suggesting the production of peroxynitrite. In agreement, DHR oxidation was prevented by the eNOS inhibitor L-NAME and Cu-Zn SOD (Fig. 5A). On a complementary assay and to assess the effect of a controlled NO source, BAECs exposed to CSE were incubated in the presence of an exogenous NO donor, NOC-18. Maximal DHR oxidation was observed in the presence of CSE and a flux of NO produced by the NO donor NOC-18. SOD inhibited DHR oxidation, indicating the need of both radicals to achieve efficient probe oxidation and, therefore, peroxynitrite formation (Fig. 5B).

In addition, BAECs exposed to CSE showed a time-dependent increase on basal protein nitration at 18 h [95% confidence interval (CI): 0.92, 1.08 vs. 1.28, 1.38; P < 0.05 control vs. CSE 18 h, respectively] (Fig. 6A). As a positive control of protein nitration, BAECs were incubated in the presence of 500 μM SIN-1, which releases near equimolar amounts of $O_2^-$, and NO, which reacts to form peroxynitrite. A time-dependent increase on protein nitration was observed during SIN-1 exposure (Fig. 6B). A parallel Western blot analysis was performed, and the nitrocellulose membrane was incubated with 100 mM dithionite before the incubation with the anti-nitrotyrosine antibody. The loss of signal indicates the specificity of the antibody used (Fig. 6C). The nitration observed after CSE exposure of BAECs was attenuated when the cells were preloaded with 35 μM α-tocopherol and 110 μM ascorbate (95% CI: 1.28, 1.38 vs. 0.9, 1.1; P < 0.05 CSE vs. antioxidants) (Fig. 6D). As an additional positive control, we challenged BAECs preloaded with 35 μM α-tocopherol and 110 μM ascorbate with peroxynitrite. Again, an inhibition on protein nitration in antioxidant-supplemented cells was seen (Fig. 6E) after a bolus addition of the nitrating agent. Finally, immunocytochemical studies using the anti-nitrotyrosine antibody showed protein nitration after an exposure to CSE, further supporting the notion of peroxynitrite formation (Fig. 6F). Collectively, these results indicate that $O_2^-$ produced by CSE exposure effectively reacts with NO and yields peroxynitrite.

eNOS Monomerization

Functional eNOS is a dimeric protein stabilized by a zinc atom tetrahedrally coordinated with four cysteines residues, two provided by each monomer. This cysteine cluster can be oxidized by strong oxidants, such as peroxynitrite, disassembling the dimer to the less active monomer (63). We explored the effect of CSE on eNOS dimer oxidation (Fig. 7). CSE alone increased the levels of the monomer (1.8-fold) compared with the control condition. This augmentation was prevented by the presence of the eNOS inhibitor L-NAME, which indicates that NO is involved in monomer formation most likely through peroxynitrite formation. The incubation of BAECs with DMNQ and NOC-18, a chemical system for the formation of peroxynitrite, also leads to eNOS monomer formation and was used as a positive control.

Clinical and Biochemical Parameters of Volunteers

Middle-aged (mean, 31 yr old) volunteers were divided into groups of nonsmokers (n = 20) and smokers (n = 5). No
Fig. 6. Exposure to CSE increases protein tyrosine nitration in BAECs. A: protein nitration by Western blot analysis of cells exposed to 10% CSE for 6 (lane 2) and 18 (lane 3) h. B: protein nitration of cells were incubated in the presence of the peroxynitrite (ONOO−) donor 1,3-morpholinosydnonimine (SIN-1) at a concentration of 500 μM, which delivers a flux of 1.3 μM/min for 60 (lane 2) and 120 (lane 3) min. C: same experimental design as in B but the nitrocellulose membrane was incubated with 100 mM dithionite for 20 min before incubation with the anti-nitrotyrosine antibody. The incubation with dithionite abolished nitrotyrosine staining, revealing the specificity of the antibody for the epitope. D: cells preloaded with 110 μM ascorbate and 35 μM α-tocopherol were exposed to 10% CSE for 18 h and probed with the anti-nitrotyrosine antibody. Control cells (lane 1) exposed to CSE (lane 2) and antioxidant (Aox)-loaded cells exposed to CSE (lane 3) are shown. E: control cells (lane 1) or cells preloaded with 110 μM ascorbate and 35 μM α-tocopherol were challenged with the nitrating agent ONOO−. Protein nitration of cells exposed to 10 (lane 2) and 20 (lane 3) μM peroxynitrite in the absence or presence of antioxidants (lanes 4 and 5). F: immunofluorescence was developed using a secondary antibody labeled with Alexa Fluor 488. Photographs were acquired with same exposure and gain settings at a magnification of ×400. F, bottom, right and left: differential interference contrast images. Data are illustrative of a n = 5 experiments, performed on independent days. See main text for 95% confidence interval.
significant differences were observed between both groups in regard to blood pressure, lipids, glucose, renal function, uric acid, ascorbate, or \( \alpha \)-tocopherol (Table 1). On the contrary, a significant decrease on FMD of the brachial artery was detected in smokers at baseline (95% CI: 10.6, 14.2 vs. 2.5, 8.3; \( P \leq 0.05 \)). Nitrated plasma proteins were also significantly increased in smokers at baseline (Table 1).

