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Mechanism of Oxidative Stress from Low Levels of Carbon Monoxide

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Includes the Commentary of the Institute's Health Review Committee

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HEI Statement

Synopsis of Research Report Number 80

Exploration of a Link Between Exposure to Carbon Monoxide and Atherosclerosis

BACKGROUND

Carbon monoxide, an indoor and outdoor air pollutant, is produced by the incomplete combustion of fossil fuels. People are exposed to carbon monoxide from sources such as automobile emissions, industrial processes, sidestream or mainstream cigarette smoke, and poorly vented space heaters and gas stoves. Exposure to high levels of carbon monoxide (500 parts per million [ppm]) can lead to respiratory failure and death. Even low-level exposures can cause adverse health effects (for example, chest pain) in some people with coronary artery disease when they exercise. For this reason, the U.S. Environmental Protection Agency has set National Ambient Air Quality Standards for carbon monoxide of 9 ppm averaged over eight hours and 35 ppm averaged over one hour.

Some researchers speculate that exposure to carbon monoxide may be related to the development of atherosclerosis, a progressive disease characterized by deposits of fat, cholesterol, cells, and connective tissue in blood vessels. These deposits, called plaques, can block blood flow. That carbon monoxide may have a role in atherosclerosis is a public health concern because this disease is the leading contributor to deaths by heart attack and stroke in the United States.

One possible mechanism by which carbon monoxide might influence the development of atherosclerotic plaques involves nitric oxide, a gas once regarded primarily as an air pollutant from combustion sources. However, we now know that nitric oxide is also produced in the body. It is involved in regulating many physiological reactions, including those in the cardiovascular, immune, and nervous systems. Nitric oxide also can be converted to toxic metabolites such as peroxynitrite, which converts cholesterol-carrying low-density lipoproteins to a form that contributes to atherosclerotic plaque formation. An earlier study by Dr. Stephen Thom and coworkers demonstrated that blood platelets isolated from rats exposed to high levels of carbon monoxide (1,000 or 3,000 ppm) released nitric oxide. This finding suggested a possible link between carbon monoxide exposure, peroxynitrite formation from nitric oxide, and atherosclerosis. The Health Effects Institute funded this pilot study to examine the effects of low concentrations of carbon monoxide on platelets and cells isolated from blood vessels.

APPROACH

Thom and Ischiropoulos exposed blood platelets (taken from rats) and endothelial cells (isolated from bovine blood vessels) to varying concentrations of carbon monoxide and measured how much nitric oxide was released. To determine if exposure to carbon monoxide causes endothelial cells to produce peroxynitrite, the investigators looked for markers of its presence in the culture medium and in the cells. They also exposed rats to carbon monoxide by inhalation, isolated platelets from the rats' blood, and measured how much nitric oxide was released.

RESULTS AND IMPLICATIONS

The investigators reported that platelets and endothelial cells exposed to carbon monoxide released nitric oxide; this was observed after exposure to carbon monoxide levels of 10 or 20 ppm, which are environmentally relevant concentrations. In addition, they found that platelets isolated from the blood of rats exposed to 20 to 1000 ppm carbon monoxide also released nitric oxide. However, concentrations of 50 or 100 ppm carbon monoxide were required to produce markers of peroxynitrite formation in endothelial cells. These concentrations approach levels of carbon monoxide that are toxic to humans; therefore, how relevant these results are to ambient exposures is uncertain.

These findings, which support the investigators' hypothesis of a possible connection between exposure to carbon monoxide and the release of nitric oxide by platelets and endothelial cells, warrant further investigation to determine their functional significance. The suggestive link to peroxynitrite formation, however, is based on experiments conducted in a cell culture system. Because such experiments do not include cellular constituents that are present and normally react with nitric oxide in vivo, thus reducing its availability for further reaction, additional work is required with experimental systems that more accurately reflect the natural cellular milieu to confirm the link.

This Statement, prepared by the Health Effects Institute and approved by its Board of Directors, is a summary of a research project sponsored by HEI from 1995 to 1996. This study was conducted by Drs. Stephen R. Thom and Harry Ischiropoulos of the University of Pennsylvania Medical Center, Philadelphia, PA. The following Research Report contains both the detailed Investigators' Report and a Commentary on the study prepared by the Institute's Health Review Committee.

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Mechanism of Oxidative Stress from Low Levels of Carbon Monoxide

Stephen R. Thom and Harry Ischiropoulos

ABSTRACT

The purpose of this study was to determine whether platelets and vascular endothelial cells would liberate nitric oxide free radical (NO)* and NO-derived oxidant species after exposure to carbon monoxide (CO) at concentrations up to 100 parts per million (ppm). We hypothesized that exposure to environmentally relevant concentrations of CO would increase production of agents that may be involved in human pathological processes, such as atherosclerosis.

Platelets obtained from rats released NO when incubated with CO, but CO did not increase platelet nitric oxide synthase activity. Platelets released comparable NO levels when they were exposed to CO in vitro and when taken from rats that had been exposed to CO. Partial pressures of CO as low as 10 ppm could successfully compete with NO for intraplatelet binding sites in in vitro studies. We conclude that CO enhanced the release of NO from platelets because it inhibited NO sequestration by intraplatelet binding sites, and that this phenomenon can occur with exposure to CO concentrations found in the environment.

Bovine pulmonary artery endothelial cells released NO in response to CO exposure. Carbon monoxide did not affect the transport of L-arginine across the plasma membrane or nitric oxide synthase activity; therefore, the mechanism appeared to be based on a disturbance of intracellular NO sequestration. Cells incubated with CO also released into the surrounding medium peroxynitrite, an NO-derived oxidant, based on oxidation of dihydrorhodamine 123 and *p*-hydroxyphenylacetic acid. Peroxynitrite-mediated oxidative stress to endothelial cells was identified as increased concentrations of nitrotyrosine in cell lysates, and by measuring the release of radioactive chromium. Carbon monoxide caused an acute injury when cells were continuously exposed for 4 hours, and a delayed injury when cells were exposed for 2 hours. Delayed injury was documented by leakage of radioactive chromium and by uptake of a vital fluorescent stain, ethidium homodimer-1, between 6 and 20 hours after CO exposure. Oxidative stress caused by CO exhibited several unique aspects because CO exposure did not alter the cellular content of reduced sulfhydryls nor did CO augment oxidative stress caused by superoxide, hydrogen peroxide, or a flux of NO. We concluded that concentrations of CO achieved in vivo when humans are exposed to CO concentrations found in the environment can cause endothelial cells to liberate NO and NO-derived oxidants, and that these products can adversely affect cell physiology.

INTRODUCTION

The health risks associated with exposure to CO from air pollution are unclear. Motor vehicle emissions are the single greatest source of outdoor CO. In urban areas levels usually vary 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 (Bevan et al. 1991; Stern et al. 1988; Wright et al. 1975). Exposure to automobile exhaust and levels of CO around 50 ppm have been associated with accelerated atherosclerosis (Stern et al. 1980, 1988); however, this association has not been shown consistently in studies with animals (Penn 1993). Epidemiological studies suggest that chronic exposure to CO, typically linked to cigarette smoking, is associated with atherosclerotic coronary artery disease (Cohen et al. 1969; Hexter and Goldsmith 1971; Kurt et al. 1978; Penn and Snyder 1993), although this relationship also has not been supported by all studies (Kuller et al. 1975).

Experimental findings indicate that oxidative stress leads to injuries to the vascular endothelium that precipi-

* A list of abbreviations appears at the end of the Investigators' Report.

This Investigators' Report is one part of Health Effects Institute's Research Report Number 80, which also includes a Commentary by the Health Review Committee, and an HEI Statement about the research project. Correspondence concerning the Investigators' Report may be addressed to Dr. Stephen Thom, University of Pennsylvania, Institute for Environmental Medicine, One John Morgan Building, 36th Street and Hamilton Walk, Philadelphia, PA 19104-6068.

Although this document was produced with partial funding by the United States Environmental Protection Agency under Assistance Award R824835 to the Health Effects Institute, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency, and no official endorsement by it should be inferred. The contents of this document also have not been reviewed by private party institutions, including those that support the Health Effects Institute; therefore, it may not reflect the views or policies of these parties, and no endorsement by them should be inferred.

tate atherosclerosis (Ross 1993). For example, modification of low-density lipoprotein by free radicals is thought to be a key event in formation of atherosclerotic plaques (Steinberg and Witztum 1990). Oxidative stress is an important signal that regulates endothelial cell surface receptors for leukocytes involved in the pathogenesis of atherosclerosis (Marui et al. 1993). Oxygen radicals also impair control over vascular contractility in atherosclerotic patients and hypercholesterolemic animals (Forstermann et al. 1988; Shimokawa et al. 1988; White et al. 1994).

A fundamental question regarding CO is whether exposure triggers biochemical mechanisms related to putative atherosclerotic processes. If so, then inconsistencies among clinical and animal studies regarding CO may be due to the complex interactions required for overt atherosclerosis. Platelets liberate NO following exposure to relatively high concentrations of CO (Thom et al. 1994). Reactive oxygen species are normally produced by mitochondria (Boveris and Cadenas 1975), and a chemical reaction between superoxide anion and NO will generate peroxynitrite, a relatively long-lived and strong oxidant (Huie and Padmaja 1993). Because the rate of this reaction is threefold greater than the reaction between superoxide anion and superoxide dismutase, production of peroxynitrite may be enhanced when the concentration of NO is increased in biological systems. Peroxynitrite can oxidize low-density lipoprotein, non-protein sulfhydryls, DNA, and membrane phospholipids (Radi et al. 1991; Hogg et al. 1993). Direct nitration of tyrosine residues can occur, and this process is catalyzed by transition metals, superoxide dismutase, and carbon dioxide (Ischiropoulos et al. 1992; Beckman et al. 1992; Gow et al. 1997). Nitrotyrosine synthesis in vivo appears to be a specific marker for peroxynitrite-mediated oxidative stress under physiological conditions (Gow et al. 1997). Nitrotyrosine has been detected in human atherosclerotic plaques (Beckman et al. 1994). In experimental CO poisoning, which involved CO concentrations well above typical environmental levels, nitrotyrosine deposits were found in a perivascular distribution in the brain microvasculature of animals, and nitric oxide synthase activity was required for the cascade of events to occur that led to brain lipid peroxidation (Ischiropoulos et al. 1996).

The aim of this study was to investigate whether environmentally relevant concentrations of CO could enhance production of NO and NO-derived oxidant species, such as peroxynitrite. Studies were performed with platelets to examine the mechanism by which CO enhances NO release. Another potential source for both superoxide anion and NO in vivo is vascular endothelial cells, and studies were conducted with bovine pulmonary artery endothelial cells to evaluate whether relatively low concentrations of CO enhanced production of NO-derived oxidants.

SPECIFIC AIMS

The objective of this research project was to examine whether platelets and vascular endothelial cells liberated NO and NO-derived oxidant species in response to exposure to environmentally relevant concentrations of CO.

Specific Aim 1: Investigate NO release by platelets.

Specific Aim 2: Investigate the effects of CO on cultured vascular endothelial cells.

METHODS AND STUDY DESIGN

This study was carried out with platelets obtained from rats or with bovine pulmonary artery endothelial cells. Studies were performed by exposing rats, isolated platelets, or endothelial cells to concentrations of CO between 0 and 1000 ppm.

ANIMALS AND REAGENTS

Wistar male rats (Charles River Laboratories, Wilmington, MA) weighing 200-290 g were fed a standard diet and water *ad libitum*. Reagents were purchased from Sigma Chemical Corp. (St. Louis, MO) unless otherwise specified.

PREPARATION OF CARBON MONOXIDE GAS SUPPLY

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 a supply of purified, compressed air to another port. Specific concentrations of CO were achieved by selecting a 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's accuracy was verified using calibration standards.

