Vascular Endothelial Cells Generate Peroxynitrite in Response to Carbon Monoxide Exposure

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Received March 19, 1997[⊗]

Carbon monoxide causes a perivascular oxidative injury in animals, and we tested the hypothesis that endothelial cells could be a source of the injurious oxidants. Studies were undertaken to assess whether exposure to carbon monoxide would cause cultured bovine pulmonary artery endothelial cells to liberate reactive species. Concentrations of carbon monoxide between 11 and 110 nM caused progressively higher concentrations of nitric oxide to be released by endothelial cells based on measurements of nitrite and nitrate. Intracellular production of peroxynitrite was indicated by elevated concentrations of nitrotyrosine, and extracellular liberation of peroxynitrite was indicated by oxidation of *p*-hydroxyphenylacetic acid and dihydrorhodamine-123. Carbon monoxide did not disturb mitochondrial function based on the rate of oxygen consumption, intracellular production of hydrogen peroxide, and the ability of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Carbon monoxide also did not alter arginine transport by cells or nitric oxide synthase activity, but it was found to increase steady state levels of nitric oxide by competing for intracellular binding sites. Acute cytotoxicity from carbon monoxide, assessed as radioactive chromium leakage, was due to nitric oxide-derived oxidants. A delayed cell death, whose mechanism is not entirely clear, was also demonstrated by chromium leakage and uptake of vital stain. These findings offer a possible mechanism for adverse health effects caused by carbon monoxide at concentrations ranging from the relatively low levels in polluted environments to levels typically encountered with life-threatening poisoning. Carbon monoxide causes oxidative stress by a novel mechanism involving a competition for intracellular binding sites which increases steady state levels of nitric oxide and allows for generation of peroxynitrite by endothelium.

Introduction

Recent clinical reports have indicated that exposure to carbon monoxide (CO)[1] precipitates a vascular injury and that characteristic aspects of CO-mediated pathology may arise secondary to hemorrhagic necrosis (*1*−*3*). In an animal model of CO poisoning, we reported a striking perivascular deposition of nitrotyrosine in the brain microvasculature and that nitric oxide synthase activity was required for the cascade of events which lead to brain lipid peroxidation after CO poisoning (*4*). Nitrotyrosine synthesis *in vivo* appears to be a specific marker for peroxynitrite-mediated oxidative stress under physiological conditions (*5*). Peroxynitrite is a relatively long-lived, strong oxidant that is generated by the near diffusion-limited reaction between nitric oxide (•NO) and superoxide (*6*). Peroxynitrite and/or its protonated form, peroxynitrous acid, have been shown to oxidize many cellular components such as proteins, non-protein sulfhydryls, deoxyribonucleic acid, and membrane phospholipids, as well as blood-borne substances such as low-density lipoproteins (*7, 8*).

Several cell types may be sources for oxidants in CO poisoning. Vascular endothelial cells can generate both superoxide and •NO (*9*). Because we found nitrotyrosine to be predominantly deposited around the vasculature, we were interested in evaluating whether exposure to CO would cause endothelial cells to generate •NO-derived oxidants such as peroxynitrite. We were also interested in evaluating the dose−response relationship to assess whether low concentrations of CO, as may be found in the environment due to air pollution, might cause endothelial cells to generate •NO-derived oxidants. In urban areas the level of CO usually varies from 2 to 40 ppm, but at times of heavy traffic the CO level on sidewalks and in underpasses may be as high as 170 ppm (*10*−*12*). The health risks of environmental CO exposures are unclear and a controversial subject.

We hypothesized that vascular endothelium would generate peroxynitrite if CO caused a local, intracellular increase in •NO in the vicinity of the superoxide normally generated by endothelial cells. In platelets, we have shown that CO enhances •NO release because CO inhibits intraplatelet binding of •NO to heme-proteins (*13*). The results from the current investigation indicate that environmentally relevant concentrations of CO not only cause production of oxidants but also have a direct cytotoxic effect on endothelial cells.

Experimental Methods

Materials. All chemicals were obtained from Sigma Chemical Corp. unless otherwise stated. All work with CO was performed in a fume hood, and a CO monitor was continuously operated in the laboratory to assure against leaks from the high-pressure tanks of gas.

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[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1997.

[1] Abbreviations: CO, carbon monoxide; •NO, nitric oxide; pHPA, *p*-hydroxyphenylacetic acid; HRP, horseradish peroxidase; DHR, dihydrorhodamine-123; L-NAME, L-nitroarginine methyl ester; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

S0893-228x(97)00041-6 CCC: $14.00

Cell Culture. Starter cultures of bovine pulmonary artery endothelial cells were a gift from Dr. E. J. Macarack (Connective Tissue Research Institute, University of Pennsylvania). Cells were grown in Medium 199 supplemented with 16% heat-inactivated fetal calf serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate on 35 mm diameter Petri dishes. Studies were performed at 1−3 days after confluence was established and at passages 4−7. Control samples, which were exposed to air plus 5% carbon dioxide, were included with each study, and both control and CO-exposed cells were split from the same parent flasks. Prior to each experiment, Krebs buffer, pH 7.4, supplemented with 10 mM Hepes, 6 mM sodium bicarbonate, and 5.6 mM glucose (Krebs-Hepes-glucose buffer) was equilibrated with either a gas mixture of air plus 5% carbon dioxide or the same gas mixture plus a desired concentration of CO. At the start of the experiment the growth medium was replaced with either air or CO-equilibrated buffer, the small gas space above the buffer was flushed with the appropriate gas mixture, and the cells were incubated at 37 °C.

Carbon Monoxide Supply. Concentrations of CO were chosen to achieve levels that endothelial cells may experience *in vivo* when blood carboxyhemoglobin levels are between 3.8% and 28%. Coburn (*14*) has demonstrated that the Haldane equation can be used to estimate interstitial CO concentrations *in vivo* when the carboxyhemoglobin level is less than 50%. Therefore, buffer was equilibrated with 10−100 ppm CO to achieve concentrations of 11−110 nM. The desired concentration of CO for each study was achieved by attaching a gas supply of compressed air containing 1000 ppm CO to one port of a gas-mixing chamber (Calibrated Instruments Inc., Ardsley, NY) and purified, compressed air containing 5% carbon dioxide (CO_2) to the second port. Specific concentrations of CO were generated by setting the mixing ratio between the CO supply and the compressed air. The concentrations of CO delivered by the fixed ratio settings of the device were verified using a gas chromatography CO detector (trace analytical reduction gas analyzer; Menlo Park, CA). The CO detector accuracy was verified using calibration standards.

