

Nitric Oxide Production and Perivascular Tyrosine Nitration in Brain after Carbon Monoxide Poisoning in the Rat

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Abstract

Nitric oxide is a short-lived free radical and physiological mediator which has the potential to cause cytotoxicity. Studies were conducted to investigate whether nitric oxide, and the potent oxidant peroxynitrite, were generated in brain during experimental carbon monoxide (CO) poisoning in the rat. Nitric oxide production was documented by electron paramagnetic resonance spectroscopy, and found to be increased by ninefold immediately after CO poisoning. Evidence that peroxynitrite was generated was sought by looking for nitrotyrosine in the brains of CO-poisoned rats. Nitrotyrosine was found deposited in vascular walls, and also diffusely throughout the parenchyma in immunocytochemical studies. The affinity and specificity of an anti-nitrotyrosine antibody was investigated and a solid phase immunoradiochemical assay was developed to quantify nitrotyrosine in brain homogenates. A 10-fold increase in nitrotyrosine was found in the brains of CO-poisoned rats. Platelets were involved with production of nitrotyrosine in the early phase of exposure to CO. However, nitrotyrosine formation and leukocyte sequestration were not decreased in thrombocytopenic rats poisoned with CO according to the standard model. When rats were pre-treated with the nitric oxide synthase inhibitor, L-nitroarginine methyl ester, formation of both nitric oxide and nitrotyrosine in response to CO poisoning were abolished, as well as leukocyte sequestration in the microvasculature, endothelial xanthine dehydrogenase conversion to xanthine oxidase, and brain lipid peroxidation. We conclude that perivascular reactions mediated by peroxynitrite are important in the cascade of events which lead to brain oxidative stress in CO poisoning. (*J. Clin. Invest.* 1996; 97:2260–2267.) Key words: peroxynitrite • nitric oxide • nitrotyrosine • electron paramagnetic resonance • oxidative injury

Introduction

Experimental evidence implicates oxidative stress as a major cause of tissue injury in a variety of human disorders and ex-

perimental models of disease. Oxidative stress is the result of excessive production of reactive species that overwhelm the cellular antioxidant capacity, leading to inactivation of key cellular functions and ultimately to cell death. Although most studies have concentrated on the contribution of oxygen-derived reactive species such as superoxide, recent reports have also described excessive production of nitric oxide in association with tissue injury (1). Nitric oxide-related tissue injury may be at least partially due to peroxynitrite, a relatively long-lived, strong oxidant that is generated by the near diffusion-limited reaction between nitric oxide and superoxide (2).

The mechanism of brain injury after carbon monoxide (CO)¹ poisoning is incompletely understood. Several recent clinical reports have suggested that the primary site of injury may be the vasculature, and that focal pathology, a hallmark of CO poisoning, may arise secondary to hemorrhagic necrosis (3, 4). We hypothesized that peroxynitrite may be produced in animals during CO poisoning because exposure to CO has been found to enhance the rates of production of both nitric oxide, and partially reduced oxygen species (5, 6). Platelets liberate large amounts of nitric oxide coincident with exposure to CO (5). Exposure to CO at concentrations sufficient to disturb cardiovascular function can also perturb mitochondrial electron transport, and increase the production of partially reduced oxygen species (6). Oxidative stress, documented as brain lipid peroxidation, occurs in rats when the exposure to CO terminates with events that include systemic hypotension, cerebral hypoperfusion and impaired mitochondrial function (7–10).

Peroxyntirite is capable of oxidizing lipids, thiols, deoxyribose, and a number of other small organic molecules (11, 12). A major product from the reaction of peroxynitrite with proteins is nitrotyrosine (13). Using antibodies that specifically recognize nitrotyrosine, extensive protein nitration has been detected in human coronary artery atherosclerotic lesions (14), in acute lung injury (15, 16), and in endotoxemia (17, 18). In this paper we have investigated the production of nitric oxide in brain and the potential for using nitrotyrosine as a marker of peroxynitrite-mediated oxidative stress. We have examined the affinity and specificity of a polyclonal anti-nitrotyrosine antibody and we have quantified the amount of nitrotyrosine in brain tissue using a solid phase immunoradiochemical assay.

Methods

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

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1. *Abbreviations used in this paper:* CO, carbon monoxide; DETC, diethyldithiocarbamate; L-NAME, L-nitroarginine methyl ester.

Exposure of rats to carbon monoxide. Exposure to CO followed our model which has been described in detail in several previous publications (7–10). In brief, rats were placed in a seven-liter Plexiglas chamber into which a small volume of pure CO was injected to achieve a concentration of 1000 ppm. A mixture of 1000 ppm CO in air was flushed through at a rate of 8 to 12 liters per minute for 40 min, at which time the gas was switched to 3000 ppm CO in air and another CO bolus was added. In day-to-day experiments, usually two out of three rats lost consciousness in this environment within 20 min and were then removed from the chamber to breath room air. Rats that did not spontaneously lose consciousness after exposure to 3000 ppm for 20 min were rendered unconscious with a 60 cc bolus of pure CO. We have found that unconsciousness is a reliable outward manifestation of systemic hypotension and cerebral hypoperfusion whether it occurs with exposure to 3000 ppm CO or with the bolus of pure CO (8, 10). In some studies rats were exposed to only 1000 ppm CO for 40 or 60 min, or to 3000 ppm CO for 1 to 2 min plus a bolus of pure CO to cause unconsciousness. In other studies rats were made thrombocytopenic by an intraperitoneal injection of 1.5 ml/kg rat plasma-adsorbed rabbit anti-rat platelet antiserum (Inter-cell Technologies, Hopewell, NJ) given 2 h before exposure to CO. Neutropenia was caused by an intraperitoneal injection of 4 ml/kg anti-neutrophil antiserum (Inter-cell Technologies, Hopewell, NJ) administered 24 h before study. L-nitroarginine methyl ester (L-NAME, 1 mg/kg s.q.) was injected 2 h before CO exposure. At the end of CO exposures, rats were killed by decapitation within 8 min. The brain was removed from the skull and homogenized with a Polytron blender using 10 ml TBS. Homogenates were diluted 1:500 (vol/vol) with TBS for analysis by solid phase radioimmunoassay. Biochemical assays of xanthine dehydrogenase, xanthine oxidase and conjugated dienes were performed on brain homogenates by following methods described in previous publications (7–9). Microvessels were isolated, and myeloperoxidase was assayed, as previously described (7).