PLATELET ISOLATION

Platelets were obtained from rats anesthetized with pentobarbital (50 mg/kg, i.p.). Blood was aspirated from the abdominal aorta using a heparinized syringe (final heparin concentration 10 units/mL blood). After prostaglandin I_2

(300 ng/mL) was added to diminish platelet interactions, the blood was centrifuged at 200 × *g* for 10 minutes. The platelet-rich plasma (PRP) supernatant was used in platelet assays for NO and nitrite plus nitrate production. The number of platelets present in individual preparations was determined by direct counting using a hemocytometer and phase-contrast microscope.

RELEASE OF NO BY PLATELETS

Samples of PRP (0.5 mL) were bubbled with CO at concentrations of 0 to 100 ppm for 30 minutes. Samples (300 μL) were then placed in 700 μL of phosphate-buffered saline (PBS) and stirred in a closed well containing the probe for a polarographic NO meter (Iso-NO, World Precision Instruments, Inc., Sarasota, FL). Selectivity of the probe measurement was verified by demonstrating that current was immediately lost when oxyhemoglobin (2 μM) was added to samples. As another control, some samples of PRP were incubated for 30 minutes with 50 μM L-nitroarginine methyl ester (L-NAME) to inhibit nitric oxide synthase before being exposed to CO for 30 minutes, and then NO flux was measured.

Experiments were also designed to measure the displacement of NO by CO. Studies were performed by first incubating PRP with 50 μM L-NAME for 30 minutes. The PRP (300 μL) was next added to 700 μL PBS in a closed well containing both the NO polarographic probe and a small plastic tube. A flow of CO was established through the tube as the PRP sample was stirred, and the peak current measured by the polarographic probe was taken to indicate the concentration of NO displaced by the incoming CO.

VASCULAR ENDOTHELIAL CELL CULTURE PROCEDURES

Bovine pulmonary artery endothelial cells (BPAEC) were grown in Medium 199 with 5% fetal calf serum (Gibco, Grand Island, N.Y.) on Petri dishes 35 mm in diameter. Studies were performed 1 to 3 days after confluence was established and at passage 4 to 7. Control cells, which were exposed to 95% air plus 5% carbon dioxide (CO_2), were included with each experiment, 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, was equilibrated with a gas mixture of air plus 5% CO_2, or the air-CO_2 mixture plus a desired concentration of CO. At the start of each experiment, the growth medium was replaced with the gas-equilibrated buffer, the small gas space above the buffer was flushed with the appropriate gas mixture, and the cells were incubated at 37°C. Throughout the text of this report, therefore, the phrases "control samples", "control cells", and "control gas mixture" all refer to exposure to 95% air plus 5% CO_2. For samples exposed to CO, the concentration of CO that was used to equilibrate the buffer and then was present in the gas space above the buffer is added to the description (e.g., "cells exposed to control gas mixture plus 100 ppm CO").

NITRITE PLUS NITRATE PRODUCTION

Suspensions of PRP were bubbled with the control gas mixture without or with CO for 30 minutes to 2 hours at room temperature, centrifuged at 10,000 × *g* for 10 minutes, and the supernatents were assayed for nitrite plus nitrate. The number of platelets in the suspension were directly measured using an automated counter (Coulter Corp., Hialeah, FL). Plates of BPAEC covered with 0.5 mL Krebs-HEPES-glucose buffer were equilibrated with the control gas mixture without or with CO and incubated for 30 minutes to 2 hours at 37°C. 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 7) for 40 seconds on ice, centrifuged at 19,000 × *g* for 10 minutes, and the protein concentration in supernatents was measured. The concentration of nitrite plus nitrate was measured spectrophotometrically using Greiss reagents (Green et al. 1982). Dissolved nitrate was first converted to nitrite by bacterial nitrate reductase, and the colored product was produced by sequential addition of sulfanilamide and then *N*-(1-naphthyl)ethylenediamine (Cayman Chemicals, Ann Arbor, MI).

L-ARGININE TRANSPORT BY BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

Measurements of L-arginine transport were carried out using techniques essentially the same as those used by Greene and coworkers (1993). Plates of BPAEC were washed with Krebs-HEPES-glucose buffer and intracellular stores of arginine were depleted by incubating cells with buffer at 37°C for 2 hours. 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 the control gas mixture without or with 100 ppm CO. Cells were incubated for up to 2 minutes, 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 minutes. The concentration of protein in this solution was measured, and ^{14}C–L-arginine was measured in a scintillation counter. Active

transport was expressed as ^{14}C–L-arginine found in the cell preparations minus ^{14}C–L-arginine present in samples co-incubated with 10 mM nonradioactive arginine.

NITRIC OXIDE SYNTHASE ACTIVITY

Suspensions of washed platelets were exposed to either the control gas mixture without or with 100 ppm CO for up to 2 hours at 37°C in the presence of 16 μM ^{14}C–L-arginine. Platelets in these studies were removed from plasma in order to limit uptake of nonradioactive arginine. This was accomplished by first centrifuging PRP at 250 × *g* for 10 minutes and suspending the platelets with 5 mL Hank's balanced salts solution (HBSS) plus 20 mM HEPES (pH 7.4) and 5.6 mM glucose. The solution was centrifuged at 250 × *g* for 10 minutes and the platelet-containing pellet was washed once more with HBSS-HEPES-glucose solution. The washed platelets were incubated with ^{14}C–L-arginine for up to 2 hours, samples were centrifuged at 4000 × *g* for 10 minutes, resuspended in 0.5 mL of 30 mM NaCl containing 8 mM dithioerythritol and 10 mM Tris-HCl buffer (pH 7.6), and lysed by subjecting them to three freeze-thaw cycles using liquid nitrogen and a 37°C heating block. The platelet lysate was passed through a 1-mL column of cation exchange resin (Dowex AG 50WX-8) to remove the ^{14}C–L-arginine, and nitric oxide synthase activity was assessed by measuring the ^{14}C–L-citrulline in the column eluate. Control samples were run by incubating some platelet samples with 250 μM nonradioactive arginine.

Plates of BPAEC were incubated with Krebs-HEPES-glucose buffer preequilibrated with the control gas mixture without or with 100 ppm CO for 2 hours. Buffer was removed from the plates before adding 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 following protease inhibitors: 50 μM phenylmethyl sulfonyl 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-second cycles (Heat Systems-Ultrasonics sonicator Model W220-F at a setting of 7). Samples were centrifuged at 12,000 × *g* for 10 minutes and nitric oxide synthase activity in the supernatant was assayed using methods previously described for platelet lysates (Thom et al. 1994). 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 ^{14}C–L-arginine by passing the lysate through Dowex columns, and enzyme activity was expressed as the difference in counts between preparations incubated without or with 100 μM L-NAME.

OXIDATION OF *p*-HYDROXYPHENYLACETIC ACID

Release of oxidants into the medium by BPAEC was assessed as horseradish peroxidase (HRP)-mediated conversion of *p*-hydroxyphenylacetic acid (PHPA) to its fluorescent dimer using methods similar to those described by Panus and coworkers (1993). In our experiments, 1.6 mM PHPA and 95 μg/mL HRP were added to the buffer overlying BPAEC, and fluorescence (excitation wavelength, 323 nm; emission wavelength, 400 nm) was measured in aliquots of the buffer taken at intervals over 2 hours. The production of hydrogen peroxide (H_2O_2) was measured by first incubating cells in buffer without reagents, and then incubating isolated samples of the buffer at room temperature for 15 minutes 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, such as H_2O_2. A standard curve was also prepared using known concentrations of H_2O_2.

ASSESSMENT OF THE EFFECT OF ILLUMINATION ON CARBON MONOXIDE–MEDIATED OXIDANT PRODUCTION

The standard arrangement for exposing BPAEC to CO was used, as outlined above. However, rather than placing cells in a 37°C incubator, they were placed on a heating plate under a light source. The temperature at the plate surface was monitored with a thermistor probe and the heating was adjusted so that cells were incubated at 37°C. Plates were exposed to light from a small quartz lamp mounted 50 cm above the plates. This lamp was similar to that used in prior studies with platelets (Thom et al. 1994).

NITROTYROSINE IN BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

The nitrotyrosine concentration in cell samples was assayed using methods described by Ischiropoulos and coworkers (1996). Bovine pulmonary artery endothelial cells were exposed to a desired concentration of CO for 2 hours using standard methods. The Krebs-HEPES-glucose buffer was removed, the cells were scraped off the plates after addition of 1 mL PBS, sonicated in an ice bath for two 30-second cycles (Heat Systems-Ultrasonics sonicator Model W220-F at a setting of 7), and centrifuged at 12,000 × *g* for 10 minutes. 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 added onto 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 hours followed by a 3-hour incubation in a solution containing a donkey anti-rabbit ^{125}I-labeled IgG (0.1 to 0.2 mCi/mL). The blot was extensively washed in Tween-containing buffer and dried. The radioactivity of each sample was measured directly by Beta scanning with 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 version 4.1, and then plotted on a semilogarithmic plot.

DETECTION OF REDUCED SULFHYDRYLS

Bovine pulmonary artery endothelial cells were incubated for 2 hours with the standard Krebs-HEPES-glucose buffer that had been preequilibrated with the control gas mixture without or with 100 ppm CO. The buffer was removed, cells were scraped off the Petri plates and lysed by sonication as described above. Cell debris was centrifuged at 12,000 × *g* for 10 minutes and aliquots of supernatant were incubated in 100 mM potassium phosphate (pH 8.1) with 70 μM 5,5′-dithio-bis(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.

DIHYDRORHODAMINE 123 OXIDATION BY BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

Plates of BPAEC were incubated with the standard Krebs-HEPES-glucose buffer plus 5 μM dihydrorhodamine 123 (DHR) for 1 hour 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 the control gas mixture without or with 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, sonicated twice for 30 seconds, and the cell debris was centrifuged at 12,000 × *g* for 10 minutes. Rhodamine concentration in the solution was measured by fluorescence (excitation wavelength, 500 nm; emission wavelength, 536 nm) and by absorbance spectroscopy (e_{500} = 7.8×10^4 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 HRP and 80 μM H_2O_2) to convert all DHR to rhodamine.

CHROMIUM RELEASE BY BOVINE PULMONARY ARTERY ENDOTHELIAL CELLS

Plates of BPAEC were exposed for 18 hours to 40 mM sodium ^{51}Cr-chromate (Amersham, Arlington Heights, IL; specific activity 300 mCi/mg, thus approximately 1 μCi/mL growth medium) to load the cells with ^{51}Cr. On the morning of the procedure, the cells were washed three times with 2 mL Krebs-HEPES-glucose buffer and then incubated with buffer equilibrated with the control gas mixture without or with a desired concentration of CO. At the completion of incubation, the buffer (identified as solution A) was removed, centrifuged at 12,000 × *g* for 10 minutes and radioactivity was counted in an automated gamma counter (Wallac, Inc., Gaithersburg, MD, model 1470). The cells adhering to the plates were removed by adding 1 mL of 0.1% (v/v) Triton X-100. The cells were scraped with a rubber policeman, collected in a tube, and the plates were washed with an additional 1 mL of 0.1% Triton X-100. 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. Other plates of cells were incubated with 50 μM L-NAME for 30 minutes before and throughout exposure to CO. After incubation of some cell preparations, the buffer was removed and replaced with 1 mL of standard growth medium equilibrated with the control gas mixture, and the cells were placed in a 37°C incubator. After 6 hours at 37°C, the medium was removed and radioactivity measured in the overlying medium as well as in cell lysates.