Nitrite/Nitrate Production as an Index of $^{\bullet}$NO Synthesis. Plates of endothelial cells were incubated for 30 min to 2 h at 37 °C with 0.5 mL of Krebs-Hepes-glucose buffer that had been equilibrated with air plus 5% CO_2 or air with CO_2 plus CO. At the end of the incubation, buffer was removed and assayed for nitrite plus nitrate. Cells were scraped off the plates with a rubber policeman, sonicated (Heat Systems-Ultrasonics sonicator Model W220-F at a setting of 6) for 40 s on ice, and centrifuged at 12000*g* for 10 min, and the protein concentration in supernatents was measured. The concentration of nitrite plus nitrate was measured spectrophotometrically using Greiss reagents (*15*). Dissolved nitrate was first converted to nitrite by bacterial nitrate reductase, and a colored azo product (λ_{max} 540 nm) was produced by sequential addition of sulfanilamide and *N*-(1-naphthyl)ethylenediamine (kit of reagents purchased from Cayman Chemicals, Ann Arbor, MI). A standard curve was generated using solutions of sodium nitrate.

Nitrotyrosine in Endothelial Cells. The nitrotyrosine concentration in cell samples was assayed using methods as described by Ischiropoulos et al. (*4*). Cells were exposed to a desired concentration of CO for 2 h using standard methods. The Krebs-Hepes-glucose buffer was removed; the cells were scraped off the plates after addition of 1 mL of phosphate-buffered saline (PBS), sonicated in an ice bath for two 30 s cycles, and centrifuged at 12000*g* for 10 min. Four to eight concentration-dependent dilutions of the supernatant were loaded in 200 μL of PBS onto nitrocellulose paper using a 96-well Bio-Dot microfiltration unit (BioRad, Hercules, CA). Eight to twelve different concentrations of peroxynitrite-modified bovine serum albumin standard were also loaded in duplicate on each blot to generate a standard curve. After blocking with 5% dry milk, the nitrocellulose was incubated with anti-nitrotyrosine antibody (a gift from Dr. Joe Beckman, University of Alabama, Birmingham, AL) for 15 h followed by a 3 h incubation in a solution containing a donkey anti-rabbit ^{125}I-labeled IgG (0.1−0.2 mCi/mL). The blot was extensively washed in Tween-containing buffer and dried. The radioactivity of each sample was measured directly by β-scanning using an Ambis 400 imaging detector. The net counts of radioactivity (corrected for background counts from a blank sample) were obtained using the AMBIS image analysis software, v4.1, and then plotted on a semilogarithmic plot.

Detection of Reduced Sulfhydryls. Endothelial cells were incubated for 2 h with the standard Krebs-Hepes-glucose buffer that had been pre-equilibrated with air/CO_2 or air/CO_2 with 110 nM CO. The buffer was removed; cells were scraped off the Petri plates and lysed by sonication as described above. Cell debris was centrifuged at 12000*g* for 10 min, and aliquots of supernatant were incubated in 100 mM potassium phosphate (pH 8.1) with 70 μM 5,5′-dithiobis(2-nitrobenzoic acid). Reduced sulfhydryls were assayed by measuring the optical density of the solution at 412 nm. A standard curve was generated using cysteine.

Oxidation of *p*-Hydroxyphenylacetic Acid (pHPA). Release of oxidants into the medium by endothelial cells was assessed as horseradish peroxidase (HRP)-mediated conversion of pHPA to its fluorescent dimer using methods similar to those described by Panus et al. (*16*). In these experiments, 1.6 mM pHPA and 95 μg/mL HRP were added to the buffer overlying the cells, and fluorescence (excitation wavelength 323 nm, emission wavelength 400 nm) was measured in aliquots of the buffer taken at intervals over 2 h. Release of hydrogen peroxide (H_2O_2) into the surrounding medium was measured by first incubating cells in buffer without reagents and then incubating isolated samples of the buffer at room temperature for 15 min in order to allow rapidly reactive oxidants, such as peroxynitrite, to spontaneously decay. After incubation, pHPA and HRP were added to the samples and fluorescence was assessed as a measurement of relatively stable oxidants. Using this procedure, changes in fluorescence were inhibited by adding catalase (500 U/mL) to the buffer, indicating that H_2O_2 was the predominant oxidizing species. A standard curve was also prepared using known concentrations of H_2O_2.

Dihydrorhodamine (DHR) Oxidation. Plates of cells were incubated with the standard Krebs-Hepes-glucose buffer plus 5 μM DHR for 1 h at 37 °C to load the cells with DHR. After this, the buffer in the plates was exchanged for fresh buffer plus 5 μM DHR equilibrated with air/5% CO_2 or air/CO_2 plus a desired concentration of CO. The small gas space in the Petri plates was flushed with the appropriate gas, and then the cells were incubated for an additional hour. Cells were scraped off the plates and sonicating twice for 30 s, and the cell debris was centrifuged at 12000*g* for 10 min. Rhodamine concentration in the solution was measured by fluorescence (excitation wavelength 500 nm, emission wavelength 536 nm) and also by absorbance spectroscopy ($\epsilon_{500} = 7.8 \times 10^4\ M^{-1}\ cm^{-1}$), and total DHR plus rhodamine content of samples was determined based on fluorescence measurements of samples first incubated with excess amounts of HRP and H_2O_2 (30 μg of HRP and 80 μM H_2O_2) to convert all DHR to rhodamine.

Oxygen Consumption Assay. The consumption of oxygen by endothelial cells was measured with a Clark-type oxygen electrode (YSI, Yellow Spring, OH) at 37 °C. Cells were grown using the standard method except they were planted onto Transwell plates (Costar Corp., Cambridge, MA) rather than plastic. The flexible membrane at the base of the Transwell plate could be easily cut out, which facilitated transfer of the cells to the oxygen electrode apparatus. Cells were incubated with 2.5 mL of Krebs-Hepes-glucose buffer that had been equilibrated with either air plus 5% CO_2 or air with 5% CO_2 and 110 nM CO, and the oxygen concentration in the system was monitored.

Determination of Intracellular H_2O_2 Concentration. Assays were conducted according to methods described by Paler-Martinez et al. (*17*). Endothelial cells were cultured under standard conditions, washed, and incubated with Krebs-Hepes-glucose buffer equilibrated with either air/5% CO_2 or air/5% CO_2 plus 110 nM CO for 1 h. The buffer was then supplemented with 10 mM 3-amino-1,2,4-triazole (ATZ), and at 10 min timed intervals the ATZ-containing buffer was removed, cells were washed three times with 5 mL of Krebs-Hepes-glucose buffer,

and 1 mL of 50 mM potassium phosphate, 0.1 mM EDTA, and 0.1% Triton X-100 (pH 7.0) was added. The cells were scraped off plates and sonicated as described above. Catalase activity in the lysates (protein concentration of approximately 0.33 μg/μL) was measured by adding 300 μL of lysate to 700 μL of 16.4 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and monitoring the disappearance of H_2O_2 with a spectrophotometer set at 240 nm. Catalase activity is defined as the amount of enzyme required to decompose 1 μmol of H_2O_2/min at 25 °C (pH 7.0) using $\epsilon_M = 43.6$. The concentration of H_2O_2 in cells was calculated using the pseudo-first-order rate constant of catalase inactivation determined from the exponential decay in cellular catalase activity versus time, as described by Royall et al. (*18*).

Reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT). MTT reduction was assayed following procedures as described by Mosmann (*19*). Cells were incubated for 90 min in Krebs-Hepes-glucose buffer equilibrated with air/CO_2 without or with 110 nM CO, and then 1.2 mM MTT was added to the cultures for the last 30 min of a 2 h incubation. The cells were then extensively washed, harvested with a rubber policeman, and sonicated in an ice bath for two 30 s cycles. Samples were centrifuged at 12000*g* for 10 min, and reduced MTT was measured at 570 nm.

Chromium Release by Endothelial Cells. Plates of cells were exposed for 18 h to 40 mM sodium [^{51}Cr]chromate (Amersham, Arlington Heights, IL; specific activity 300 mCi/mg, thus approximately 1 μCi/mL of growth medium) to load the cells with ^{51}Cr. On the morning of the study, the cells were washed three times with 2 mL of Krebs-Hepes-glucose buffer and then incubated with buffer equilibrated with air/5% CO_2 or air/CO_2 and a desired concentration of CO. At the completion of incubation, the buffer (identified as solution A) was removed and centrifuged at 12000*g* for 10 min, and radioactivity was counted in an automated gamma counter (Wallac, Inc., Gaithersburg, MD; Model 1470). The adherent cells were removed from the plates by adding 1 mL of 0.1% (v/v) Triton X-100. The cells were scraped with a rubber policeman and collected in a tube and the plates washed with an additional 1 mL of 0.1% Triton. The radioactivity in the combined sample was measured (identified as solution B). Leakage of chromium was defined as the radioactive counts in solution A divided by the counts in solution A plus solution B. Where indicated, plates of cells were exposed to 100 μM L-NAME for 30 min prior to and also throughout the time of incubation with gas-equilibrated buffer. After incubation of some cell preparations the buffer was removed and replaced with 1 mL of standard growth medium equilibrated with air/5% CO_2, and the cells were placed in a 37 °C incubator. After 6 h at 37 °C the medium was removed and radioactivity measured in the overlying medium as well as in cell lysates.

Ethidium Homodimer-1 Uptake. Cells were exposed to standard growth medium that was pre-equilibrated with either air plus 5% CO_2 or air with CO_2 plus a desired concentration of CO for a period of 2 h. The medium was removed and replaced with fresh medium equilibrated with air and 5% CO_2 that contained 15 nM ethidium homodimer-1 (Molecular Probes, Eugene, OR). The cells were placed in a 37 °C incubator and examined at intervals under a Nikon Diaphot-TND epifluorescence inverted microscope. Ethidium uptake was quantified in cells incubated for 24 h after CO exposure. The overlying medium was removed, cells were washed twice with PBS and removed from the plastic plates by incubating cells for 5 min with a solution of 0.05% (w/v) trypsin and 0.2% (w/v) EDTA, a small volume of suspension was placed on a hemocytometer, and cells were counted in a phase-contrast microscope; cells were sonicated twice for 30 s on ice using a Heat Systems-Ultrasonics sonicator (Model W220-F) at setting 6. The cell lysate was centrifuged at 1000*g* for 10 min, fluorescence was measured in a Perkin Elmer LS 50 B fluorimeter, and the results are expressed as relative fluorescence units per 1×10^4 endothelial cells.

L-Arginine Transport by Endothelial Cells. L-Arginine uptake was measured under conditions approaching initial entry rates using techniques essentially the same as those used by Greene et al. (*20*). Plates of endothelial cells were washed with Krebs-Hepes-glucose buffer, and intracellular stores of arginine were depleted by incubating cells with buffer at 37 °C for 2 h. The air-equilibrated buffer was removed and replaced with fresh buffer containing 50 μM [^{14}C]-L-arginine (New England Nuclear; specific activity 55.6 mCi/mmol) equilibrated with either air/CO_2 (control) or air/CO_2 plus 110 nM CO. Cells were incubated for up to 2 min, washed three times with Krebs-Hepes-glucose buffer, left to air-dry, and then treated with 300 μL of 0.2% sodium dodecyl sulfate in 0.2 N NaOH. Plates were incubated at room temperature for 30 min. The concentration of protein in this solution was measured, and [^{14}C]arginine was measured in a scintillation counter. Active transport is expressed as [^{14}C]-L-arginine found in the cell preparations minus [^{14}C]-L-arginine present in samples coincubated with 10 mM nonradioactive arginine.

Nitric Oxide Synthase Activity. An initial series of studies was performed by first incubating plates of cells with Krebs-Hepes-glucose buffer pre-equilibrated with air/5% CO_2 or air/CO_2 plus 110 nM CO for 2 h. Buffer was removed from the plates followed by addition of 0.5 mL of lysis buffer (Krebs buffer plus 10 mM Hepes (pH 7.4) containing 0.1 mM EDTA, 1 mM dithioerythritol, and the protease inhibitors: 50 μM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 2 μg/mL aprotinin). Cells were scraped off the plates using a rubber policeman and sonicated in an ice-bath for two 30 s cycles. Samples were centrifuged at 12000*g* for 10 min, and nitric oxide synthase activity in the supernatant was assayed using methods previously described for platelet lysates (*13*). In brief, cell lysates were incubated at 37 °C with 2 mM NADPH, 230 μM $CaCl_2$, 3 μM tetrahydrobiopterin, and 10 mM [^{14}C]-L-arginine. At intervals, the [^{14}C]-L-citrulline was separated from the remaining arginine by passing the lysate through Dowex columns, and enzyme activity is expressed as the difference in counts between preparations incubated without or with 0.1 mM L-nitroarginine methyl ester (L-NAME).

Increases in Steady State Nitric Oxide by CO. Endothelial cells grown on 35 mm plastic plates were incubated for 30 min with 100 μM L-NAME in Krebs-Hepes-glucose buffer to inhibit nitric oxide synthase activity. The cells were washed three times with fresh buffer and covered with 0.5 mL of buffer containing 100 μM L-NAME that had been pre-equilibrated with air plus 5% CO_2 or air with 5% CO_2 plus 110 nM CO. After incubation for 30 min, buffer was removed, and the concentration of nitrite plus nitrate was measured.

As an alternative technique, •NO displacement was also monitored using a polarographic •NO meter (Iso-NO, World Precision Instruments, Inc., Sarasota, FL). Endothelial cells were plated onto 24-well microplates, rather than the usual 35 mm plates, in order to provide a smaller surface area and therefore reduce the rate of efflux of aqueous •NO. Before each study the growth medium on a microwell plate of cells was removed and replaced with Krebs-Hepes-glucose buffer equilibrated with air/5% CO_2 as well as 100 μM L-NAME. Cells were incubated at 37 °C for 30 min to inhibit cellular nitric oxide synthase, and then the polarographic probe was placed in the buffer. After the current stabilized a flow of gas was begun using a needle immersed in the buffer. A flow meter in the gas line was used so that gas flow was the same for every experiment. The turbulence produced by the bubbling gas mixed the sample, and the change in current was monitored on a chart recorder. Gas flows were established with air/5% CO_2 (control) or air/CO_2 with 50 or 100 ppm CO. Control experiments were carried out using the identical techniques but in microplates containing no endothelial cells and by observing a loss of current when 2 μM oxyhemoglobin was added to wells with cells exposed to air/CO_2 plus 100 ppm CO.