Antioxidant pharmacokinetic profile of the supplemented population. Ascorbate (1 g) plus \( \text{all-rac-} \alpha \)-tocopherol (800 IU) were administered daily to volunteers of both groups. The dose was divided in two intakes every 12 h to maximize ascorbate absorption that saturates at 500 mg, seeking to maximize the plasma steady-state concentrations of ascorbate over a day that has a half-life of ca. 6 h (24, 36).

To evaluate the efficacy of the pharmacological preparation to increase plasma levels of ascorbate and \( \alpha \)-tocopherol, we measured the concentration of both antioxidants in plasma at different time intervals during the study in all subjects (Fig. 1). Since no differences in antioxidant levels at baseline (Table 1) or during the supplementation phase were detected between smokers and nonsmokers (not shown), all data were pooled to construct the kinetic profiles of ascorbate and \( \alpha \)-tocopherol (Fig. 8). The plasma concentration of both compounds showed a similar behavior. A plateau in blood levels with the dosage used was reached after 30 days of treatment. At this stage, the levels of ascorbate and \( \alpha \)-tocopherol were 105 (SD 9.1) and 35.5 (SD 2.5) \( \mu \text{M} \), respectively, which represent a 3.0- and 2.4-fold increase for ascorbate and \( \alpha \)-tocopherol, respectively. The plasma levels remained constant during the supplementation period. The pharmacological intervention was stopped after 165 days, taking 30 days of washout (Fig. 1) for the antioxidants to return to baseline levels (Fig. 8).

Reversal of ED After Antioxidant Supplementation

FMD of the brachial artery was reassessed after 2 mo of reaching the plasmatic plateau for both antioxidants. The ED present at baseline in smokers (95% CI: 2.5, 8.3 vs. 10.6, 14.2;
P < 0.05) was completely restored, whereas no further improvement was detected in nonsmokers (95% CI: 10.6, 14.2 vs. 11.4, 14.8) (Fig. 9).

DISCUSSION

Mainstream tobacco smoke reaches the lungs, solubilizing a myriad of chemicals present in tobacco or formed during its combustion. Most of the particulate tar fraction (>90%), which includes stable organic carbon-centered free radicals as well as conjugated quinones and semiquinones present in tobacco smoke, are retained by the filter present in commercially available filtered cigarettes (8). Nevertheless, the fraction of polinuclear quinones and semiquinones escaping the filter can solubilize in the lung-lining fluid and lead to the formation of $\text{O}_2^-$ by redox cycling. Additionally, alkyl, alkyloxyl, and peroxy radicals together with $\cdot\text{NO}_2$-dependent free-radical chemistry can be involved in the cellular damage attributed to CSE (8).