Electron paramagnetic resonance measurement of nitric oxide. Rats were exposed to CO according to our standard model. After exposure to 1000 ppm CO for 20 min, rats were briefly removed and injected subcutaneously with diethyldithiocarbamate (DETC) (500 mg/kg) and, at a second site, with a ferrous sulfate (50 mg/kg)-sodium citrate (250 mg/kg) mixture (19). The rats were replaced in the CO environment, exposed to 1000 ppm CO for an additional 20 min and then to 3000 ppm CO for 20 min. Unconscious rats were removed from the CO environment, decapitated 8 min later and one cerebral hemisphere was extruded into a quartz EPR tubing (4 mm diameter). The specimen was immediately frozen in liquid nitrogen. EPR spectra were recorded using a Varian E-109 spectrometer equipped with a liquid flow system (temperature was set at -130°C). The EPR settings were: 9.22 GHz microwave frequency, 20 mW microwave power, 100 KHz modulation frequency, 0.32 mT modulation and 0.25 s time constant. Two types of EPR signals are observed in the rat brain: a quartet hyperfine spectrum of Cu-DETC and a nitric oxide-Fe-DETC spectrum which is similar to the Cu-DETC spectrum but reveals two distinct peaks at $g = 2.047$ and $g = 2.025$ (19, 20). The nitric oxide-Fe-DETC spectrum was observed only when rats were injected with both Fe and DETC and not with either Fe or DETC alone, consistent with previous reports (19, 20).

Immunohistochemistry. Staining was carried out with an affinity purified polyclonal anti-nitrotyrosine antibody obtained from Dr. J.S. Beckman, University of Alabama at Birmingham. The polyclonal antibody was raised by immunizing rabbits with peroxynitrite-treated keyhole limpet hemocyanin as described previously (13). The binding characteristics of this antibody were investigated using peroxynitrite-modified BSA that had been immobilized onto nitrocellulose using the 96 well Bio-Dot microfiltration unit (BioRad, Hercules, CA). Fatty acid free bovine serum albumin (4 mg/ml) was reacted with peroxynitrite in 100 mM potassium phosphate buffer that contained $100\text{ }\mu\text{M}$ of the metal chelator diethylenepentaacetic acid (DTPA), pH 7.4. Peroxynitrite was synthesized as described previously (1), or by utilizing 3-morpholinysydnnonimine (SIN-1) which decomposes to release

nitric oxide as well as superoxide (21). The yield of nitration was determined by measuring the absorbance at pH 11.5, ($\epsilon_{428\text{ nm}} = 4,400\text{ M}^{-1}\text{ cm}^{-1}$).

After CO poisoning, rats were anesthetized by an intraperitoneal injection with ketamine (73.5 mg/kg) and xylazine (1.5 mg/kg). Brains were fixed in situ by infusing 4% paraformaldehyde in a 0.1 M sodium cacodylate buffer through a catheter in the left ventricle under 50 cm of water pressure. The brains were removed, left in fixative for three hours, and then cryoprotected by 1 h incubations in 10, 20, and 30% sucrose in 0.1 M sodium cacodylate buffer. The brains were placed on OTC-coated tissue holders, covered with a thin layer of OTC, and frozen with distilled freon 22 in liquid nitrogen. Eight micron thin sections were prepared and placed onto poly-L-lysine coated slides. Before staining, slides were soaked twice for 5 min to remove excess fixative. When a peroxidase-based staining method was to be used, endogenous peroxidase was quenched by incubating sections with 0.3% methanolic hydrogen peroxide (1:25 vol/vol 30% hydrogen peroxide:methanol) for 20 min. Slides were rinsed with PBS and incubated for 20 min with blocking solution (4% fatty acid free BSA, 10% goat serum and 3% Triton X-100 in 0.1 M PBS, pH 7.2). Slides were rinsed with PBS and incubated for 1.5 h with either anti-nitrotyrosine polyclonal antibody at $10\text{ }\mu\text{g/ml}$, with antigen-competed primary antibody, with an irrelevant rabbit IgG, or with anti-CD31 polyclonal antibody at $20\text{ }\mu\text{g/ml}$. The competed reagent for the anti-nitrotyrosine antibody was prepared by diluting the primary antibody at $10\text{ }\mu\text{g/ml}$ in 0.1 M PBS that contained 10 mM nitrotyrosine (Aldrich). Slides used for visualizing the presence of antibody on tis-