Statistical Methods. Statistical significance was determined by ANOVA followed by Scheffe's test. The level of significance was taken as $p < 0.05$. Results are expressed as mean ± standard error.

Results

Nitrite Plus Nitrate as an Index of •NO Production by Endothelial Cells. When endothelial cells were

Table 1. Oxidation of pHPA by Vascular Endothelial Cells Exposed to CO[a]

	CO concentration, nM			
	0 (air)	44	77	110
relative fluorescence, units min^{-1} (mg of protein)$^{-1}$	1.91 ± 0.18 (20)	3.04 ± 0.26 (6)	5.01 ± 0.9* (4)	6.37 ± 0.74* (13)

[a] Values reflect pHPA dimer formed in buffer overlying endothelial cells exposed to selected concentrations of CO in addition to air/5% CO_2. Numbers in parentheses below the measurements indicate the number of trials with different plates of cells; *p < 0.05.

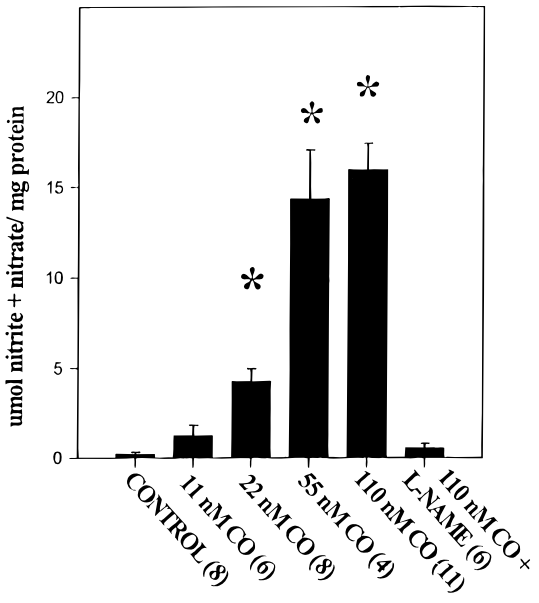

Figure 1. Nitrite + nitrate production by vascular endothelial cells. Bovine pulmonary artery endothelial cells were exposed to buffer-equilibrated air/5% CO_2 and CO at various concentrations. Values reflect the concentration of nitrite and nitrate measured in the medium after 1 h. Number of trials are indicated (n); *p < 0.05 versus control.

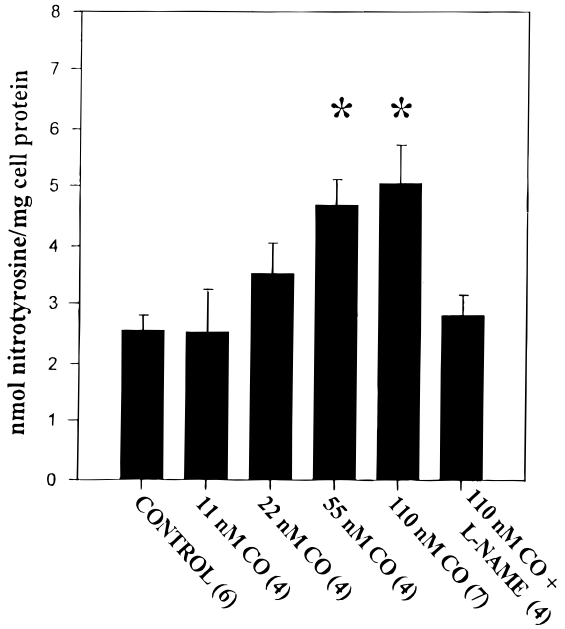

Figure 2. Nitrotyrosine in endothelial cell lysates. Values indicate the concentration of nitrotyrosine measured in lysed cell supernatants. Where indicated, cells were incubated with 100 μM L-NAME for 30 min before and throughout the CO exposure. Number of trials are indicated (n), *p < 0.05, versus control. Results with L-NAME + 110 nM CO were significantly less than results with 55 and 110 nM CO.

exposed to CO there was a concentration-dependent increase in nitrite + nitrate production (Figure 1). To confirm that the increase was due to nitric oxide synthase activity, cells were preincubated with 100 μM L-NAME for 30 min and then exposed to air/5% CO_2-equilibrated buffer containing 110 nM CO and 100 μM L-NAME. As shown in Figure 1, nitrite + nitrate production was significantly reduced.

Nitrotyrosine in Cells. Lysates from cells exposed to CO were analyzed for content of nitrotyrosine. There was a significant increase in lysates prepared from cells exposed to 55 or 110 nM CO (Figure 2). Additionally, the elevation in nitrotyrosine was inhibited when cells were incubated in the presence of 100 μM L-NAME. We conclude from these data that CO exposure caused cells to increase production of •NO-derived oxidants.

Cellular Content of Sulfhydryls. The concentration of reduced sulfhydryls in control cell lysates was 254 ± 28 (SE, n = 9) μmol/mg of cell protein, and in lysates from cells exposed to 110 nM CO the concentration was 303 ± 44 (n = 11) (no significant difference). We conclude that although CO appeared to cause an oxidative stress based on an increase in nitrotyrosine content, there was no gross cellular oxidative stress which would be expected to have caused a depletion of cellular reduced sulfhydryls.

Exposure to CO Increases Production of Agents Capable of Oxidizing pHPA. We next investigated whether CO caused cells to release oxidant species into the medium. Endothelial cells normally release oxidants capable of dimerizing pHPA (*16*). Oxidation of pHPA in the buffer above cultures was increased in a dose-dependent fashion when cells were incubated with CO (Table 1). Enhanced fluorescence was inhibited when cells were incubated with 100 μM L-NAME for 30 min before beginning the experiment. Control cells preincubated with L-NAME exhibited production of oxidants at a rate insignificantly different from cells in the absence of L-NAME, 2.35 ± 0.4 (n = 5). Cells exposed to 110 nM CO after incubation with L-NAME exhibited a rate of 2.15 ± 0.5 (n = 5) units min^{-1} (mg of protein)$^{-1}$ (no significant difference from control). We conclude from these findings that activity of nitric oxide synthase is necessary for enhanced extracellular oxidant production by CO.