Previous data have shown that CSE impairs the endothelium-dependent relaxation of aortic (30, 34) and carotid rings (33) by CSE-triggered $\text{O}_2^-$ production. In the present report, we expand those observations and provide evidence for the formation of peroxynitrite arising from CSE-triggered $\text{O}_2^-$ formation and $\cdot\text{NO}$. First, we conducted EPR spin-trapping experiments with DMPO to evaluate $\text{O}_2^-$ formation by BAECs (Fig. 2) in the presence of 10% CSE, observing the typical DMPO-$\text{OH}$ signal, compatible with previous results obtained with quinones that undergo redox cycling in BAECs (50). BAECs in the presence of CSE produced $\text{O}_2^-$ to the extracellular space as shown by SOD-inhibitable cytochrome $c^\text{3+}$ reduction, an extracellular probe (Fig. 3). $\text{O}_2^-$ was also produced at enhanced rates intracellularly, as evaluated by the loss of aconitase activity (Fig. 4). Under our experimental conditions, consisting of relatively mild and short-term (≤24 h) CSE exposure, the intracellular formation of $\text{O}_2^-$ was mainly cytosolic as judged from the relative inactivation patterns of cytosolic and mitochondrial aconitase that was corroborated by the lack of MitoSox oxidation (not shown); however, it has just been shown that under a more sustained or stronger CSE challenge to vascular endothelial cells, mitochondria become important intracellular sources of $\text{O}_2^-$ (11) as revealed by a mitochondrial-targeted redox probe (29, 42).

Endothelial cells express mainly NOX-1 and the constitutively NOX-4 isofrom, although NOX-2 can be observed under some circumstances (2). The effects of apocynin on endothelial cells are difficult to predict given that NOX isoforms show differential sensitivity to this inhibitor (20). Recent data on cultured rat vessels, including the carotid artery, had provided evidence that an exposure to CSE triggers a $\text{O}_2^-$ and hydrogen peroxide formation by NOX with the concomitant activation of NF-κB and the subsequent inflammatory process in an apocynin-inhibitable manner (33); in this model, however, CSE-triggered $\text{O}_2^-$ production was most likely due to the participation of the immune system and smooth muscle cells. In this study that uses cultured vascular endothelial cells, no effects of apocynin or diphenylene iodonium (a general flavoenzyme inhibitor) were observed (see, for example, Fig. 2). Therefore, we favor the concept that the compounds present in CSE, in particular, redox active quinones, mediate endothelial cytosolic and extracellular $\text{O}_2^-$ production in BAECs in the short term (6 h) (50), although the activation of an apocynin-insensitive NOX or the induction of NOX after 6 h cannot be totally ruled out.

Previous data (1, 31, 49, 58) and this work indicate that $\cdot\text{NO}$ bioavailability is decreased in smokers. We therefore explored whether CSE could interfere with $\cdot\text{NO}$ production by BAECs through the reaction with $\text{O}_2^-$ and divert it to peroxynitrite formation. Data obtained with the redox-sensitive probe DHR indicated that peroxynitrite was formed when BAECs were stimulated to produce $\cdot\text{NO}$ or even if $\cdot\text{NO}$ was arising from an extracellular source in the presence of CSE (Fig. 5). Peroxynitrite was formed intra- and extracellularly, as suggested by the partial inhibition by SOD on DHR oxidation (Fig. 5) and the intracellular detection of nitrated proteins (Fig. 6). Importantly, it was reported that CSE can decrease eNOS activity; for instance, CSE decreases eNOS mRNA and protein levels (54) after 24 h. Herein, we found that eNOS is oxidized after only 4 h of CSE exposure to BAECs (Fig. 7), which may lead to an early decrease on $\cdot\text{NO}$ levels on top of its oxidative inactivation by $\text{O}_2^-$, closing a negative feedback loop. Moreover, the oxidation of eNOS favors its uncoupling with the subsequent production of $\text{O}_2^-$ and peroxynitrite by the enzyme (16, 59). Therefore, as a consequence of CSE-triggered $\text{O}_2^-$ production, $\cdot\text{NO}$ bioavailability is shifted from signal transduction to nitrooxidative stress. In regard to the nature of the nitrated proteins observed in BAECs, future studies should be aimed to identify and assess the biological relevance of this posttranslational modification. In addition, the magnitude of the increase assessed by the semiquantitative antibody-based methodology used in the present work should be taken cautiously due to the intrinsic limitations of this technique. Further work with potent analytical methodology such as liquid chromatography tandem mass spectrometry is needed to unambiguously evaluate the extent of the changes observed.