Several experiments were carried out to evaluate whether exposure to CO enhanced the oxidative stress caused by other agents. Oxidants were added to Krebs-HEPES-glucose buffer preequilibrated with the control gas mixture without or with 100 ppm CO at the start of a 4-hour incubation. Some experiments were performed by incubating cells with 0.2 mM pterine and 55 mU xanthine oxidase to generate superoxide anion and H_2O_2. Others were performed by directly adding H_2O_2 to BPAEC. To assess the effect of excess NO in the system, cells were exposed to spermine NONOate, which spontaneously dissociated to a free amine and NO at physiological pH. A concentration of spermine NONOate was chosen to sustain an NO flux that might occur in vivo from platelets due to CO. We found that the rate of NO production by platelets, based on nitrite plus nitrate formation, was 4.5 nmol NO/minute/1 × 10^8 platelets in the presence of 100 ppm CO (Figure 1). It is likely that the only NO produced by platelets in vivo that would be available to interact with endothelium would be from platelets in close proximity to the vascular wall. A relevant rate for in

vitro studies was considered to be 4 to 5 nmol/minute. The half-life of spermine NONOate at pH 7.4 and room temperature is 144 minutes, according to the manufacturer (Cayman Chemical Co., Ann Arbor, MI). Hence, we chose to do studies with 1 μM spermine NONOate in order to examine the impact of a flux of 5.7 nmol NO/minute.

ETHIDIUM HOMODIMER-1 UPTAKE

Cells were exposed to standard growth medium that was preequilibrated with the control gas mixture without or with a desired concentration of CO for a period of 2 hours. The medium was removed and replaced with fresh medium equilibrated with the control gas mixture containing 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.

STATISTICAL METHODS

Statistical significance was determined by ANOVA followed by Scheffe's test. The level of significance was taken as $p < 0.05$ and results are expressed as mean ± SE. Analyses were primarily focussed on comparisons between the results obtained with various concentrations of CO and the control samples. When studies included use of nitric oxide synthase inhibitors, post hoc analysis included comparisons of results between samples with and without inhibitor. Where appropriate, comparisons of the results obtained from several different CO concentrations were performed using the Mann-Whitney Rank Sum Test.

The primary aim of this one-year investigation was an initial examination of possible mechanisms for CO-mediated oxidant production. Hence, when a mechanistic conclusion could be reached from an experiment undertaken, that line of work was discontinued and the next experiment begun. Tables 1 through 4 list the sequence of studies undertaken and sample sizes for each investigation.

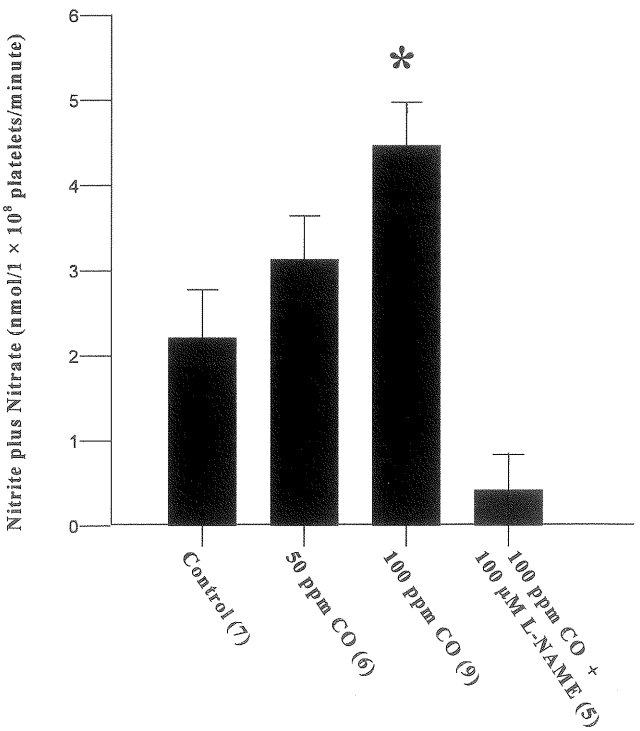


Figure 1. Nitrite plus nitrate from platelets exposed to CO. Samples of PRP were incubated for 2 hours with the control gas mixture without or with selected concentrations of CO, and then nitrite plus nitrate was measured. The number of samples is given in parentheses. An asterisk (*) indicates significant ($p < 0.05$) results when compared with control samples.

Table 1. Overview of Studies Performed with Platelets Under Specific Aim 1

Type of Study	CO Concentration (ppm)	Sample Size
Platelet release of NO following in vitro exposure to CO	0	6
	20	6
	50	6
	80	6
	100	6
	100 + L-NAME	4
Nitric oxide synthase activity in platelets	0	11
	100	11
	100 + 250 μM L-arginine	4
NO displacement from platelets after inhibition of endogenous nitric oxide synthase	0	5
	10	6
	20	6
	50	8
	100	8
Nitrite plus nitrate production by platelets exposed to CO in vitro	0	7
	50	6
	100	9
	100 + L-NAME	5
NO liberated by platelets taken from rats exposed to CO	0	6
	20	3
	100	6
	1000	4

Table 2. Overview of Studies Performed with Vascular Endothelial Cells Under Specific Aim 2

Type of Study	CO Concentration (ppm)	Sample Size
Nitrite plus nitrate produced by BPAEC	0	8
	10	6
	20	8
	50	4
	100	11
	100 + L-NAME	6
Nitrite plus nitrate production by cells exposed to light source	0	2
	100	8
L-Arginine transport by BPAEC	0	4
	100	4
Nitric oxide synthase activity in cell lysates	0	4
	100	4
Oxidation of *p*-hydroxyphenyl-acetic acid		
• Reagents in buffer during incubation with cells	0	20
	40	6
	70	4
	100	13
	0 + L-NAME	5
	100 + L-NAME	5
• Reagents added to buffer after removal from cells	0	6
	100	9
Nitrotyrosine in cells after CO exposure	0	4
	10	3
	20	6
	50	4
	100	5
	100 + L-NAME	3
Reduced sulfhydryl content of cells	0	9
	100	11
Dihydrorhodamine 123 oxidation		
• Rhodamine 123 formation	0	10
	20	6
Nitrite plus nitrate produced by BPAEC	0	8
	100	6
	100 + L-NAME	5
Cytotoxicity of CO assessed as ^{51}Cr leakage		
• Incubation for 4 hours	0	8
	50	3
	80	3
	100	9
	100 + L-NAME	7
• Incubation for 2 hours with CO, analysis immediately	0	4
	100	5
• Incubation for 2 hours with CO, analysis after 6-hour incubation in growth medium	0	5
	100	8
	100 + L-NAME	5
Cytotoxicity due to CO and other sources of oxidants		
• Pterine and xanthine oxidase, 4-hour incubation	0	3
	100	3
• 50 μM H_2O_2, 4-hour incubation	0	3
• 10 μM H_2O_2, 4-hour incubation	0	3
	100	3
• 1 μM spermine NONOate	100	3
Cytotoxicity assessed as ethidium homodimer-1 uptake	0	3
	100	3

(Continued next column)

Table 3. Overview of Studies Performed Under Specific Aim 2, Dihydrorhodamine 123 Oxidation: Intracellular Rhodamine 123 Concentration as a Function of Duration of Incubation[a]

Duration (min)	0 ppm CO	0 ppm CO + L-NAME	100 ppm CO	100 ppm CO + L-NAME
5	6	0	6	3
10	6	0	7	1
20	6	0	8	3
30	6	0	6	0
60	18	6	8	4

[a] Values indicate the number of samples used for each experiment.

Table 4. Overview of Studies Performed Under Specific Aim 2, Dihydrorhodamine 123 Oxidation: Total Dihydrorhodamine in Buffer and Cell Lysates (Entire Experimental System) as a Function of Duration of Incubation[a]

Duration (min)	0 ppm CO	100 ppm CO
5	6	5
10	6	7
20	4	6
60	7	7

[a] Values indicate the number of samples used for each experiment.

RESULTS

SPECIFIC AIM 1: PLATELET STUDIES

Platelets Release NO in Response to Carbon Monoxide Exposure

When PRP was exposed to CO at concentrations up to 100 ppm, there was a dose-dependent increase in release of NO from the platelets (Table 5). When samples were incubated with the nitric oxide synthase inhibitor, L-NAME, at 50 μM and then exposed to 100 ppm CO, the NO flux measured was 0 ± 0 ($n = 4$). The absence of current was taken as an indication that the NO signal measured was entirely due to endogenous nitric oxide synthase activity.

Exposure to Carbon Monoxide Does Not Enhance the Activity of Nitric Oxide Synthase

The activity of nitric oxide synthase in aliquots of platelets incubated with ^{14}C–L-arginine in the presence of the control gas mixture was 6.3 ± 0.8 (SE, $n = 11$) pmol ^{14}C–L-citrulline/min/1×10^8 platelets. When platelets were incubated with the control gas mixture plus 100 ppm CO, enzyme activity was 7.0 ± 0.7 ($n = 11$) (no significant difference). Production of ^{14}C–L-citrulline was diminished more than 96% when either control or CO-exposed platelet preparations were incubated in the presence of 250 μM nonradioactive arginine.

Carbon Monoxide Can Displace NO from Endogenous Binding Sites in Platelets

The data described above demonstrate that exposure to CO increases the release of NO from platelets, but that nitric oxide synthase activity does not increase. In previous studies, we have shown that CO enhances release of NO from platelets by binding to intraplatelet heme-proteins, thus blocking intraplatelet scavenging of NO (Thom et al. 1994). We set up an alternative experiment to test whether CO and NO competed for the same intraplatelet binding site: first we inhibited endogenous NO production with L-NAME, and then evaluated whether platelets released NO when exposed to CO (see the Methods section). Nitric oxide synthase has been shown to be active in isolated rat platelets (Thom et al. 1994). Therefore, some fraction of intraplatelet NO binding sites (e.g., heme-proteins) may be expected to have bound NO. We hypothesized that after

Table 5. NO Released from Platelets Exposed to Carbon Monoxide in Vitro[a]

CO Concentration (ppm)	NO (nmol/1×10^8 platelets)
0	0.3 ± 0.3
20	15.7 ± 8.1
50	21.6 ± 5.3
80	27.0 ± 3.3
100	53.9 ± 9.9[b]

[a] NO was measured by electrode, as described in Methods, and is expressed as mean ± SE; $n = 6$ for all measurements. Significance was considered at $p < 0.5$. Values obtained with 20, 50, and 80 ppm CO were not significantly different from each other.

[b] Significantly greater than 20, 50, and 80 ppm CO values.

Table 6. Displacement of NO from Platelets Exposed to Carbon Monoxide[a]

CO Concentration (ppm)	*n*	NO (nmol/1 × 10^8 platelets)
0	5	0 ± 0
10	6	23.4 ± 8.5
20	6	23.7 ± 6.7
50	8	40.1 ± 10.8
100	8	49.5 ± 9.9

[a] Platelets were preincubated with L-NAME to inhibit ongoing synthesis of NO, and then exposed to CO at different concentrations. Values are expressed as mean ± SE. *n* values indicate the number of trials with platelet preparations from different rats. Results for 10 through 100 ppm CO were all significantly greater than control (0 ppm CO), but none of the CO-related values were significantly different from each other.

on-going NO synthesis was inhibited with L-NAME, we might be able to detect displacement of this bound NO with an NO-selective electrode. After nitric oxide synthase was inhibited, NO release from platelets could be detected by polarographic probe when preparations were exposed to CO (Table 6). Displacement was detected when platelets were exposed to even 10 ppm CO, and there was only a marginal increase in release of NO with higher concentrations of CO.

Nitrite plus Nitrate Production by Platelets Exposed to Carbon Monoxide

Nitrite plus nitrate, stable end-products from NO, were measured after suspensions of PRP were exposed to CO. Again, inhibition of production when platelets were exposed to L-NAME was used as an indication that nitric oxide synthase activity was required (Figure 1). The advantage of these measurements versus polarographic measurements was that a true rate of NO synthesis could be determined. The rate of nitrite plus nitrate found in control samples was greater than the activity of nitric oxide synthase measured as ^{14}C–L-citrulline production. This suggests that endogenous stores of arginine in platelets cause less ^{14}C–L-arginine to be converted to L-citrulline in the assays with radioisotopes. We concluded that L-citrulline production was a reflection of nitric oxide synthase activity, but not a true measure of total enzyme activity.