These assays were conducted with the reagents in buffer over the cells; hence the agent(s) responsible for pHPA oxidation could be a short-lived, reactive agent such as peroxynitrite (*21*). As an alternative technique to assess whether a relatively stable oxidant such as H_2O_2 was produced, the cells were exposed to air/CO_2 plus CO and the reagents, pHPA and HRP, were not initially included (see Experimental Methods). As the half-life of peroxynitrite in an aqueous solution is on the order of 0.9 s (*22*), it should rapidly react with substances in the buffer and be undetectable by the pHPA/HRP assay. Aliquots of the buffer were removed from the solution overlying the cells and incubated for 10−15 min at room temperature, and then pHPA and HRP were added. We found no difference in the fluorescence signal in preparations from control cells and cells exposed to 110 nM CO. The changes in fluorescence detected in this study were virtually eliminated by coincubating samples with 500 U/mL catalase, leading to the conclusion that H_2O_2 was the principal oxidant detected. Based on a standard

Table 2. Oxidation of DHR by Endothelial Cells[a]

sample	[rhodamine], pM
control	44 ± 8 (10)
22 nM CO	55 ± 2.2 (6)
110 nM CO	99 ± 15 (6)*
110 nM CO + 100 μM L-NAME	40 ± 10 (5)

[a] Values reflect the concentration of rhodamine measured in buffer overlying endothelial cells exposed for 5 min to air/5% CO_2 (control) or air/5% CO_2 plus the indicated concentration of CO. Number of samples is in parentheses; *p < 0.05.

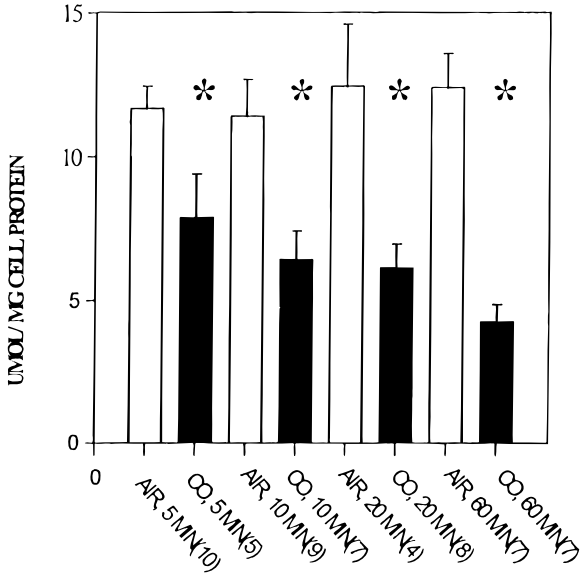

Figure 3. Concentration of rhodamine plus dihydrorhodamine in cell lysates. Values reflect the concentration in lysates from cells exposed to air/5% CO_2 or air/CO_2 plus 110 nM CO. Number of trials are indicated (n); *p < 0.05 versus control.

curve generated with H_2O_2, control cells exposed to air plus 5% CO_2 produced 1.91 ± 0.17 (n = 6) nmol of H_2O_2 min^{-1} (mg of protein)$^{-1}$. Cells exposed to air/CO_2 plus 110 nM CO produced 1.98 ± 0.28 (n = 9) nmol of H_2O_2 min^{-1} (mg of protein)$^{-1}$ (no significant difference). Hence, exposure to CO caused the release of a rapidly reactive agent into the medium, but CO did not cause endothelial cells to increase the release of H_2O_2 into the medium.

Dihydrorhodamine Oxidation. Conversion of DHR to fluorescent rhodamine is a sensitive method for detecting peroxynitrite, and H_2O_2 and •NO will not confound the results because they do not react with DHR (*23*). The concentration of rhodamine in the buffer was increased when endothelial cells were exposed to 110 nM CO (Table 2). This effect was due to the activity of nitric oxide synthase, based on the inhibitory effect of 100 μM L-NAME.

We hypothesized that measurements of rhodamine in cell lysates would be indicative of production of oxidants within the cells, as DHR only slowly diffuses out of intact cells (*24*). However, we found that exposure to CO caused a progressive decrease in the total concentration of DHR + rhodamine detected in the cell system over the course of the experiment. This did not occur in cells exposed to only air/5% CO_2 (control) (Figure 3). Samples were surveyed both by fluorescence spectroscopy and by standard absorbance spectroscopy, and the same results were found. It should be noted that when cell-free solutions of 5 μM DHR were incubated with 110 nM CO, no reduction in detectable DHR occurred. The loss of detectable DHR + rhodamine with cells exposed to CO was not changed if the cells were incubated in the presence of 100 μM L-NAME; hence we conclude that the

Table 3. Total DHR + Rhodamine in the Experimental System[a]

CO, nM	% of control
22	98 ± 13 (6)
55	60 ± 8 (6)*
77	54 ± 10 (5)*
110	48 ± 7 (8)*

[a] Values reflect the relative concentration of DHR plus rhodamine in buffer and cell lysates from preparations exposed to various concentrations of CO in relation to the concentration in control cell preparations from the same day. Number of samples is in parentheses; *p < 0.05.

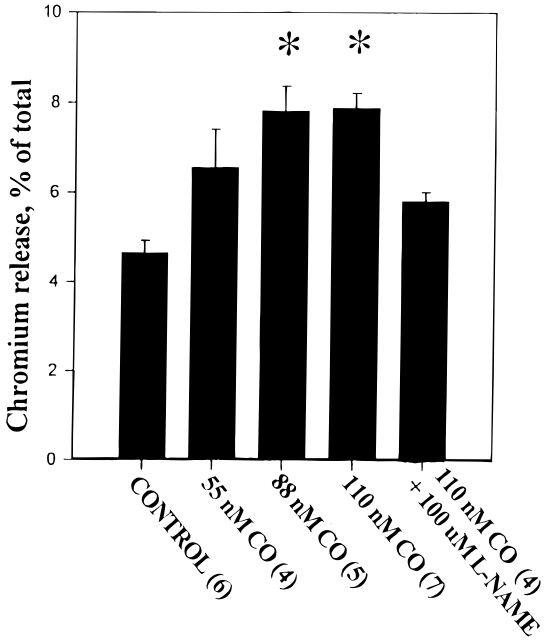

Figure 4. Chromium release by cells incubated for 4 h. Values reflect the leakage of ^{51}Cr from endothelial cells exposed to air/CO_2 or air/CO_2 plus selected concentrations of CO. n = number of samples; *p < 0.05 versus control. Results with L-NAME + 110 nM CO were significantly less than results with 88 and 110 nM CO.

effect was independent of nitric oxide synthase activity. The decrease in detectable DHR plus rhodamine occurred in a dose-dependent manner over a range of CO concentrations. This is shown in Table 3 as a percent of the concentration found in matched control cell preparations from experiments done on the same day.

Cytotoxicity of CO Exposure. Exposure to CO for 4 h caused a dose-dependent increase in ^{51}Cr release which was inhibited if cells were also incubated with L-NAME (Figure 4). When cells were incubated with 110 nM CO for 2 h, there was no detectable release of ^{51}Cr immediately after the exposure (Figure 5). However, if the CO-containing buffer was removed and cells were incubated with growth medium equilibrated with air/5% CO_2, ^{51}Cr release was evident 6 h later (Figure 5). Of note, when cells were incubated with L-NAME during the CO exposure, they were not protected from the delayed ^{51}Cr release.