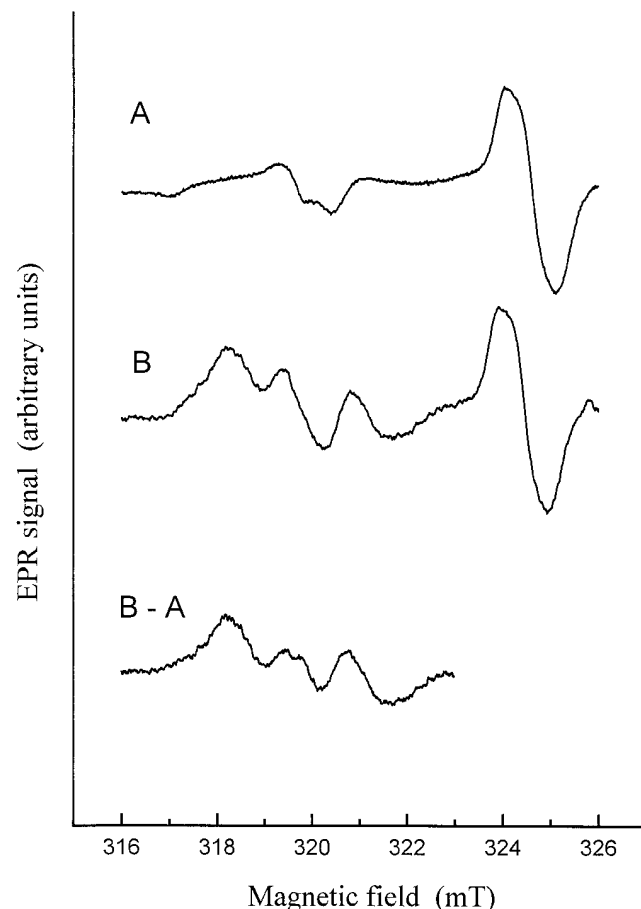


Figure 1. Electron spin resonance spectra of rat cerebral cortex from a control rat (A), from a CO poisoned rat (B) and difference spectrum (B-A).

sue sections by immunofluorescence were incubated for 1 h with an anti-rabbit IgG conjugated to Texas red (1:100 dilution in TBS) and examined under a Nikon Diaphot-TND epifluorescence inverted microscope. Antibody was also visualized using a peroxidase based method by following the manufacturer's instructions (Elite Vectastain Kit, Vector Labs, Burlingame, CA). Sections were stained with biotinylated anti-rabbit IgG for 30 min, washed for 20 min with PBS, and incubated for 30 min with a solution of Avidin DH and biotinylated horseradish peroxidase. Slides were rinsed with PBS and incubated with a solution of 1.4 mM diaminobenzidine tetrachloride and 0.3% (vol/vol) hydrogen peroxide for 3 min, washed with water, and counterstained with Harris-modified hematoxylin.

Solid phase immunoradiochemical assay. Tissue proteins were immobilized onto nitrocellulose using the 96 well Bio-Dot microfiltration unit (BioRad, Hercules, CA). 8–12 different concentrations of peroxynitrite-modified BSA standard were also loaded in duplicate on each blot to generate a standard curve. 4–8 concentration-dependent dilutions of the tissue protein samples were loaded in 400 μ l TBS. After blocking with either 2% gelatin or 5% dry milk, the nitrocellulose was incubated with the anti-nitrotyrosine antibody for 15 h followed by a 3-h incubation in a solution containing a donkey anti-rabbit 125 I-labeled IgG (0.1–0.2 mCi/ml). The blot was extensively washed in Tween-TBS and dried. The radioactivity of each sample was measured directly by Beta scanning using an Ambis 400 imaging detector. The net counts of radioactivity (corrected for background counts from a sample blank) were obtained using the AMBIS image analysis software v4.1 and then plotted on a semi-logarithmic plot.

Reduction of nitrotyrosine with dithionite for solid phase immunoradiochemical assay. Nitrotyrosine was reduced to aminotyrosine

with dithionite under alkaline conditions. The dithionite solution was made fresh daily by placing dithionite crystals in a plastic tube that had first been flushed with nitrogen and then adding deaerated buffer to dissolve the crystals. The pH of 1 ml brain homogenate was adjusted to 9.0 by the addition of sodium hydroxide and a freshly prepared dithionite solution was added to a final concentration of 0.5 M in order to reduce nitrotyrosine to aminotyrosine. Dithionite-reduced samples were prepared fresh because of the time dependent auto-oxidation of aminotyrosine back to nitrotyrosine. The radioactive counts obtained from Dot-blot of dithionite-reacted samples were subtracted from the counts of unreacted samples, and net counts were used in calculations of brain nitrotyrosine concentration.

Statistics. Statistical significance was determined by ANOVA followed by Scheffe's test (22). The level of significance was taken as $P < 0.05$. Results are expressed as mean \pm SE.