NO Release by Platelets Obtained from Rats Exposed to Carbon Monoxide

We have previously demonstrated that platelets from rats exposed to 1000 or 3000 ppm CO liberate NO (Thom et al. 1994). In the present studies, rats were exposed for 1 hour to CO at concentrations from 0 to 1000 ppm, PRP was obtained, and NO release was measured by polarographic probe. Results in Table 7 indicate that in vivo exposure to CO will cause platelets to liberate NO.

SPECIFIC AIM 2: VASCULAR ENDOTHELIAL CELL STUDIES

Nitrite plus Nitrate as an Index of NO Production by Endothelial Cells

Exposure to CO caused a dose-dependent increase in nitrite plus nitrate production by BPAEC (Figure 2). To confirm that the increased concentration of nitrite plus nitrate generated during CO exposure was due to nitric oxide synthase activity, cells were preincubated with 50 μM L-NAME for 30 minutes and then exposed to the control gas mixture plus 100 ppm CO in buffer containing 50 μM L-NAME. As shown in Figure 2, nitrite plus nitrate production was significantly less than in samples without L-NAME.

Assessment of the Effect of Illumination on Carbon Monoxide–Mediated Nitrite plus Nitrate Production

In previous studies (Thom et al. 1994), exposure to light prevented CO-mediated NO release from platelets. We believe this phenomenon is due to photo-dissociation of CO

Table 7. NO released by Platelets Taken from Rats Exposed to Carbon Monoxide[a]

CO Concentration (ppm)	*n*	COHb Level (%)	NO[b] (nmol/1 × 10^8 platelets)
0	6	0.7 ± 0.2	0.3 ± 0.3
20	3	3.2 ± 1.1	18.9 ± 5.5[c]
100	6	7.8 ± 1.2[c]	56.7 ± 8.6[c]
1000	4	51.0 ± 2.0[c,d]	74.0 ± 2.5[c]

[a] NO release was measured in PRP obtained from blood after rats were exposed to CO at the indicated concentration for 1 hour. COHb (shown as mean ± SE) indicates the carboxyhemoglobin levels in rats.

[b] The values for NO release (shown as mean ± SE) between 20, 100, and 1000 ppm CO were not significantly different from each other.

[c] $p < 0.05$ compared with control values.

[d] Significantly greater than 20 and 100 ppm CO.

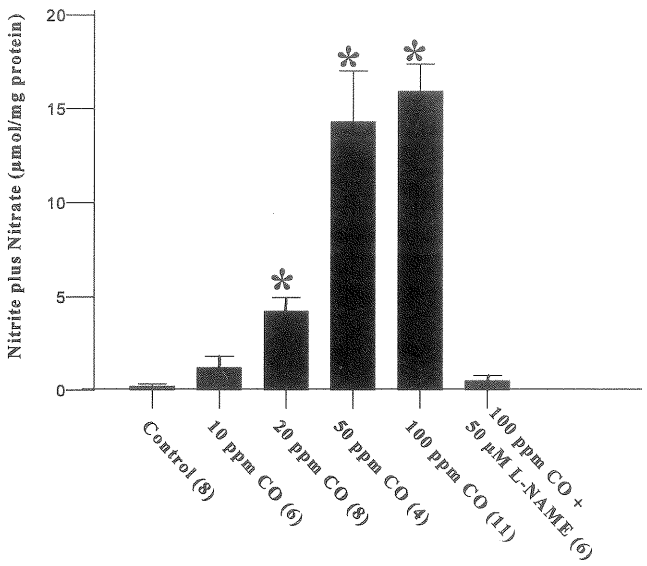


Figure 2. Nitrite plus nitrate production by vascular endothelial cells. BPAEC were exposed to buffer equilibrated with the control gas mixture without or with selected concentrations of CO. Bars indicate the concentration of nitrite plus nitrate in the buffer after incubation for 1 hour. The number of samples is given in parentheses. An asterisk (*) indicates significant ($p < 0.05$) results when compared with control samples. Other significant differences were 50 ppm CO compared with 10 ppm and 20 ppm, and 100 ppm CO compared with 10 ppm and 20 ppm.

from heme-proteins that would normally function to bind intraplatelet NO. We hypothesized that exposure to light would act in a similar way with endothelial cells, and prevent the enhanced production of nitrite plus nitrate when endothelial cells were exposed to CO. Plates of BPAEC were exposed to light from a small quartz lamp suspended 50 cm above the culture plates (see Methods). Exposure to light for 2 hours injured the cells, and they became rounded and some lifted off the plastic plates. Therefore, we reduced the exposure time. Plates of cells were exposed to three 5-minute periods of light, one at the start of incubation, one at 45 minutes, and again at 90 minutes. With this protocol, the structure of cells was normal; however, nitrite plus nitrate concentration in samples exposed to the control gas mixture increased by nearly two thirds, to 1.1 ± 0.2 (n = 3) µmol/mg protein per hour ($p < 0.05$ versus standard control). In eight trials with cells incubated with the control gas mixture plus 100 ppm CO, the nitrite plus nitrate concentration was not significantly different from samples that had not been exposed to light. The concentration was 14.7 ± 0.7 µmol/mg protein per hour. We concluded that exposure to light at a level that only mildly injured BPAEC, based on an enhanced nitrite plus nitrate production in control cells, was an insufficient exposure to affect the CO-mediated process. Therefore, due to the sensitivity of BPAEC to light, the impact of light on CO-mediated nitrite plus nitrate production could not be adequately investigated.

Enhanced NO Production Does Not Involve Alteration in L-Arginine Transport by Vascular Endothelial Cells

With the inability to directly evaluate whether CO led to NO release because of impairment of intracellular NO scavenging, we examined the L-arginine uptake and nitric oxide synthase activity. Nonspecific binding was determined to be 1.20 ± 0.09 (n = 5) nmol/mg protein. Active transport in 2 minutes by control cells was 0.35 ± 0.04 (n = 4) nmol/mg protein, and in cells exposed to the control gas mixture plus 100 ppm CO, it was 0.34 ± 0.04 (n = 4) pmol/µg protein (no significant difference). We concluded that CO did not affect the ability of cells to transport L-arginine across the cell membrane.

Carbon Monoxide Exposure Does Not Alter Nitric Oxide Synthase Activity

Nitric oxide synthase activity in cytosolic preparations from control cells was 6.4 ± 0.8 (n = 4) nmol L-citrulline/mg protein when samples were incubated for 1 hour. In the preparations from cells exposed for 2 hours to the control gas mixture plus 100 ppm CO, activity was 7.0 ± 0.9 (n = 4) nmol L-citrulline/mg protein/hour (no significant difference).

Exposure to Carbon Monoxide Increases Production of Agents Capable of Oxidizing *p*-Hydroxyphenylacetic Acid

Endothelial cells release oxidants capable of dimerizing PHPA (Panus et al. 1993). Oxidation of PHPA in the buffer above BPAEC was increased in a dose-dependent fashion when cells were incubated with CO (Table 8). Enhanced fluorescence was inhibited when cells were incubated with 50 µM L-NAME for one hour before beginning the exposure. Control cells preincubated with L-NAME exhibited production of oxidants at a rate insignificantly different from cells

Table 8. Oxidation of PHPA by Vascular Endothelial Cells Exposed to Carbon Monoxide[a]

CO Concentration (ppm)	n	Relative Fluorescence (units/min/mg protein)
0	20	1.91 ± 0.18
40	6	3.04 ± 0.26
70	4	5.01 ± 0.9[b]
100	13	6.37 ± 0.74[b]

[a] Values (shown as mean ± SE) reflect PHPA dimer formed in buffer overlying BPAEC exposed to the control gas mixture plus selected concentrations of CO. n values indicate the number of trials with different plates of cells.

[b] $p < 0.05$ compared with control values.

in the absence of L-NAME, 2.35 ± 0.4 (n = 5) arbitrary fluorescence units/min/mg protein. Cells exposed to 100 ppm CO after incubation with L-NAME exhibited a rate of 2.15 ± 0.5 (n = 5) arbitrary fluorescence units/min/mg protein (no significant difference from control). We concluded from these findings that activity of nitric oxide synthase is necessary for enhanced 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 or a more stable agent such as H_2O_2. As an alternative technique to assess whether a more stable oxidant was produced, the cells were exposed to the control gas mixture plus CO; the reagents PHPA and HRP were not initially included (see Methods). As the half-life of peroxynitrite in an aqueous solution is approximately 0.1 seconds (Goldstein and Czapski 1995), peroxynitrite should rapidly react with substances in the buffer and be undetectable by the PHPA and HRP assay. Aliquots of the buffer were removed from the solution overlying the cells, incubated for 10 to 15 minutes at room temperature, and then PHPA and HRP were added. Control cells produced 0.23 ± 0.02 (n = 6) relative fluorescence units/min/mg protein. Cells exposed to the control gas mixture plus 100 ppm CO produced 0.25 ± 0.03 (n = 9) relative fluorescence units/min/mg protein (no significant difference). We concluded that exposure to CO does not increase endothelial cell release of stable oxidant species such as H_2O_2.

Nitrotyrosine in Cells

Lysates from cells exposed to CO were analyzed for their content of nitrotyrosine. There was a significant increase in lysates prepared from cells exposed to 50 or 100 ppm CO (Table 9). In addition, the elevation in nitrotyrosine was inhibited when cells were incubated in the presence of 100 μM L-NAME (Table 9). We concluded 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 cell protein, and in lysates from cells exposed to the control gas mixture plus 100 ppm CO the concentration was 303 ± 44 (n = 11) (no significant difference). We concluded that although, based on an increase in nitrotyrosine content, CO appeared to cause oxidative stress, there was no gross cellular oxidative stress that would be expected to have caused a depletion of cellular reduced sulfhydryls.

Dihydrorhodamine 123 Oxidation

Conversion of DHR to fluorescent rhodamine is a sensitive method for detecting peroxynitrite, and H_2O_2 will not confound the results because it does not react with DHR (Kooy et al. 1994). The rate of DHR oxidation in the buffer overlying endothelial cells increased by exposure to the control gas mixture plus 100 ppm CO (Table 10). 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 the production of oxidants within the cells, because DHR slowly diffuses out of intact cells (Royall and Ischiropoulos 1993). We found, however, that the concentration of rhodamine was insignificantly different in control samples and in lysates from cells exposed to the control gas mixture plus 100 ppm CO (Figure 3). The apparent lack of intracellular DHR oxidation would have significant implications for the mechanism of CO-induced oxidative stress, except that exposure to CO was found to cause a progressive loss of detectable DHR and rhodamine 123 from the cell system. This loss of

Table 9. Nitrotyrosine in Endothelial Cell Lysates[a]

CO Concentration (ppm)	n	Nitrotyrosine (μmol/mg cell protein)
0	4	2.4 ± 0.4
10	3	2.5 ± 1.3
20	6	3.5 ± 0.5
50	4	4.7 ± 0.4[b]
100	5	4.2 ± 0.4[b]
100 + L-NAME	3	3.5 ± 0.5

[a] Values (shown as mean ± SE) indicate the concentration of nitrotyrosine measured in lysed cell supernatents. n indicates the number of trials.

[b] $p < 0.05$ compared with control values.

Table 10. Increase in Buffer Rhodamine 123[a]

CO Concentration (ppm)	n	Rhodamine 123 (pM)
0	10	44 ± 8
20	6	55 ± 2.2
100	6	99 ± 15[b]
100 + L-NAME	5	40 ± 10

[a] Values (shown as mean ± SE) reflect the concentration of rhodamine 123 measured in buffer overlying BPAEC exposed for 5 minutes to the control gas mixture without or with the indicated concentration of CO. Where indicated, cells were incubated with 100 μM L-NAME (see Methods section). n indicates the number of samples.