Smokers are under direct and indirect nitrooxidative stress as indicated by the presence of elevated levels of nitrooxidative biomarkers such as plasma levels of F-isoprostanes (28), protein 3-nitrotyrosine (39); and this work, see Table 1) and protein

![Graph](http://ajpheart.physiology.org/)

Fig. 9. Restoration of endothelial function by antioxidant supplementation in smokers. FMD of the brachial artery was performed at baseline and after 165 days of supplementation with ascorbic acid and all-rac-α-tocopherol. Graph shows 95% confidence interval for each group.
carbonyls (14), which make it a suitable population to test the effects of antioxidants on NOS bioavailability. The effect of antioxidants on smokers has relied mainly on short-term intravenous or oral supplementation with ascorbate (32, 58). Ascorbate is a soluble antioxidant, whereas α-tocopherol dissolves well in hydrophobic compartments acting synergistically to prevent oxidative damage (57). Additionally, ascorbic acid and α-tocopherol play other biological functions. Indeed, ascorbic acid reduces the eNOS cofactor tetrahydrobiopterin to maximize NO output (19), and α-tocopherol has an anti proliferative-action via interactions with protein kinase C (4) and may also affect processes in vascular tissue. Interestingly, it has been recently communicated that a combined treatment with ascorbic acid and α-tocopherol had beneficial effects on FMD and arterial stiffness in untreated, essential hypertensive patients, with this effect associated with changes in plasma markers of oxidative stress (40).

In our work the study population was supplemented with a commercial preparation of 500 mg ascorbate plus 400 IU all rac-α tocopherol every 12 h for 165 days to test whether a biochemical synergy on antioxidant and NO-dependent responses could be achieved at the clinical level. We studied a population that consisted in age- and sex-matched normal subjects and otherwise healthy smokers to avoid other pathologies that can cause ED (e.g., hypercholesterolemia and hypertension) and medication that could revert ED (e.g., statins and angiotensin-converting enzyme inhibitors) that could mask the beneficial effects of antioxidants. The preparation used raised the plasma levels of ascorbate and α-tocopherol by 3.0- and 2.4-fold, respectively, reaching a steady-state concentration that was sustained during the supplementation phase (Fig. 8). After 30 days of cessation, both antioxidants returned to basal levels. After 2 mo at the maximum plasma concentration, the value of FMD of smokers returned to that of the control population (Fig. 9), in line with previous reports in the literature (55, 58) and consistent with the experiments at the cellular level reported herein (Figs. 3 and 6) where the combination of ascorbate and α-tocopherol inhibited CSE-triggered O2•− and peroxynitrite formation and therefore undoubtedly spared NO from oxidative inactivation. Some limitations of the intervention trial are the relatively small number of participants, the disparity in the size between groups, and the absence of placebo. However, the ED of smokers has been already reported on several clinical trials. Endothelial function is a relative measure, and no reference value for a healthy population for this variable has been reported. Thus, in this context, it is most important to establish a solid reference value for the healthy population for comparison of the endothelial function of smokers; the data presented herein after antioxidant supplementation were solid for all smokers and broadened the significance of the data obtained at the cellular level. Increased levels of ascorbate and α-tocopherol may block redox cycling of quinones present in CSE, stabilizing its reduced form. No additional effect of antioxidant supplementation was observed in control subjects. Dot blot analysis of plasma-nitrated proteins showed that the basal level of this biomarker is increased in smokers in agreement with the nitrooxidative stress expected in this population. Antioxidant supplementation did not significantly decrease 3-nitrotyrosine levels in smokers within the time frame of the study. A likely explanation is that a relatively modest increase on NO bioavailability has a larger impact in its signaling functions than on the more stable nitrooxidative modifications such as plasma protein 3-nitrotyrosine. In fact, only a modest decrease (ca. 25%) on plasma 3-nitrotyrosine levels on patients with coronary artery disease after a 12 wk statin treatment was reported using highly sensitive mass spectrometry-based methodologies (52).

In summary, CSE, used as a surrogate of tobacco smoke, led to O2•− production in the cytosol and the extracellular space on BAECs and led to peroxynitrite formation. The increased cellular O2•− and peroxynitrite formation was prevented by the addition of ascorbate and α-tocopherol to the culture media at the same plasma concentrations achieved in the clinical trial. In addition, CSE caused an oxidation and disruption of eNOS, which can amplify the already-altered NO bioavailability. The cellular data provide mechanistic information to explain the decreased NO-dependent FMD in smokers and the modulatory action of long-term oral supplementation with ascorbate and α-tocopherol. Our data indicate that elements in tobacco smoke divert NO toward peroxynitrite by inducing O2•− production in endothelial cells both in vitro and in vivo.

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