Results

Detection of nitric oxide in brain after CO poisoning by electron paramagnetic resonance spectroscopy (EPR). Rats were poisoned with CO in a manner known to cause brain oxidative injury (7–9, see Methods) and the relative concentration of nitric oxide in brains was evaluated using EPR. A triplet nitric oxide signal, characteristic for the nitric oxide-iron-DETC adduct (20), was found in the brains of rats immediately after they were poisoned with CO (Fig. 1). The height of this signal averaged ninefold greater than the signal found in control rats in three

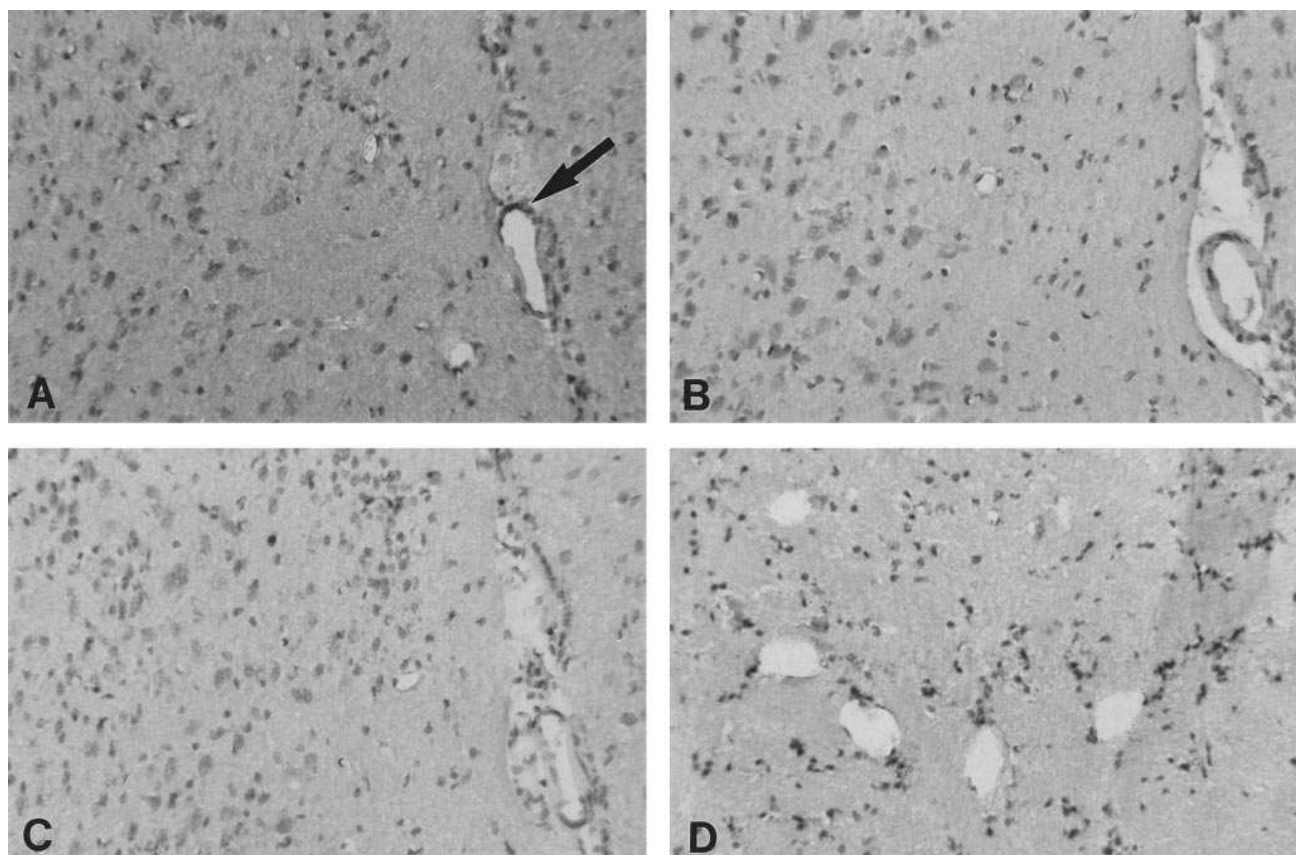


Figure 2. Photomicrographs from immunoperoxidase-stained rat cerebral cortex. A–C show thin sections from a rat poisoned with CO. (A) Section stained with anti-nitrotyrosine antibody. Positive staining is indicated by dark coloration, especially around the vessel (arrow). (B) Stained with antigen-competed anti-nitrotyrosine antibody. (C) Stained with non-specific IgG. (D) A section from a control rat brain stained with anti-nitrotyrosine antibody. $\times 250$.