[b] $p < 0.05$ by ANOVA compared with control values.

DHR and rhodamine 123 was not observed in control cells (Figure 4). Samples were surveyed both by fluorescence spectroscopy and by standard absorbance spectroscopy, and the same results were found. When cell-free solutions of 5 μM DHR were incubated with 100 ppm CO, no reduction in detectable DHR occurred. The effect of CO was not changed when cells were incubated in the presence of 100 μM L-NAME, hence we concluded that the effect was independent of nitric oxide synthase activity.

The loss of DHR and rhodamine 123 occurred in a dose-dependent manner over a range of CO concentrations. This is shown in Table 11 as a percentage of the concentration found in matched control-cell preparations from experiments done on the same day. When cell-free suspensions of 5 μM DHR were incubated with the peroxynitrite generator 3-morpholinosyndnonimine, conversion of DHR to rhodamine 123 was observed and all of the decrease in DHR could be accounted for by the increase in rhodamine. When solutions of DHR were incubated with diethylamine NONOate to generate NO, or with xanthine oxidase plus pterine to generate superoxide anion and H_2O_2, there was no loss of detectable DHR or conversion of DHR to rhodamine 123.

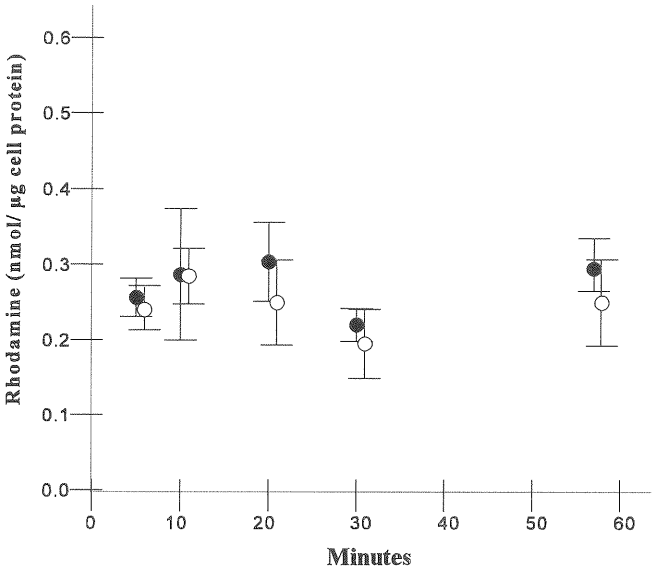


Figure 3. Concentration of rhodamine 123 in cell lysates. Data points are means ± SE for rhodamine 123 concentration in lysates from control (●) cells and from cells exposed to 100 ppm CO (○). $n = 6$ to 18 for each data point.

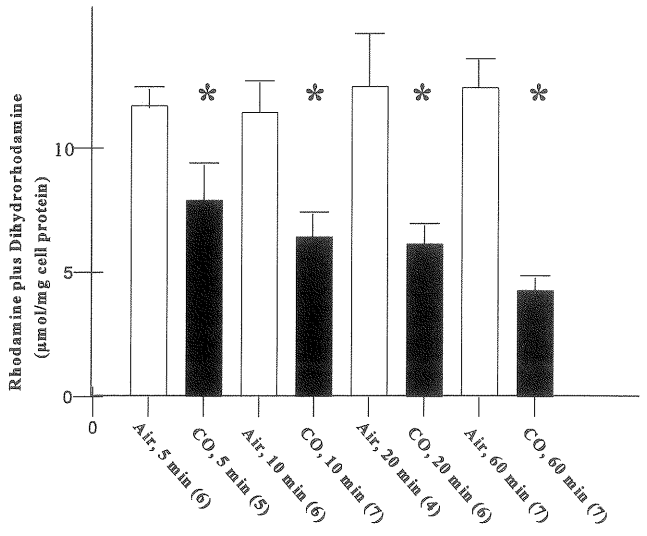


Figure 4. Concentration of rhodamine 123 and DHR in cell lysates. Values reflect the concentration in lysates from cells exposed to the control gas mixture without or with 100 ppm CO. The number of samples is given in parentheses. An asterisk (*) indicates significant ($p < 0.05$) results when compared with control samples.

Cytotoxicity of Carbon Monoxide Exposure

The cytotoxicity of CO was evaluated by comparing the release of ^{51}Cr from control cells with that released from cells exposed to the control gas mixture plus a range of concentrations of CO. Exposure to CO for 4 hours caused a dose-dependent increase in ^{51}Cr release (Figure 5).

When cells were incubated with 100 ppm CO for just 2 hours, there was no detectable release of ^{51}Cr immediately after the exposure (Figure 6). However, if the CO-containing buffer was removed and cells were incubated with growth medium equilibrated with just the control gas mixture after the 2-hour CO exposure, ^{51}Cr release was evident 6 hours later (Figure 6). When cells were incubated with L-NAME during the CO exposure, they were not protected from the delayed ^{51}Cr release, although incubation with the nitric oxide synthase inhibitor did have a protective effect if cells were continuously exposed to CO for 4 hours (Figure 5).

A series of examinations also was undertaken to assess whether CO would accentuate BPAEC sensitivity to oxida-

Table 11. Total Dihydrorhodamine 123 in Endothelial Cell Lysates[a]

CO Concentration (ppm)	n	% of Control
20	6	98 ± 13
50	6	60 ± 8[b]
70	5	54 ± 10[b]
100	8	48 ± 7[b]

[a] Values (shown as mean ± SE) reflect the relative concentration of DHR in cell preparations in relation to the DHR concentration in control cell preparations from the same day.

tive stress from other agents. In some studies, cells were incubated for 4 hours with pterine and xanthine oxidase to generate both superoxide anion and H_2O_2. Chromium leakage significantly increased to 63 ± 10% (SE, n = 3) versus the leakage from cells exposed only to the control gas mixture (5.1 ± 0.3%, n = 10, Figure 5). When cells were incubated with pterine, xanthine oxidase, the control gas mixture, and 100 ppm CO, the leakage of chromium was somewhat higher, 81 ± 3% (n = 3). This value is significantly greater than with control cells, but not significantly greater than the leakage seen with cells exposed to the control gas mixture, pterine, and xanthine oxidase. Other cells were incubated with 50 μM H_2O_2 in addition to the control gas mixture, and chromium leakage increased to 14.2 ± 4.6 %(SE, n = 3) (significantly greater than control, ANOVA). When the concentration was decreased to 10 μM H_2O_2, chromium leakage in samples exposed to the control gas mixture was 5.8 ± 1.0% (n = 3, no difference from control). If cells were incubated with 10 μM H_2O_2, the control gas mixture, as well as 100 ppm CO, chromium leakage was 9.9 ± 2.2% (n = 3), which was not significantly different than when cells were exposed to the control gas mixture and CO, but without the H_2O_2 (Figure 5). Similarly, when cells were exposed to the control gas mixture plus 100 ppm CO, in combination with 1 μM spermine-NONOate to generate free NO (see Methods), chromium leakage was 9.6 ± 0.6% (SE, n = 3). We concluded that, for the time intervals used in our studies, CO did not accentuate oxidative stress caused by superoxide anion, H_2O_2, or free NO.

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. Fluorescent cells were not evident in cultures during the first 12 hours after exposure to the control gas mixture plus 20 or 100 ppm CO for 2 hours. However, by 20 hours, uptake of the fluorescent dye was clearly seen in samples exposed to 20 ppm CO, and uptake was very large after exposure to 100 ppm CO (Figures 7 and 8). These studies were repeated three separate times and similar results were seen with each experiment.

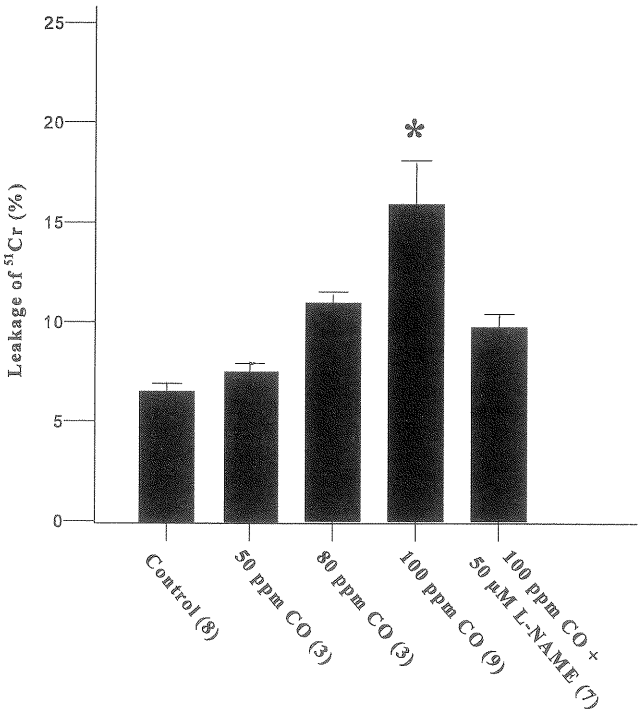


Figure 5. ^{51}Cr released from BPAEC incubated with the control gas mixture without and with selected concentrations of CO for 4 hours, and then incubated for 4 hours in buffer. The number of samples is given in parentheses. An asterisk (*) indicates significant ($p < 0.05$) results when compared with control samples.

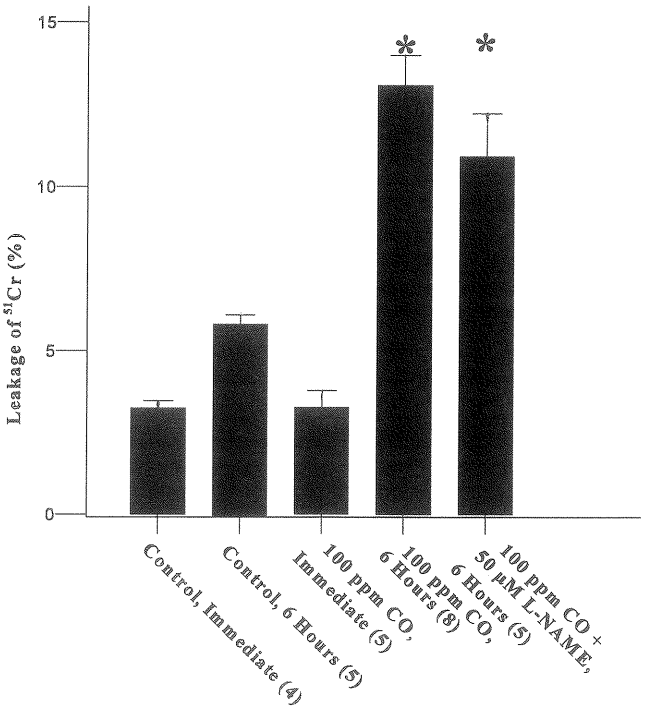
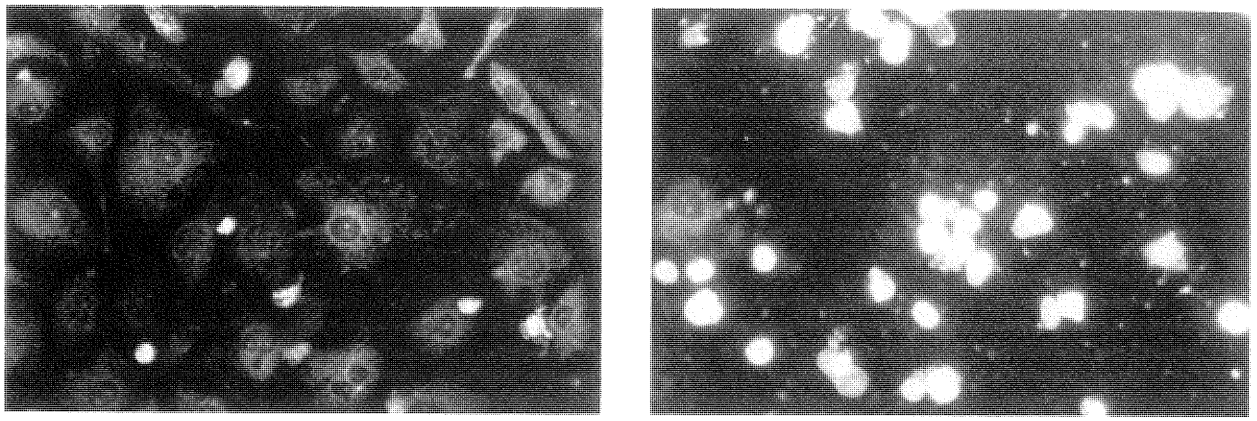