Vital Staining with Ethidium Homodimer-1. The loss of membrane integrity with time after CO exposure was also evident when cells were incubated with the vital stain ethidium homodimer-1. After cells were exposed to CO for 2 h, uptake of the fluorescent marker was not evident. However, by 20 h, uptake of the fluorescent dye was clearly seen with samples exposed to as little as 22 nM CO. Uptake occurred in more cells with higher concentrations of CO. Examples of cells incubated for

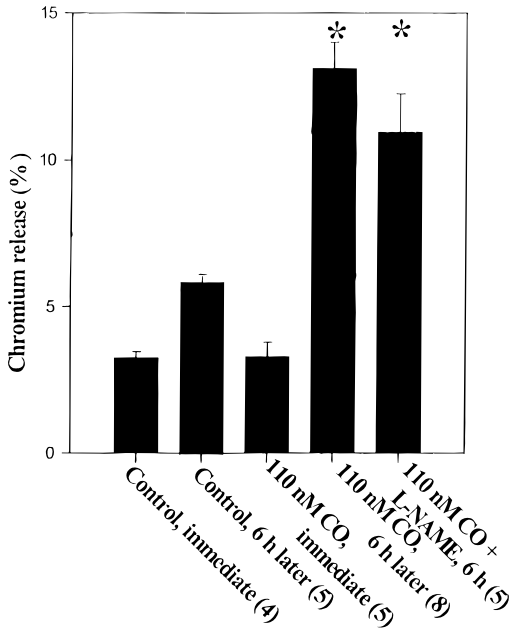

Figure 5. Chromium release by cells in 6 h after air or CO exposure. Values reflect the leakage of ^{51}Cr from endothelial cells exposed to air/CO_2 or air/CO_2 plus 110 nM CO for 2 h and then analyzed immediately or after incubation with air/CO_2-equilibrated growth medium for 6 h. Where indicated cells were preincubated with 100 μM L-NAME for 30 min before and throughout the 2 h exposure to CO. n = number of samples; *p < 0.05 versus control.

20 h after exposure to air (control) or 110 nM CO are shown in Figure 6. Many of the cells exposed to CO were lifted off of the plate, and many that still remained partially attached exhibited nuclear blebbing. Uptake of vital stain and morphological changes were not observed in cells incubated with 100 μM L-NAME during the exposure to 110 nM CO. Ethidium uptake by cells was quantified (see Experimental Methods), and results are shown in Table 4. We conclude that exposure to CO causes a form of delayed death in endothelial cells that is mediated by •NO-derived oxidants.

Effect of CO on Cellular Oxygen Consumption, Intracellular H_2O_2 Concentration, and Redox State. The data indicate that CO caused endothelial cells to increase production of peroxynitrite. This could occur if CO increased production of superoxide, •NO, or both radicals in the cells. While there is precedence to consider that exposure to CO may disturb mitochondrial function, which may enhance generation of $O_2^{\bullet -}$ as well as H_2O_2, this would not be expected in the well-aerated cultures used in these experiments (*25*).

Oxygen consumption by endothelial cells exposed to air with 5% CO_2 was 24 ± 7 (n = 4) nmol of O_2 min^{-1} (mg of protein)$^{-1}$, and the rate with cells incubated with air/CO_2 plus 110 nM CO was 25 ± 3 (n = 4) nmol of O_2 min^{-1} (mg of protein)$^{-1}$ (no significant difference). The concentration of H_2O_2 inside endothelial cells was measured based on ATZ-mediated inactivation of catalase after cells had been exposed to 110 nM CO for 1 h. Catalase was not inhibited when cells were exposed to up to 110 nM CO. The activity of catalase in control cells (exposed to buffer equilibrated with air plus 5% CO_2) was 3.4 ± 0.2 (n = 7) mU/mg of protein, and in cells exposed to air/5% CO_2 plus 110 nM CO it was 3.2 ± 0.3 (n = 7) mU/mg of protein (no significant difference). The concentration of H_2O_2 inside control cells was 52 ± 2 (n = 6) pM, and in cells exposed to 110 nM CO it was 50 ± 5 (n = 6) pM (no significant difference). We conclude that 110 nM CO had no significant effect on endothelial cell oxygen consumption or production of intracellular H_2O_2.

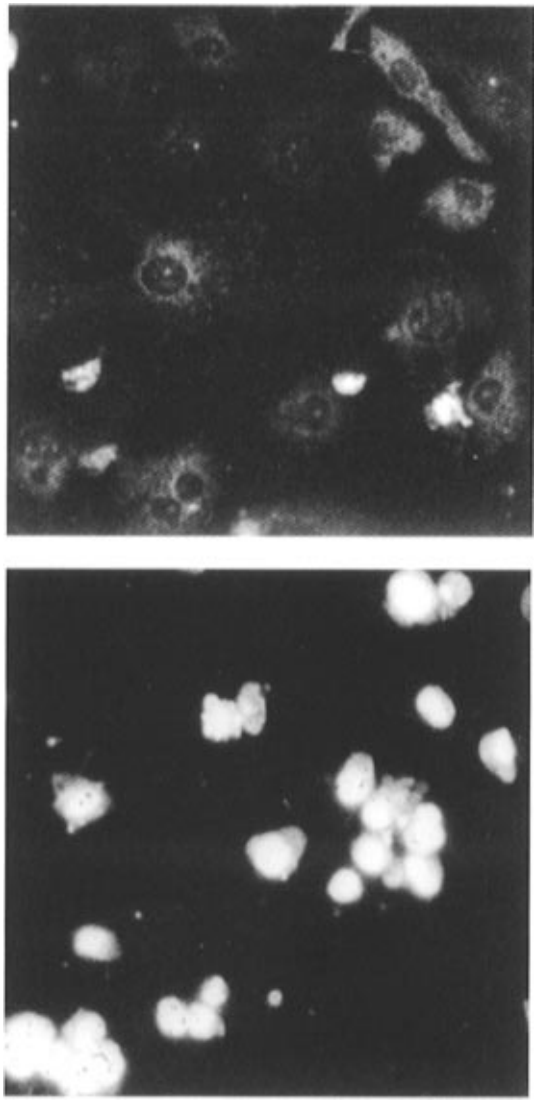

Figure 6. Ethidium homodimer-1 uptake by endothelial cells exposed to air/CO_2 (top) or air/CO_2 plus 110 nM CO in buffer for 2 h and then incubated with air/CO_2-equilibrated medium containing 15 nM ethidium homodimer-1 for 18 h (bottom).

Table 4. Ethidium Homodimer Uptake by Endothelial Cells[a]

sample	ethidium in cells
control	0.16 ± 0.02 (6)
110 nM CO	0.29 ± 0.02 (6)
110 nM CO + L-NAME	0.18 ± 0.01 (6)$^{+}$

[a] Values reflect mean ± SE of relative fluorescence units/1 × 10^4 endothelial cells. Cells were exposed to 110 nM CO for 2 h and then incubated for 24 h in fresh growth medium equilibrated with air/5% CO_2. Where indicated cells were exposed to 100 μM L-NAME beginning 30 min before exposure to CO. Number of samples is in parentheses; *p < 0.05 versus control, ^{+}p < 0.05 versus 110 nM CO.