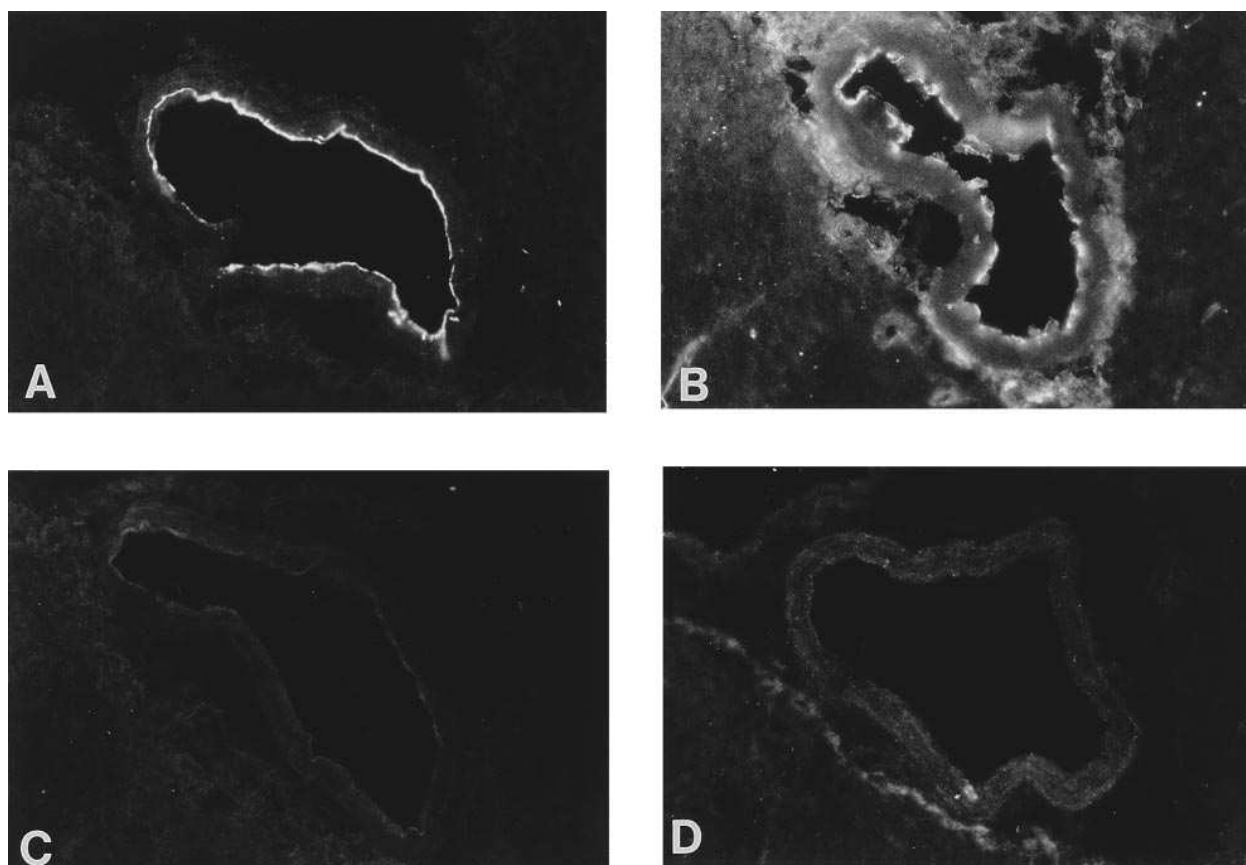


Figure 3. Immunofluorescence photomicrographs from cerebral cortex. Panels show serial thin sections from a rat poisoned with CO. (A) Stained with anti-nitrotyrosine antibody. (B) Stained with anti-CD31 antibody. (C) Stained with antigen-competed anti-nitrotyrosine antibody. (D) Stained with non-specific IgG. $\times 875$.

paired trials. The triplet nitric oxide signal is clearly seen in difference spectra (Fig. 1). When rats were treated before CO poisoning with the nitric oxide synthase inhibitor, L-NAME, the nitric oxide-Fe-DETC adduct was not found. The nitric oxide signal diminished in intensity in brains obtained > 8 – 10 min after CO poisoning, and was not detectable in brains beyond 20 min after CO poisoning.

Immunocytochemical studies of brain. Brains from control and CO-poisoned rats were sectioned and stained with anti-nitrotyrosine antibody. A diffuse pattern of staining, with no localization to specific structures such as the basal ganglia, was present in brains from rats poisoned with CO (Fig. 2). Staining was not found in brains from control rats. On close examination of serial brain sections, relatively dense deposits of nitrotyrosine were frequently found in a perivascular location. While this could be appreciated to some degree with the immunoperoxidase staining, it was most clearly discerned in fluorescence micrographs (Fig. 3). One panel in Fig. 3 also shows a section stained with antibodies to CD31, or PECAM-1, which is expressed on endothelial cells and has been used to highlight vascular structures (23).

Binding characteristics of the polyclonal antibody against nitrotyrosine. In the process of developing a method to quantify tissue nitrotyrosine, we first established the binding characteristics of the polyclonal affinity purified anti-nitrotyrosine antibody. Binding by the antibody was found to be saturable and dependent on the concentration of both the antibody and antigen (Fig. 4, A and B). When antibody was used at a con-

centration of $2 \mu\text{g/ml}$, binding of nitrotyrosine increased linearly with increasing antigen concentration up to $1 \mu\text{g}$ of nitrotyrosine. Using solutions of antibody ($2 \mu\text{g/ml}$) and 50 ng of immobilized antigen, we determined the K_d for nitrotyrosine inhibition of binding to be $2.5 \mu\text{M}$ (Fig. 4 C). The binding of the antibody under the same conditions was not inhibited by the presence of 10 mM of aminotyrosine ($115 \pm 4\%$), phosphotyrosine ($126 \pm 3\%$), methyltyrosine ($141 \pm 14\%$) and tyrosine ($91 \pm 4\%$).

Nitrotyrosine quantification in brain. Nitrotyrosine formation in brain after CO poisoning was quantified by using the solid phase radiochemical assay (Fig. 5). A 10-fold increase in nitrotyrosine was found after CO poisoning and the nitrotyrosine level was not significantly different in neutropenic rats (circulating neutrophil counts $< 200 \text{ cells}/\mu\text{l}$ blood). However, nitrotyrosine was not increased in brains from rats that had been pretreated with a competitive nitric oxide synthase inhibitor, L-NAME.