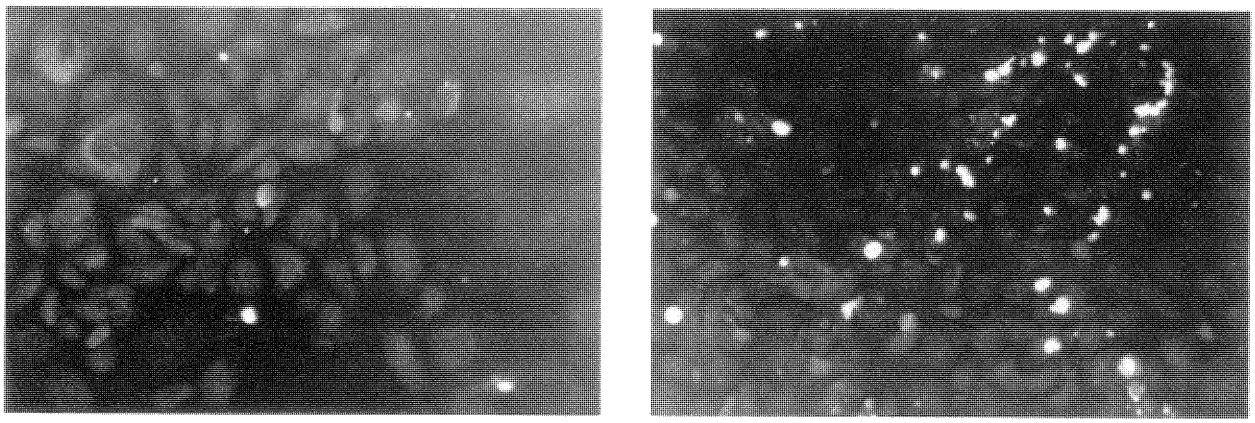
Figure 6. ^{51}Cr released from BPAEC incubated with the control gas mixture without and with 100 ppm CO for 2 hours, and then incubated with growth medium for 6 hours. Samples analyzed immediately following the 2-hour incubation are labeled "immediate". For other samples, buffer was removed, replaced with growth medium equilibrated with the control gas mixture, and cells were incubated at 37°C for 6 hours before analysis. Where indicated, cells were preincubated with 50 μM L-NAME for 30 minutes before and throughout the 2-hour exposure to CO, and then incubated with growth medium equilibrated with the control gas mixture for 6 hours. The number of samples is given in parentheses. An asterisk (*) indicates significant ($p < 0.05$) results when compared with control samples.



Control Exposure

100 ppm CO Exposure

Figure 7. Ethidium homodimer-1 uptake by endothelial cells exposed to medium equilibrated with the control gas mixture without or with 100 ppm CO for 2 hours, and then incubated with medium equilibrated with the control gas mixture for 18 hours.



Control Exposure

20 ppm CO Exposure

Figure 8. Ethidium homodimer-1 uptake by endothelial cells exposed to medium equilibrated with the control gas mixture without or with 20 ppm CO for 2 hours, and then incubated with medium equilibrated with the control gas mixture for 18 hours.

DISCUSSION

The results of this study provide information on a mechanism for oxidant production due to exposure to CO. The studies with isolated platelets demonstrated that concentrations of CO as low as 20 ppm cause NO to be liberated from platelets. In previous studies we showed that when platelets were exposed to 1000 ppm CO, nitric oxide synthase activity was unchanged in lysate experiments (Thom et al. 1994). Studies with intact platelets were performed to assess whether CO may disturb enzyme regulation. Activity of the constitutive enzyme, which is present in platelets, requires calmodulin, several flavin groups, and in some isotypes, phosphorylation by specific protein kinases (Marletta et al. 1988; Palmer et al. 1987; Bredt and Snyder 1990). We found that enzyme activity in intact platelets was not altered by exposure to CO. However, the drawback with the studies reported here relates to the relatively low enzyme activity measurable as ^{14}C–L-citrulline production in comparison with the rate of nitrite plus nitrate production and the flux of NO liberated. We believe that this discrepancy is related to competition between endogenous L-arginine stores and the added ^{14}C–L-arginine that must be taken up and metabolized by the platelets. It appears that produc-

tion of ^{14}C–L-citrulline is useful to assess relative enzyme activity, but it obviously does not accurately measure total nitric oxide synthase activity.

Based on our previous studies, we hypothesized that CO caused platelets to release NO because CO impairs normal intraplatelet NO binding (Thom et al. 1994). Additional support for this hypothesis was provided by the NO displacement studies carried out as part of this project. Nitric oxide synthase was inhibited by preincubating platelets with L-NAME, and NO was released from these platelets when they were exposed to just 10 ppm CO. However, we failed to find a significantly higher flux of NO when platelets were exposed to CO concentrations above 10 ppm. When platelets were used that had not been incubated with L-NAME, some differences in NO release were observed in association with higher concentrations of CO. Therefore, we suspect that the apparent absence of differences with higher CO concentrations in the displacement studies may be related to the relatively low signal produced when NO could not be continuously generated, and the sensitivity of the NO-selective electrode. Additional studies will be necessary to properly evaluate this question.

Another surprising aspect to the platelet studies was the similarity in NO flux seen whether platelets were exposed to CO in vitro or in vivo (comparison of Tables 5 and 7). The only location where platelets would experience conditions in vivo similar to those found in the in vitro experiments is the alveolar capillary, suggesting that platelets may take up CO from the environment on passage through the lungs, as do the erythrocytes. The interesting physiological question from this result is whether platelets undergo gas exchange at the capillary wall despite the seemingly overwhelming number of erythrocytes that are also present. Again, additional studies will be necessary to address this issue.

Vascular endothelial cells liberate NO and NO-derived oxidants in response to CO. A fraction of the oxidants are expected to interact with cellular tyrosine residues; therefore, detection of nitrotyrosine (Table 9) indicates that CO causes production of peroxynitrite, or another transnitrosating agent such as a nitrosothiol. It was surprising that CO did not decrease the content of reduced sulfhydryls in cells. Oxidative stress mediated by a number of agents, including H_2O_2, have been shown to diminish the concentration of reduced sulfhydryls (Schuppe et al. 1992). It is possible that CO exerts its effect at discrete sites, possibly dependent on the intracellular localization of nitric oxide synthase. Endothelial cell nitric oxide synthase appears to be localized predominantly to the plasma and organelle membrane structures, including mitochondria (Pollack et al. 1993).

L-Arginine uptake by cells and nitric oxide synthase activity in cell lysates were not affected by CO. It would be desirable to investigate the activity of nitric oxide synthase in intact endothelial cells, as was done with the platelet studies. However, this was not part of the HEI contract and time ran out before these studies were done. The results obtained in this contract period suggest that the mechanism for NO release by CO, which was quantified based on the liberation of nitrite plus nitrate, may have been a disturbance of the normal intracellular scavenging system for NO. As we have discussed, some of the data suggests that peroxynitrite may have been generated in response to CO. This could have occurred if the relative intracellular concentration of NO were increased, due to impaired heme-protein scavenging of NO, which would leave the NO available to react with superoxide anion radicals that are normally generated by mitochondria and other organelles. It would be desirable to also investigate the rate of oxygen consumption by cells, and the intracellular rate of production of reduced oxygen species under our experimental conditions. At concentrations considerably higher than those used in our study, CO can disturb mitochondrial function (Chance 1975), which could lead to enhanced generation of superoxide anion and H_2O_2. We did not find CO exposure to increase the concentration of relatively stable oxidants, such as H_2O_2, in the extracellular space. Oxidation of DHR and PHPA in the buffer above cells exposed to CO suggests that peroxynitrite was generated and diffuses into the medium. However, the results with DHR should be considered cautiously because we do not understand the intracellular biochemistry and why CO appears to cause cells to metabolize DHR to undetectable products.

Release of chromium when cells were exposed to CO demonstrates that CO-mediated oxidative stress had a direct adverse effect on cells. Because L-NAME prevented chromium release during the 4-hour exposures, we concluded 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-hour exposure to CO when cells were incubated for an additional 6 hours in growth medium equilibrated with the control gas mixture. This delayed chromium leakage was not inhibited when cells were exposed to L-NAME. However, the results in Table 9 suggest that L-NAME did not provide complete protection against CO-mediated oxidative stress. There was a small elevation of nitrotyrosine in cells exposed to CO and L-NAME. Hence, persistent ^{51}Cr leakage in the 6-hour delayed studies when cells were incubated with L-NAME does not necessarily mean that cell injury was caused by processes unrelated to NO-derived oxidants.

We observed delayed uptake of ethidium homodimer-1 in cells 20 hours after exposure to CO. Whether this is

apoptosis is not clear from the experiments performed to date. It is intriguing to note that the blebbing of the plasma membrane seen in some cells exposed to 100 ppm CO in Figure 7 is a characteristic of apoptosis. Moreover, NO-derived oxidizing species (such as peroxynitrite) and some nitrosothiol compounds have been shown to trigger apoptosis in certain cell types (Lin et al. 1995; Bonfoco et al. 1995; Palluy and Rigaud 1996).

IMPLICATIONS

These results demonstrate production of NO and NO-derived oxidants by platelets and vascular endothelial cells in response to exposure to CO. The linkage between oxidative stress and illness has been well established, and the association between oxidative damage and atherosclerosis also has been widely accepted (Cross et al. 1987; Ross 1993). Therefore, studies carried out in this contract offer a possible biochemical mechanism for CO-mediated pathology.

A major issue pertaining to the physiological relevance of our data relates to the concentrations of CO that various cells are likely to experience in vivo when humans are exposed to environmentally relevant concentrations of CO. The data indicate that the ambient or environmental CO level is the relevant concentration to consider regarding disturbances in the NO flux from platelets. We measured the same flux of NO whether platelets were exposed to CO in vitro or in vivo. We interpret these results as suggesting that platelets must respond to the alveolar CO level as they pass through the pulmonary microcirculation. The pathophysiological impact of excess NO from platelets has not been established. However, with regard to exposures to high CO concentrations, we have shown that platelet-derived NO contributes to vascular oxidative stress (Ischiropoulos et al. 1996).

Although it is not entirely clear how best to estimate the concentration of CO that the vascular endothelium may experience in vivo due to a particular environmental exposure, it is probably close to the interstitial CO concentration. Absorption and elimination of CO in humans has been studied for decades. Uptake of CO depends on many variables, including a number of endogenous physiological parameters, the ambient CO concentration, and the duration of exposure (Coburn et al. 1965). If one assumes that the highest relevant levels of environmental CO are in the range of 50 to 100 ppm, then corresponding carboxyhemoglobin levels may be expected to be between 3% and 8% when exposures are from 1 to 4 hours (Peterson and Stewart 1970). Coburn (1970) demonstrated that the Haldane equation could be used to predict the interstitial CO level when blood carboxyhemoglobin was 50% or lower. With a carboxyhemoglobin level of 8%, the interstitial CO level would be approximately 22 ppm. Our results indicate that CO levels of even 20 ppm have adverse effects on endothelial cells.