We also investigated redox activity of cells by examining reduction of MTT. MTT reduction is mediated by both mitochondrial and cellular dehydrogenases, and hence, it is dependent on the availability of reduced pyridine nucleotides in the cell. We found the magnitude of MTT reduction was the same in cells exposed to either air plus 5% CO_2 (control) or air plus CO_2 and 110 nM CO for up to 2 h. Reduced MTT in lysates of control cells at the end of a 2 h incubation (see Experimental Methods) was 0.17 ± 0.01 (n = 5) absorbance unit/mg of cell

protein, and in lysates from cells exposed to CO the value was 0.19 ± 0.03 (n = 5) absorbance unit/mg of cell protein (no significant difference).

Enhanced •NO Production Does Not Involve Alteration in L-Arginine Transport by Vascular Endothelial Cells. Nitric oxide synthesis requires the availability of arginine, a substrate for nitric oxide synthase. Therefore, we investigated whether CO altered arginine uptake by endothelial cells. Nonspecific binding was determined to be 1.20 ± 0.09 (n = 5) nmol/mg of protein. Active transport by cells exposed to air/5% CO_2 was 0.35 ± 0.04 (n = 4) nmol (mg of protein)$^{-1}$ min^{-1} and in cells exposed to 110 nmol of CO plus air/CO_2 it was 0.34 ± 0.04 (n = 4) nmol (mg of protein)$^{-1}$ min^{-1} (no significant difference). We conclude that CO did not affect the ability of cells to transport L-arginine across the cell membrane.

CO Exposure Does Not Alter Nitric Oxide Synthase Activity. Nitric oxide synthase activity was initially measured as the accumulation of radioactive citrulline in cytosolic preparations made from cells that had been exposed to buffer equilibrated with air and 5% CO_2 for 2 h (control) or air and CO_2 plus 110 nM CO. Using lysates from control cells, 6.4 ± 0.8 (n = 4) nmol of L-citrulline/mg of protein accumulated during a 1 h incubation, and with samples from CO-exposed cells, accumulation was 7.0 ± 1.8 (n = 4) nmol of L-citrulline/mg of protein (no significant difference). We also assessed the nitric oxide synthase activity in intact cells. We found that [^{14}C]arginine uptake was negligible unless cells had been starved by replacing the complex medium with buffer for 2 h before the experiment began. After this manipulation, cells accumulated 2.2 ± 0.5 (n = 5) pmol of L-citrulline/mg of cell protein when incubated in the presence of air plus 5% CO_2 for 2 h and 1.8 ± 0.7 (n = 5) pmol/mg of protein when cells were exposed to air with CO_2 and 110 nM CO (no significant difference). It should be noted that these enzyme activities were lower than rates estimated by nitrite plus nitrate production. This may be related to further metabolism of citrulline, versus nitrate which is relatively stable, or because cell stores of arginine were not entirely depleted in intact cells. However, the results are important as an indication of relative enzyme activity in the two experimental groups. We conclude that exposure to CO did not cause an increase in nitric oxide synthase activity.

Elevation in Steady State •NO by CO. The data indicate that CO did not increase synthesis of either superoxide or •NO. In platelets, CO increases the steady state level of •NO in suspensions because it binds to heme-proteins that normally scavage intraplatelet •NO, and exposure to light reverses this effect (*13*). We attempted to test whether a similar mechanism occurred in endothelial cells by investigating whether nitrite + nitrate production was increased after cells were exposed to bright light (see Experimental Methods). Endothelial cells were found to be extremely sensitive to light, however, and levels of exposure that only mildly injured cells were insufficient to affect the CO-mediated process (data not shown).

As an alternative, to evaluate the potential for competition, we investigated whether CO could displace intracellular •NO. Endothelial cells were first incubated with 100 μM L-NAME for at least 30 min to inhibit nitric oxide synthase. In one series of studies, cells were incubated with 100 μM L-NAME in buffer that had been equilibrated with air/CO_2 or air/CO_2 plus 110 nM CO,

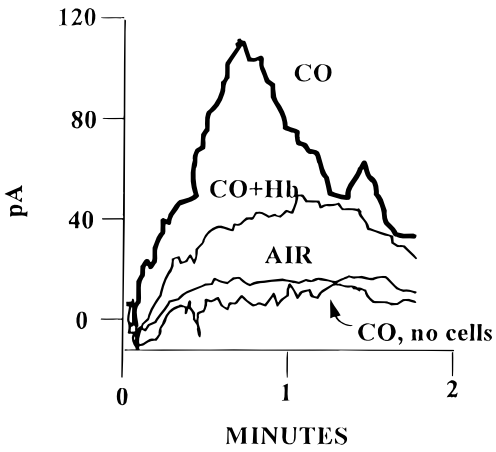

Figure 7. Displacement of intracellular NO• by CO. Bovine pulmonary artery endothelial cells were preincubated with 100 μM L-NAME to inhibit nitric oxide synthase, and then cells were exposed to flows of gas containing air/5% CO_2 or air/CO_2 and 100 ppm CO. In the sample indicated, 2 μM oxyhemoglobin was added to the buffer approximately 20 s after starting the gas flow. Curves shown are representative of studies performed at least four times with each condition.

and nitrite plus nitrate concentration was measured after 30 min. The concentration in buffer, normalized to the amount of cell protein present in each preparation, was 5.6 ± 2.7 (n = 3) μmol/mg of cell protein in samples exposed to air/CO_2 and 30.0 ± 5.5 (n = 4, p < 0.05) μmol/mg of cell protein in samples exposed to air/CO_2 plus 110 nM CO.

A second series of studies was conducted using an •NO selective electrode (see Experimental Methods). After cells were preincubated with 100 μM L-NAME for 30 min, either air plus 5% CO_2 or air plus CO_2 with CO was bubbled into the buffer overlying the cells, and aqueous •NO concentration was assayed. As shown in Figure 7, a current was detectable for approximately 1.5 min in the CO-exposed samples, and this was blunted when the sample was injected with 2 μM oxyhemoglobin. The current was negligible when cells were exposed to air/5% CO_2 or when buffer was exposed to a flow of CO in the absence of cells. In replicate testing with different preparations of cell monolayers, the •NO peak height measured with exposures to a gas flow of air/5% CO_2 was 3 ± 2 nmol (n = 4), whereas the flux of •NO from cells exposed to air/CO_2 plus 50 or 100 ppm CO was, respectively, 37.2 ± 6.9 nmol (n = 4, p < 0.05) and 113.5 ± 4.0 nmol (n = 4, p < 0.05). We interpret these results as evidence that CO could compete for intracellular binding sites, and the •NO displaced was detected until it diffused into the overlying gas phase. There was no steady state •NO signal because there was no continuous •NO synthesis by the cells, which were incubated in the presence of L-NAME, and the gas flow removed the •NO from solution to the gas phase where it could not be detected by the probe.