In previous studies we have found that oxidative injury in the brain depends on the pattern of CO poisoning (8). Whereas the standard model (see Methods) will precipitate changes including brain lipid peroxidation, exposure to just 3000 ppm CO will cause a temporary drop in blood pressure that is manifested as a loss of consciousness, but lipid peroxidation does not occur (8, 10). Nitrotyrosine was not increased in the brains of rats exposed to 3000 ppm CO for $< 2 \text{ min}$ and to a pulse of pure CO to cause unconsciousness (Fig. 5). The mean carboxy-hemoglobin level in these rats was $81 \pm 1\%$, a level somewhat

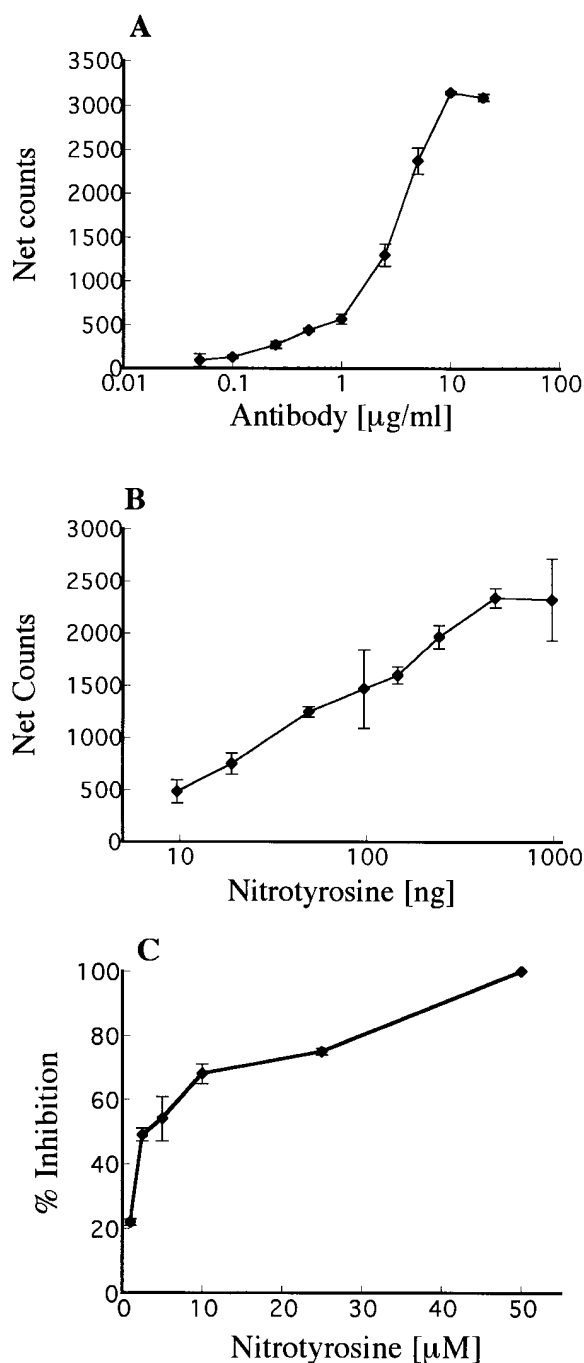


Figure 4. Characterization of the polyclonal affinity-purified anti-nitrotyrosine antibody. (A) Antibody binding to 50 ng of immobilized antigen (peroxynitrite-modified fatty acid free BSA). (B) Antigen concentration dependent binding of anti-nitrotyrosine antibody (2 $\mu\text{g/ml}$). (C) Competition of antibody binding with free nitrotyrosine. Peroxynitrite-modified fatty acid free BSA containing 50 ng of nitrotyrosine was immobilized onto nitrocellulose. Antibody solutions (2 $\mu\text{g/ml}$) were incubated for 1 h at room temperature with different concentrations of nitrotyrosine dissolved in PBS, pH 7.5 ($n = 4$). The antibody binding was visualized with the addition of ^{125}I -labeled donkey anti-rabbit IgG.

higher than the carboxyhemoglobin level in rats exposed to the standard model $66 \pm 3\%$ ($n = 8$, $P < 0.05$). This indicates that nitrotyrosine production was not due simply to an hypoxic stress precipitated by CO.

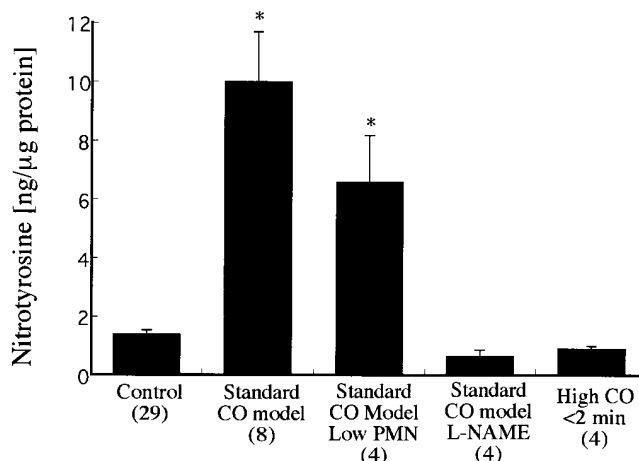


Figure 5. Nitrotyrosine concentration in rat brain homogenates. Rats were exposed to the standard CO model or to a bolus of CO to cause unconsciousness in < 2 min, as described in Methods. L-NAME (1 mg/kg i.p.) was injected 2 h before CO poisoning. Neutropenic rats had fewer than 200 polymorphonuclear leukocytes/ μl blood. (n) = number of rats; * significantly greater than control.