Among the limitations of our study, the most obvious one relates to potential differences in physiological responses of endothelial cells. We do not expect that human arterial endothelium would respond much differently to CO than bovine pulmonary artery endothelium. However, this is unknown and several groups have reported differences in the sensitivity of cells toward peroxynitrite (Bolanos et al.1995; Lin et al.1995). Additional unanswered questions from our study include whether repetitive CO exposures cause additive endothelial injury, and whether the NO-derived oxidants released by endothelial cells may augment injuries in vivo because they disturb blood-borne substances such as lipoproteins.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Thom SR, Xu YA, Ischiropoulos H. 1997. Vascular endothelial cells generate peroxynitrite in response to carbon monoxide exposure. Chem Res Toxicol 10:1023–1931.

ABBREVIATIONS

ANOVA	analysis of variance
BPAEC	bovine pulmonary artery endothelial cells
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
CO	carbon monoxide
COHb	carboxyhemoglobin
^{51}Cr	chromium-51
DHR	dihydrorhodamine 123
e_{500}	extinction coefficient
EDTA	ethylaminediaminetetraacetic acid
HBSS	Hank's balanced salts solution
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
IgG	immunoglobulin G
L-NAME	L-nitroarginine methyl ester
NaCl	sodium cloride
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NO	nitric oxide
PBS	phosphate-buffered saline
PHPA	*p*-hydroxyphenylacetic acid
ppm	parts per million
PRP	platelet-rich plasma

INTRODUCTION

Each year, the Health Effects Institute issues Requests for Preliminary Applications (RFPAs), inviting brief innovative research proposals on topics outside those defined by the more targeted Requests for Applications. In response to an RFPA issued in 1993, Drs. Steven R. Thom and Harry Ischiropoulos of the University of Pennsylvania Medical Center submitted a preliminary application, entitled *Vascular Oxidative Stress Due to Low Concentrations of Carbon Monoxide*. Their earlier results (Thom et al. 1994) indicated that inhaled carbon monoxide (CO)* competes with newly synthesized nitric oxide (NO) for binding sites in cells, resulting in release of NO free radicals. Drs. Thom and Ischiropoulos proposed to investigate whether the available NO might undergo further reactions that may be involved in atherosclerosis. The Health Research Committee requested a full application, and on the basis of the evaluation of outside reviewers, its own review, and the investigators' preliminary evidence supporting their hypothesis, they funded a one-year pilot study to examine the effect of environmentally relevant levels of CO on NO release by blood platelets and vascular endothelial cells.†

SCIENTIFIC BACKGROUND

Carbon monoxide is a colorless, odorless, and poisonous gas derived mainly from the incomplete combustion of fossil fuels. Transportation sources account for approximately 80 percent of the nation's CO emissions, with the largest contribution coming from motor vehicle exhaust (U.S. Environmental Protection Agency 1993).

The current National Ambient Air Quality Standards (NAAQSs) for CO are 9 ppm averaged over eight hours, and 35 ppm averaged over one hour, neither to be exceeded more than once a year. Studies have demonstrated that CO emissions decreased after emission control devices were added to automobiles (Flachsbart 1995; Yu et al. 1996). Data from fixed-site monitoring stations indicate that the one-hour NAAQS for CO of 35 ppm is rarely exceeded (U.S. Environmental Protection Agency 1991). Therefore, environmentally relevant concentrations of CO are between 9 and 35 ppm. However, although nationwide emissions of CO are decreasing, in some areas of the United States CO levels still exceed the EPA standards (especially during winter, when fuel combustion is less efficient because of low temperatures) (U.S. Environmental Protection Agency 1993).

When CO is inhaled, it combines with hemoglobin in red blood cells to form carboxyhemoglobin (COHb), which decreases the number of binding sites on hemoglobin for oxygen and reduces the oxygen-carrying capacity of blood. The lethal consequences of exposure to high concentrations of CO are well known. Exposure to concentrations of CO above 500 ppm (producing COHb levels above 45%) can cause fatal hypoxemia (Ilano and Raffin 1990). However, the effects of low-level CO exposures are also of public health and regulatory concern because these concentrations may adversely affect patients with cardiovascular disease. For example, an inspired concentration of 100 ppm (producing 14% COHb) can cause angina pectoris (chest pain) in some patients with coronary artery disease (Dwyer and Turino 1989; Ilano and Raffin 1990). Exposure to even lower levels of COHb (2% to 6%) limits the ability of people with coronary artery disease to exercise and shortens the time to the onset of exercise-induced angina pectoris (Adams et al. 1988; Allred et al. 1989, 1991; HEI Multicenter CO Study Team 1989; Kleinman et al. 1989). In addition, as discussed in the remainder of this section, some researchers speculate that long-term exposure to CO and the development of atherosclerosis are related.

THE DEVELOPMENT OF ATHEROSCLEROSIS

Atherosclerosis is characterized by progressive thickening of the arterial wall caused by a buildup of fat, cholesterol, cells, and connective tissue deposits that develop into fibrous plaques. Plaques can bulge into blood vessel lumina, impede blood flow, and may ultimately completely occlude the artery (Ross 1993). Current evidence suggests that injury to the endothelial cell layer lining the arteries causes endothelial cell loss and initiates atherosclerosis (reviewed by Steinberg and Wiztum 1990; Ross 1993). The

* A list of abbreviations appears at the end of the Investigators' Report for your reference.

† Dr. Thom's final report, Mechanism of Oxidative Stress from Low Levels of Carbon Monoxide, was received for review on June 21, 1996. A revised report, received on February 18, 1997, was accepted for publication by the Health Review Committee on April 14, 1997. The cost of the one-year study, which began in May 1995, was $120,032. During the review of the Investigators' Report, the Review Committee and the investigators had the opportunity to exchange comments and to clarify issues in the Investigators' Report and the Health Review Committee's Commentary. The Commentary is designed to place the Investigators' Report in perspective as an aid to the sponsors of the Health Effects Institute and to the public.

earliest recognizable lesion of atherosclerosis, called a "fatty streak," appears in the arterial intima (the inner lining of the blood vessel that is normally a single layer of endothelial cells) and is composed of aggregations of lipid-rich macrophages (foam cells) and lymphocytes. A critical step in plaque formation appears to be the oxidation of low-density lipoproteins, which carry cholesterol in the blood. Macrophages take up oxidized low-density lipoproteins to form foam cells that invade the arterial wall and become a part of fatty streaks. In addition, oxidized low-density lipoproteins are believed to injure endothelial cells.

ASSOCIATION BETWEEN CARBON MONOXIDE EXPOSURE AND ATHEROSCLEROSIS

Whether or not prolonged exposure to CO is involved in the pathogenesis of progressive cardiovascular diseases, such as atherosclerosis, is an important public health concern because atherosclerosis is the leading contributor to deaths from heart attack and stroke in the United States (Myerburg and Castellanos 1988). The results of two large epidemiologic studies suggest a positive association between some indices of air pollution and cardiopulmonary disease (Dockery et al. 1993; Pope et al. 1995). A few epidemiologic and toxicologic studies have addressed CO directly. Studies of motor vehicle examiners (Stern et al. 1980) and tunnel officers (Stern et al. 1988) suggest that prolonged exposure to CO may be associated with an elevated risk of death from atherosclerotic heart disease; however, other pollutants are also present in vehicle exhaust. Although historical monitoring reports indicated that the tunnel officers were exposed to levels of CO in excess of 50 ppm, neither study provided information on actual CO exposures or the possible confounding effect of smoking cigarettes (a significant source of CO). Koskela (1994) compared the incidence of mortality from cardiovascular disease among male foundry workers exposed regularly, occasionally, or infrequently to CO. (For most of the study period, the mean CO level in the most highly polluted foundry air was 82 ppm, which later was reduced to a mean level of 36 ppm CO.) A follow-up study of workers who underwent a health examination led Koskela (1994) to conclude that the extent of occupational CO exposure and smoking independently increased mortality from heart disease.

Studies with laboratory animals provide some evidence that atherosclerosis is accelerated in rabbits, monkeys, and pigeons when fed diets high in cholesterol or cholesterol and fat and exposed to high levels of CO leading to 15% to 20% COHb (Astrup 1967; Davies et al. 1976; Webster et al. 1968; Thomsen 1974; Armitage et al. 1976; Turner et al. 1979). Two studies indicate that the development of atherosclerosis increased in rabbits when fed cholesterol and exposed to environmental tobacco smoke (also a source of inhaled CO) for ten weeks (Sun et al. 1994; Zhu et al. 1993). In contrast, Rogers and coworkers (1988) reported little difference in the incidence of atherosclerosis between baboons fed a diet enriched in cholesterol and saturated fat who puffed on smoking machines for two to three years and animals fed the same diet who puffed air.

NITRIC OXIDE, PEROXYNITRITE, AND ATHEROSCLEROTIC PLAQUE FORMATION

Thom and Ischiropoulos suggested a new mechanism, involving the highly reactive compound NO, by which prolonged exposure to CO might lead to the development of atherosclerotic plaques. Although once regarded primarily as an air pollutant (NO, like CO, is produced by the combustion of fossil fuels), research over the past twelve years has shown that NO is produced in biological systems and is a ubiquitous regulator of the cardiovascular system, the immune system, and both the central and peripheral nervous systems (reviewed by Moncada et al. 1991; Feldman et al. 1993; Moncada and Higgs 1995). Nitric oxide is produced by the amino acid L-arginine reacting with oxygen catalyzed by nitric oxide synthases, enzymes found in many cells, including macrophages, blood platelets, and endothelial cells (reviewed by Kobzik et al. 1993). In the cardiovascular system, NO (Palmer et al. 1987; Ignarro et al. 1987) or an NO-precursor compound (reviewed by Kelley and Smith 1996) diffuses from the vascular endothelium to the vascular smooth muscle, where it causes vasodilation by a process called signal transduction. (Signal transduction by NO is also important in the central and peripheral nervous systems.) Blood platelets also contain NO, which helps to maintain blood fluidity by preventing platelets from aggregating and adhering to the vascular endothelium (Moncada and Higgs 1995).

Beckman and Koppenol (1996) proposed that NO has three main biochemical reactions, each of which occurs rapidly under physiological conditions. In addition to its role in signal transduction, NO rapidly diffuses to red blood cells where it is destroyed by reacting with oxyhemoglobin. A third reaction, which is critical to the rationale behind Thom's study, is the formation of the powerful oxidant peroxynitrite from NO and superoxide anion. Peroxynitrite can contribute to the development of atherosclerotic plaques because it oxidizes low-density lipoproteins (Hogg et al. 1993). Peroxynitrite also modifies proteins containing the amino acid tyrosine by forming nitrotyrosine; thus, the presence of nitrotyrosine in proteins is an indicator of peroxynitrite formation (reviewed by Crow and Beckman 1996). The demonstration that proteins in human atherosclerotic lesions contain nitrotyrosine suggests that peroxynitrite

participates in plaque formation (Beckman et al. 1994). Therefore, Thom and Ischiropoulos hypothesized that the possible link between exposure to CO and the development of atherosclerosis lay in the ability of CO to interfere with the normal intracellular binding of NO and the consequent ability of free NO to react with superoxide anion to produce peroxynitrite.

JUSTIFICATION FOR THE STUDY

During the 1980s, HEI conducted a number of clinical and laboratory studies on the effects of CO on cardiovascular disease. Although research on CO was not a high priority when Thom and Ischiropoulos submitted their proposal in 1993, the Health Research Committee thought that they proposed to examine an interesting and potentially important mechanism that might link prolonged CO exposure to atherosclerosis and other pathological processes. Because of the quality of the research team and their proposal's strength, the Committee funded a one-year pilot study that would extend the earlier demonstration of NO release by blood platelets isolated from rats exposed to 1,000 or 3,000 ppm CO (Thom et al. 1994) to environmentally relevant CO levels.