Discussion

The results of this study indicate that vascular endothelial cells liberate •NO and cytotoxic •NO-derived oxidants in response to CO. A fraction of the oxidants can be expected to interact with cellular tyrosine residues, and detection of nitrotyrosine in cell samples strongly implicates peroxynitrite as the oxidizing species. Oxidation of pHPA in the buffer above CO-exposed cells indicates that peroxynitrite also diffuses into the medium. The results with DHR indicate production of

peroxynitrite when cells are exposed to CO. However, conversion of DHR and/or rhodamine to undetectable products suggests that our measurements may underestimate the rate of oxidation, and this also indicates that •NO-independent effects are initiated by CO exposure.

Exposure to CO at concentrations up to 110 nM for 2 h did not alter mitochondrial function based on measurements of oxygen consumption, H_2O_2 production, and MTT reduction. Arginine uptake by cells and nitric oxide synthase activity were also unaffected by CO. It appears that CO competes for intracellular sites that normally bind •NO, which increases the steady state level of unbound •NO available to undergo reactions with superoxide. This mechanism is supported by elevations in nitrite/nitrate concentration and also detection of •NO in the buffer overlying cells exposed to CO after •NO synthesis was inhibited. We have reported a similar mechanism for the elevation in steady state •NO concentration in suspensions of platelets exposed to CO (*13*). In our previous studies, exposure to light reversed the effect of CO on platelets, indicating that photodissociation of CO from heme-proteins restored normal intraplatelet •NO binding (*13*). A similar effect could not be shown with endothelial cells, however, due to their sensitivity to light.

Hemoproteins are likely to play a major role in binding •NO in endothelial cells. CO can successfully compete against •NO and bind to heme moieties when there is an alternative target molecule available to bind the •NO (*26*). This is especially true if the concentration of CO exceeds that of •NO, which was the case in our investigation. Cells were exposed to nanomolar concentrations of CO, and the intracellular concentration of •NO is likely to be in the picomolar range. Using a •NO selective electrode, Tsukahara et al. (*27*) estimated that the basal level of •NO released from a confluent layer of human endothelial cells on a 35 mm dish was 260 nM. Assuming that the electrode surface area was about 1% of the surface of the dish and that there were about 2×10^6 cells on the dish, each cell released approximately 13 pM in the time of the measurement (between 0.5 and 1 min). We can estimate the intracellular steady state concentration of •NO in our air-exposed endothelial cells based on the nitrite/nitrate concentration that was measured (Figure 1). Control cell preparations (approximately 2×10^6 cells) accumulated about 0.7 μmol of nitrite + nitrate in the 0.5 mL of buffer over 1 h. Therefore, we estimate that •NO was produced at about 12 pmol cell^{-1} min^{-1}. A similar analysis of data reported by Schmidt et al. (*28*) provides an estimate of 33 pmol of nitrite + nitrate cell^{-1} min^{-1}. Therefore, it is quite feasible for CO to have competed successfully against •NO for intracellular hemeproteins, although our data do not exclude an effect of CO on other •NO-binding agents.

Peroxynitrite synthesis can be increased when the intracellular concentration of superoxide is increased, and the result is enhanced cell death (*29*). Our data indicate that CO exposure increased peroxynitrite synthesis by rendering •NO more available to undergo chemical reactions, although effects on antioxidant defenses were not investigated. Release of chromium when cells were exposed to CO demonstrates that CO had a direct adverse effect. Because L-NAME prevented chromium release during the 4 h exposures, we conclude that •NO-derived oxidative stress was the principal cause of enhanced membrane permeability. Exposure to CO also had delayed effects on endothelial cells. Chromium leakage was clearly evident in response to just a 2 h exposure to CO when cells were incubated for an additional 6 h in air-equilibrated growth medium. This delayed chromium leakage was not inhibited when cells were exposed to L-NAME. However, the results in Figure 5 suggest that L-NAME did not provide complete protection against CO-mediated oxidative stress. Hence, it is unclear whether delayed chromium leakage after CO exposure for 2 h was due to mechanisms other than •NO-mediated oxidative stress.

The delayed toxicity of CO for endothelial cells was also observed as ethidium homodimer-1 uptake in cells 20 h after exposure to CO. Whether this is an example of apoptotic cytotoxicity is not clear from the experiments performed to date. The blebbing of the plasma membrane seen in some cells exposed to 110 nM CO in Figure 6 is a characteristic of apoptosis. Peroxynitrite has been shown to trigger apoptosis in some cell types (*30, 31*), and hallmarks of apoptosis have been detected in cultured endothelium after peroxynitrite exposure (*32*).

Exposure to CO did not decrease the content of reduced sulfhydryls in cells, although thiols are targets for peroxynitrite (*7*). We may have failed to detect sulfhydryl oxidation if thiol recycling were rapid, especially in view of the fact that pyridine nucleotides were not diminished by CO exposure. Alternatively, the presence of abundant CO_2 in the system could have redirected the reactivity of peroxynitrite, causing increased nitration of protein tyrosine moieties and decreased thiol oxidations (*33*). It is also possible that CO may exert effects at discrete sites in the cell due to the intracellular localization of nitric oxide synthase. Endothelial cell nitric oxide synthase appears to be associated predominantly with plasma and organelle membrane structures, including mitochondria (*34*). Additional studies will be needed to explain the apparent preservation of reduced thiol groups despite oxidative stress from peroxynitrite.

Enhanced production of •NO-derived oxidants by endothelium provides a possible explanation for the perivascular distribution of nitrotyrosine in brain after animals are exposed to CO (*4*). It may also be of epidemiological significance that oxidant release occurs with exposure to rather low concentrations of CO. Delayed cell death occurred following exposure to 22 nM CO, which is the concentration expected with an interstitial CO partial pressure of about 20 ppm and a blood carboxyhemoglobin level of 7% (see Experimental Methods and ref 14). Oxidative stress leads to injuries to the vascular endothelium that precipitate atherosclerosis (*35*), and nitrotyrosine has been detected in human atherosclerotic plaques (*36*). The health risks associated with exposure to CO from air pollution and in tobacco smoke remain controversial. Exposure to automobile exhaust and levels of CO around 50 ppm have been associated with accelerated atherosclerosis in some (*11, 37*) but not all studies (*38*). Similarly, chronic exposure to CO, typically linked to cigarette smoking, is associated with atherosclerotic coronary artery disease (*39*−*41*), although this relationship too has not been supported by all studies (*42*). Evidence that low concentrations of CO can cause oxidative stress provides a biochemical basis for considering the contribution of CO to health risks. Further investigations are necessary to examine whether events we have observed in bovine vascular endothelium occur in human cells and the precise mechanisms involved with cell death.

Acknowledgment. We are grateful to Drs. J. S. Beckman and Y. Z. Ye for providing the anti-nitrotyrosine

antibody. This work was supported by a grant from the National Institute of Health (ES-05211) and by funds provided under contract to the Health Effects Institute (HEI), an organization jointly funded by the United States Environmental Protection Agency (EPA) (Assistance Agreement X-816285) and certain motor vehicle and engine manufacturers. The contents of this article do not necessarily reflect the views of HEI, or its sponsors, nor do they necessarily reflect the views and policies of the EPA or motor vehicle and engine manufacturers. H.I. is an Established Investigator of the American Heart Association.

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