Effect of platelets on nitrotyrosine formation. Based on the immunocytochemical findings and the quantitative results summarized in Fig. 5, we hypothesized that a perivascular oxidative stress may be an early event during CO poisoning. Therefore, we examined whether exposure to CO at 1000 ppm, the first portion of the standard CO model, led to nitrotyrosine formation. We found the nitrotyrosine concentration to be significantly increased in brains of rats exposed to 1000 ppm CO for 40 min (Fig. 6). Moreover, when the number of circulating platelets was decreased to $15.6 \pm 8\%$ ($n = 6$) of the control level by injecting rats with anti-platelet antiserum (see Methods), no significant increase in nitrotyrosine was observed.

When the length of exposure to 1000 ppm CO was increased to 60 min, the nitrotyrosine concentration in brain was significantly higher than after 40 min, and the concentration of nitrotyrosine was actually higher in rats with low numbers of platelets (Fig. 6). Hence, mechanisms unrelated to platelet-derived oxidants must cause nitrotyrosine formation during the hour-long exposures. In this situation, platelets appear to antagonize the oxidative process; a phenomenon reported in several models of oxidative stress (24, 25). The concentration of nitrotyrosine was the same whether rats were exposed to 1000 ppm CO for 1 h or to the full, standard CO model which involved exposure to 1000 ppm for 40 min and then to 3000 ppm for 20 min (Fig. 5). Thrombocytopenic rats exposed to the standard CO model exhibited nearly the same increase in nitrotyrosine as did rats with normal platelet counts, 13.9 ± 6.0 ng nitrotyrosine/ μg protein ($n = 5$).

Role of platelets and nitric oxide in leukocyte sequestration. In previous studies we found that CO poisoning caused leukocyte sequestration in brain microvessels, and we measured this effect as an increase in myeloperoxidase activity (7). In a new series of studies (Table I) we found a sixfold increase in myeloperoxidase activity in microvessels from CO poisoned rats, and no significant difference in thrombocytopenic rats poisoned with CO. However, treatment with L-NAME (1 mg/kg i.p. 2 h before CO poisoning) inhibited leukocyte sequestration.

Role of nitric oxide in biochemical indices of CO-mediated brain oxidative stress. The results of previous studies indicate

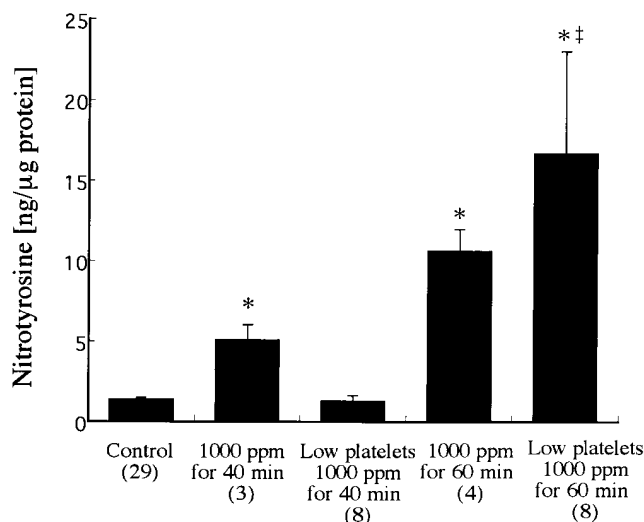


Figure 6. Nitrotyrosine concentration in rat brain homogenates. Rats were exposed to 1000 ppm CO for 40 min or 1 h. Circulating platelets were decreased by injecting anti-platelet antiserum 2 h before CO exposure. (n) = number of rats, **P* < 0.05 significantly greater than control ‡*P* < 0.01 significantly greater than value for rats exposed to 1000 ppm CO for 1 h.

that following their sequestration in the brain microvasculature, leukocytes become activated and cause endothelial xanthine dehydrogenase to be converted to xanthine oxidase (7). Xanthine oxidase activity is responsible for causing brain lipid peroxidation (7, 9). We were therefore interested in assessing the effect of L-NAME treatment on these indices, as it appeared effective in inhibiting leukocyte sequestration. As shown in Table II, L-NAME was effective in preventing CO-mediated biochemical effects. CO poisoning caused xanthine oxidase activity to increase to 51% of the total dehydrogenase plus oxidase activity, virtually all of the increase was due to a change in the sulfhydryl-irreversible form, and the brain conjugated diene level was increased 2.5-fold. The brains of rats treated with L-NAME did not show these changes.

Discussion

The results of this study provide information on early oxidative changes that occur in brain following CO poisoning. A burst of NO· was detected in brain at the termination of CO exposure when unconsciousness occurred. We have shown that unconsciousness is associated with cerebral hypoperfusion, and that blood flow is decreased to ~ 50% of control for several minutes (8, 10). It is likely that this vascular stress contributed to the production of NO·, as the concentration of NO· in brain has been found to increase in response to a reduction in blood pressure (20).