TECHNICAL EVALUATION OF THE STUDY

ATTAINMENT OF OBJECTIVES

Thom and Ischiropoulos had two objectives. The first was to determine whether platelets and endothelial cells release NO following exposure to what the investigators considered environmentally relevant levels of CO. The second was to determine whether endothelial cells produce oxidants derived from NO.

The investigators carried out a careful and logical series of experiments that allowed them to successfully attain their stated objectives. Given the limited duration of the study, their findings could not be pursued further. One problem, however, is that the investigators chose to use different units of measure in similar experiments. This creates difficulty when comparing their experiments with each other and trying to integrate their results.

METHODS AND RESULTS

The researchers exposed rat blood platelets and bovine pulmonary artery endothelial cells in vitro to CO levels from 0 (control cells) to 100 ppm for periods ranging from two minutes to four hours, and measured NO release, the production of oxidants, and CO toxicity. In one experiment, they isolated platelets from rats exposed to 0, 20, 100, or 1,000 ppm CO for one hour and measured NO release in vitro.

The investigators used state-of-the-art methods to measure NO release by platelets. They first measured NO release electrochemically with an NO-selective electrode. They substantiated release of NO by also measuring nitrite plus nitrate ions, which are stable end products derived from NO. Some investigators have found it difficult to obtain reliable data with the NO-selective electrode; therefore, Thom and Ischiropoulos are to be commended for checking their electrochemical measurements with a second technique. The investigators used standard techniques (described below with their key results) to determine oxidant formation and cytotoxicity in endothelial cells exposed to CO.

Thom and Ischiropoulos determined statistical significance by using one-way analysis of variance followed by Scheffe's test. Scheffe's test was applied primarily to compare the results from various concentrations of CO to the control level of 0 ppm. To compare data between and among noncontrol concentrations, they used the Mann-Whitney Rank Sum test. This test does not control for multiple comparisons, however, and may indicate a greater degree of statistical significance than the data warrant.

Platelets

Electrochemical determination indicated that the levels of NO released by rat blood platelets exposed to 20, 50, 80, or 100 ppm CO were significantly elevated compared with those released by control platelets exposed to 0 ppm CO. Platelets exposed to 100 ppm CO released significantly more NO than those exposed to the three lower CO levels; however, the amounts of NO released after exposure to 20, 50, or 80 ppm CO were not significantly different from each other. The lack of a strong dose-response effect may be due to the lack of sensitivity of the NO-selective electrode. Nevertheless, the electrochemical assay proved to be more sensitive than measuring nitrite plus nitrate, which indicated that NO was released from platelets exposed to 100 ppm, but not to 50 ppm CO.

The investigators concluded that NO synthesis was required for CO-mediated NO release because preincubating platelets with L-nitroarginine methyl ester (L-NAME), which inhibits nitric oxide synthase, eliminated the effect of CO. Furthermore, they concluded that the increase in NO was not due to its increased synthesis because exposure to 100 ppm CO did not stimulate nitric oxide synthase activity. As the investigators propose, it is likely that CO competes with newly synthesized NO for binding sites on platelet proteins

and the reduced number of available binding sites causes NO release from cells. In addition, when the synthesis of new NO was inhibited, exposure to 10 to 100 ppm CO displaced NO that had been previously bound by platelets. The electrochemical assay also provided evidence that platelets isolated from rats exposed to 20, 100, or 1,000 ppm CO released NO. The presence of NO (which is presumed to be short-lived because of its high reactivity) after the time required to isolate platelets from the animals for the in vitro assay is surprising because free NO should undergo rapid reaction in vivo.

Endothelial Cells

Bovine pulmonary artery endothelial cells exposed to 20 to 100 ppm CO in culture also released NO. As with platelets, NO release depended on the activity of nitric oxide synthase, and CO did not stimulate nitric oxide synthase activity. Competition of CO with NO for binding sites was again proposed as the mechanism for NO release.

An important goal of this study was to determine whether endothelial cells exposed to CO produce oxidants derived from NO. One method of determining this was to measure the oxidation of *para*-hydroxyphenylacetic acid (PHPA) to its fluorescent derivative. Culture medium isolated from cells exposed to 70 or 100 ppm CO showed significantly greater fluorescence than control cells. Inhibiting NO synthesis by preincubating cells with L-NAME before exposure to 100 ppm CO reduced the fluorescence. Thom and Ischiropoulos next obtained evidence that the putative oxidant was short-lived, rather than a relatively stable compound such as hydrogen peroxide.

The researchers used two methods to implicate peroxynitrite as the oxidant formed after exposure to CO. First, they determined that exposure to 50 or 100 ppm CO elevated nitrotyrosine levels in endothelial cell proteins compared with cells incubated in the absence of CO. Second, they measured the oxidation of dihydrorhodamine 123 (DHR) to its fluorescent derivative, rhodamine 123. Because hydrogen peroxide does not react with DHR, the investigators interpreted the increased fluorescence in the medium from endothelial cells exposed to 100 ppm CO, compared with controls, as evidence of peroxynitrite formation.

Peroxynitrite formation is considered to reflect oxidative stress in cells. Another indicator of oxidative stress is the conversion of reduced sulfhydryl compounds to their oxidized derivatives. Because the level of reduced sulfhydryl compounds was similar in endothelial cells exposed to 0 or 100 ppm CO, Thom and Ischiropoulos concluded that the level of CO-induced oxidative stress was not high.

The investigators measured the toxicity of CO to vascular endothelial cells by (1) incorporating radioactive chromium (^{51}Cr) into the cells and measuring how much the cells release into fresh medium after being exposed to CO, and (2) adding the fluorescent dye ethidium homodimer-1 to cells after they've been exposed to CO and measuring how much the cells take up. Both of these methods are effective for assessing cellular integrity because cells will release ^{51}Cr and will take up ethidium homodimer-1 if the cell membranes are damaged, but not if the membranes are intact.

Cells exposed to 100 ppm CO (but not to 50 or 80 ppm) for four hours released significantly more ^{51}Cr immediately after exposure ceased than control cells. Because cells with L-NAME in the medium released less ^{51}Cr than cells without L-NAME, the investigators proposed that CO-induced damage was likely due to NO or its products. When cells were exposed to 100 ppm CO for only two hours, the amount of ^{51}Cr released immediately after exposure ceased did not differ from that released by control cells. However, when the CO-exposed cells remained in medium (with no CO) for an additional six hours after exposure ceased, they released more ^{51}Cr at the end of the six hours than control cells did. In contrast to the protective effect of L-NAME on the immediate release of ^{51}Cr after four hours of exposure to CO, its ineffectiveness in reducing the delayed release of ^{51}Cr after two hours of exposure to CO is unexplained. Ethidium homodimer-1 appeared in cells twenty hours after exposure to 20 or 100 ppm CO for two hours.

The investigators propose that CO-induced cell toxicity was caused by NO-derived oxidants. However, the delay in both ^{51}Cr release and uptake of ethidium homodimer-1 would not be expected if cell membranes were damaged by a short-lived oxidant, such as peroxynitrite. In addition, the lack of protection by L-NAME in delayed ^{51}Cr release weakens the case for cell damage caused by NO-derived oxidants. An alternative explanation for oxidant-induced cell damage is that CO blocks electron transport in cell organelles (such as mitochondria or microsomes), which then release toxic oxygen species. Although recent work by Thom and coworkers (1997) indicates that endothelial cells' mitochondrial function was not affected by exposure to 100 ppm CO, the delayed toxicity and lack of protection by L-NAME require further exploration.

PHYSIOLOGICAL SIGNIFICANCE

Two factors need to be considered when interpreting Thom and Ischiropoulos' findings that platelets and endothelial cells exposed to CO in vitro release NO. First, in vitro

experiments are likely to exaggerate the cytotoxicity and chemical reactivity of NO because these systems typically do not include components that remove NO from further reaction in vivo (Beckman and Koppenol 1996). For example, in the intact organism, NO produced in vascular endothelial cells diffuses into blood vessels and is inactivated by reacting with oxyhemoglobin faster than it reacts with endothelial cell components. The level of free NO is also reduced when it diffuses to smooth muscle and is destroyed by reacting with oxymyoglobin (Beckman and Koppenol 1996). Thus, NO's rapid diffusion from endothelial cells makes it less available to form peroxynitrite by reaction with endothelial cell superoxide anion.

Second, the reactivity of NO with other cellular targets is low. Beckman and Koppenol (1996) estimate that "enormous" amounts of oxygen are necessary to sustain nitric oxide synthase activity at levels needed to produce enough NO to react with these targets. In this study, Thom and Ischiropoulos determined that platelets from rats exposed to an unrealistically high concentration of CO (1,000 ppm) liberated only 74 nmol of NO per 10^8 cells. This is a negligible amount of NO compared to the concentrations that are considered necessary to produce oxidative damage. Based on this result, displacement of relatively small amounts of NO cannot necessarily be considered to be an important cytotoxic mechanism. These two factors need to be considered when assessing the physiological relevance of the investigators' findings.

Another issue is the levels of CO that may be considered environmentally relevant. The current allowable limits for CO are designed to result in no more than 2% to 3% blood COHb, corresponding closely to the eight-hour standard of 9 ppm CO. The investigators demonstrated that exposure to environmentally relevant levels of 10 to 20 ppm CO released NO from platelets and endothelial cells. In contrast, oxidant production by vascular endothelial cells and cell damage measured by ^{51}Cr release occurred predominantly after exposure to 50 to 100 ppm CO. The threshold for CO toxicity to cardiac muscle is thought to be 100 ppm (Turino 1981); because few environments expose people to this level of CO, these results may be of questionable physiological relevance.

IMPLICATIONS FOR FUTURE RESEARCH

The ability of environmentally relevant levels of CO to mediate platelet and vascular endothelial cell activities is potentially important in the pathogenesis of atherosclerosis. The investigators' in vitro findings are provocative; however, future research with experimental systems should limit exposure levels to 35 ppm CO (the one-hour NAAQS) and below. Because atherosclerotic plaque formation is not common in the pulmonary artery, cells from systemic arteries, such as the coronary artery or the aorta, may be preferable for future studies. An important consideration for future research is that experimental designs more accurately reflect the natural cellular milieu. For example, because NO diffusion reduces its endothelial cell concentration, future research should determine if sufficient NO remains to form peroxynitrite at levels that can cause toxicity.

CONCLUSIONS

Thom and Ischiropoulos found that exposure to 10 or 20 ppm CO in vitro caused platelets and endothelial cells to release NO. Platelets isolated from rats that inhaled 20 ppm CO and higher also released NO in vitro. Carbon monoxide did not stimulate the activity of nitric oxide synthase in platelets or endothelial cells; therefore, elevated enzymatic production of NO is probably not the mechanism for its increased release. The investigators reasonably suggest that NO is released because CO binds to platelet and endothelial cell proteins and reduces the number of binding sites for newly synthesized NO. The nature of these NO binding sites remains to be elucidated.

Vascular endothelial cells exposed in vitro to concentrations of CO that approach toxic levels for humans (50 and 100 ppm) produced at least one short-lived oxidant. Because nitric oxide synthase activity is necessary for oxidant production, NO was implicated as its precursor. The investigators obtained suggestive evidence that the oxidant produced was peroxynitrite.

Thom and Ischirpoulos speculate that because CO prevents the normal intracellular binding of NO, it can react with superoxide anion and produce peroxynitrite. The possible significance of this reaction is that peroxynitrite oxidizes cholesterol-carrying low-density lipoproteins to a form implicated in the development of atherosclerotic plaques. However, because even high levels of CO (1,000 ppm) released negligible amounts of NO, this cannot necessarily be considered as an important cytotoxic mechanism.

Experiments performed in vitro may exaggerate the toxic effects of NO because they do not include cellular constituents that normally react with NO in vivo and reduce its availability for peroxynitrite formation. This study provides provocative results; however, determining their physiological significance requires experimental conditions that more accurately reflect the natural cellular milieu.

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