The immunohistochemical studies indicated that nitrotyrosine was deposited predominantly in the perivascular region. This suggested that the vascular endothelium and/or blood-borne elements may be responsible for generation of oxidants. We developed a solid phase immunoradiochemical assay to quantify nitrotyrosine in tissue using polyclonal anti-nitrotyrosine antibodies. Using this method we found a marked rise in nitrotyrosine in brains of rats exposed to CO. Neutrophils did not appear to be involved in nitrotyrosine formation, based

Table I. Myeloperoxidase Activity in Brain Microvessels from Rats Killed Immediately after CO Poisoning

Group	n	Enzyme Activity
Control	4	2.6±0.4
CO	5	13.0±3.3*
CO (Low plts)	3	11.7±2.3*
CO + L-NAME	5	2.3±0.7

Values are mean±SE. Myeloperoxidase activity was expressed as absorbance at 460 nm (A_{460}) × 1000/min per gram brain. Thrombocytopenia (low plts) was induced with anti-platelet antibodies as described in Methods. **P* < 0.05 relative to control, n = number of rats.

on the results with neutropenic rats. We investigated the involvement of neutrophils because these cells can generate both superoxide and nitric oxide (26, 27), and we have shown that neutrophils are involved with conversion of xanthine dehydrogenase to xanthine oxidase (7).

Based on findings with thrombocytopenic rats, platelets were one source of oxidants responsible for nitrotyrosine formation when animals were exposed to 1000 ppm CO for 40 min. However, there does not appear to be a large role for platelets in brain oxidative stress associated with the standard CO model. Nitrotyrosine levels in brain were not significantly different in thrombocytopenic rats poisoned with CO and leukocyte adherence to microvessels, which was assessed as myeloperoxidase activity, was not changed. Cells intrinsic to the brain, such as neurons and the microvascular endothelium, are possible alternative sources for oxidants that generate nitrotyrosine. However, it is intriguing to consider a possible role for platelets in CO exposures that do not result in overt brain injuries. Although controversial, some epidemiological studies have suggested that exposure to CO can accelerate atherosclerosis (28). Superoxide and peroxynitrite may be mediators of atherosclerosis, and nitrotyrosine deposits have been found in atherosclerotic fatty streaks (14, 29).

The data suggest that oxidative stress mediated by peroxynitrite, or other NO·-related oxidants, may be required for the cascade of events which lead to brain lipid peroxidation in CO poisoning. Oxidative stress is known to cause expression of adherence molecules for leukocytes on endothelial cells (30). It is also feasible that NO·-related oxidants may cause generation of other pro-adhesive substances, such as platelet activating factor (31). We hypothesize that NO·-related oxidative

Table II. Xanthine Dehydrogenase, Xanthine Oxidase, and Conjugated Diene Levels in Brain Homogenates from Rats Killed 90 min after CO Poisoning

Group	n	XD + XO	Total XO	Irreversible XO	CD
Control	6	0.95±0.17	0.21±0.05	0.14±0.04	2.4±0.6
CO	6	0.97±0.11	0.49±0.11*	0.38±0.05*	6.2±0.8*
CO + L-NAME	4	1.04±0.12	0.14±0.02	0.09±0.01	2.1±0.3

Values are mean±SE. Enzyme activity was expressed as mU/mg protein; conjugated diene level was expressed as μmol/mg lipid. XD, xanthine dehydrogenase; XO, xanthine oxidase; CD, conjugated diene. **P* < 0.05 relative to control, n = number of rats.

stress causes expression of adherence molecules on endothelial cells, and that leukocytes bind to these substances when cerebral blood flow decreases during exposure to 3000 ppm CO in the standard model. This hypothesis is supported by two observations. We have found that exposure to 1000 ppm CO for 40 min will generate perivascular nitrotyrosine, but brief exposures to high concentrations of CO will not cause significant formation of nitrotyrosine. In previous studies we found that when rats were exposed to CO in a pattern reversed from the standard model, a high concentration of CO to cause unconsciousness and then 1000 ppm CO for 40 min, conversion of xanthine dehydrogenase to xanthine oxidase and lipid peroxidation did not occur (8, 9). Hence, cerebral hypoperfusion occurred before the development of vascular oxidative changes. The second observation is based on work with L-NAME. Injections of L-NAME prevented perivascular nitrotyrosine deposition, as well as all subsequent steps linked with brain lipid peroxidation: leukocyte sequestration, conversion of xanthine dehydrogenase to xanthine oxidase, and lipid peroxidation. These results do not contradict reports which show that NO \cdot can inhibit leukocyte B $_2$ integrin adherence (32, 33). It is important to keep in mind the temporal pattern of events with CO poisoning. Vascular changes, such as nitrotyrosine deposition, precede leukocyte sequestration that initially does not appear to depend on B $_2$ integrins (7). Indeed, we have shown that the high flux of NO \cdot from platelets in the first 45 min after poisoning inhibits the function of leukocyte B $_2$ integrins (5).

Further work is necessary to elucidate the agents responsible for leukocyte adherence, and to clarify the consequences of brain lipid peroxidation. This work may lead to a better understanding of CO poisoning as a reperfusion-like injury, and greater insight into possible treatments. The presence of nitrotyrosine in the brain microvasculature is indicative of production of a NO \cdot -related oxidant, but it is not synonymous with peroxynitrite. Hence, the direct contribution of peroxynitrite to CO-related neuropathology remains unknown. It is interesting to note, however, that peroxynitrite can inhibit DOPA synthesis (34). Excessive neuronal production of an NO \cdot -derived oxidant appears responsible for the neurotoxicity associated with N-methyl-D-aspartate receptor activation (35), and inhibitors of receptor activation have been shown to decrease neurotoxicity in one model of CO poisoning (36).

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