Intravascular Neutrophil Activation Due to Carbon Monoxide Poisoning

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Rationale: We hypothesized that platelet–neutrophil interactions occur as a result of acute carbon monoxide (CO) poisoning, and subsequent neutrophil activation triggers events that cause neurologic sequelae.

Objectives: To identify platelet–neutrophil interactions and neutrophil activation in patients and in animal models, and to establish the association between these intravascular events and changes linked to CO-mediated neurologic sequelae in an animal model.

Measurements and Main Results: Blood was obtained from 50 consecutive patients. Abnormalities were variable depending on the carboxyhemoglobin level at study admission and duration of CO exposure. Platelet–neutrophil aggregates were detected and plasma myeloperoxidase (MPO) concentration was significantly elevated in those with confirmed CO poisoning. Among patients exposed to CO for over 3 h, flow cytometry scans of neutrophils revealed increased surface expression of CD18 and, in some groups, MPO on the cell surface. Animal models revealed consistent evidence of platelet–neutrophil aggregates, neutrophil activation and surface MPO, and plasma MPO elevation. MPO was deposited along the brain vascular lining and colocalized with nitrotyrosine. CO poisoning caused abnormalities in the charge pattern of myelin basic protein (MBP), changes linked to adaptive immunologic responses responsible for neurologic sequelae in this model. Changes did not occur in thrombocytopenic rats, those receiving tirofiban to inhibit platelet–neutrophil interactions, or those receiving L-nitroarginine methyl ester to inhibit nitric oxide synthesis. Alterations in MBP did not occur in CO-poisoned knockout mice lacking MPO.

Conclusions: Acute CO poisoning causes intravascular neutrophil activation due to interactions with platelets. MPO liberated by neutrophils mediates perivascular oxidative stress, which is linked to immune-mediated neurologic sequelae.

Keywords: myelin basic protein; myeloperoxidase; neuropathology

Carbon monoxide (CO) is the leading agent of injury and death by poisoning worldwide (1). An elevated carboxyhemoglobin (COHb) level can precipitate tissue hypoxia due to direct effects of CO on hemoglobin and because of impaired perfusion from cardiac dysfunction. High COHb levels impair mitochondrial electron transport because CO binds to cytochrome c oxidase. In brain, this has been shown to impair ATP synthesis and increase mitochondrial production of reactive oxygen species (2, 3). Energy production and mitochondrial function are restored after COHb levels decrease, but the transient changes can cause

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Pathophysiologic mechanisms for CO-mediated brain injury are known to extend beyond hypoxic stress due to carboxyhemoglobin, but they are poorly understood.

What This Study Adds to the Field

Platelet–neutrophil activation due to CO poisoning was shown to occur in patients and animal models and platelet–neutrophil aggregation with intravascular neutrophil degranulation was required for one form of CO-mediated brain injury in an animal model.

neuronal necrotic or apoptotic death (4–6). These processes are likely to be responsible for neurologic abnormalities that are manifested during initial hospitalization of some patients (7–9).

Neurologic dysfunction may occur in a delayed fashion. Impairments of concentration and learning, dementia, cog-wheel rigidity, amnesia, and/or depression develop in 23 to 76% of patients from several days to approximately 4 wk after poisoning (9–15). The pathophysiology for these sequelae remains unclear. COHb values correlate poorly with clinical outcomes, and even when CO poisoning appears to be relatively mild, delayed neurologic sequelae may still occur (1, 10–18). Therefore, additional pathophysiologic mechanisms beyond COHb-mediated hypoxia are believed to exist.

The goal of this investigation was to determine whether CO poisoning triggers physical interactions between circulating platelets and neutrophils, and whether this association initiates events that can be related to neuropathology. Consideration that CO may stimulate heterotypic (platelet–neutrophil) aggregation was fueled by observations that CO increases the flux of nitric oxide ($\cdot$NO) released from platelets, apparently because CO and $\cdot$NO compete for hemoprotein targets (19). When stimulated platelets synthesize $\cdot$NO or when platelets are artificially loaded with $\cdot$NO-donating compounds, platelets and neutrophils aggregate (20). This process is believed to be related to production of reactive $\cdot$NO-associated species, such as peroxynitrite, due to neutrophil-derived superoxide reacting with $\cdot$NO produced by platelets. Platelet adhesion molecules can be activated by direct addition of peroxynitrite to platelet suspensions (21). Once a physical linkage between platelets and neutrophils is established, neutrophils exhibit a marked increase in oxidative burst and synthesis of additional reactive $\cdot$NO-derived species (20). Physical associations between neutrophils and platelets can also precipitate neutrophil degranulation (22). We hypothesized that these relatively complex platelet–neutrophil interactions are triggered by CO exposure, and that intravascular neutrophil degranulation will cause additional perivascular changes that mediate neuropathology.

CO can inhibit homotypic (platelet–platelet) adhesion. At concentrations of 10 to 600 μM, CO inhibits aggregation of platelets in purified suspensions through pathways involving cyclic GMP, impaired calcium entry, and inhibition of cytochrome P-450 (23–25). The effect of CO on heterotypic adhesion between platelets and leukocytes has not been investigated in depth. Cigarette smoke, although clearly a more complex stimulus than pure CO, was reported to induce aggregates between leukocytes and platelets in hamsters (26). In experimental settings where homotypic adhesion is inhibited, leukocyte–platelet aggregation is accelerated when platelets are activated (27).

In this article, we show that exposure to CO triggers intravascular platelet–neutrophil interactions that lead to neutrophil degranulation in both experimental animals and patients suffering acute CO poisoning. In the animal model, we show that the myeloperoxidase (MPO) concentration in brain increases due to CO poisoning, MPO is deposited along the vascular lining, and it appears to cause vascular oxidative stress based on colocalization with nitrotyrosine. MPO can catalyze the reaction between nitrite and H_2O_2 to form nitrogen dioxide ($\cdot NO_2$), which will nitrate local protein tyrosine residues, cause lipid peroxidation, and stimulate expression of endothelial adhesion molecules (28–31). Colocalization of MPO and nitrotyrosine along the subendothel-ial lining has been found in human tissues after several inflammatory diseases (32).

Recently, we demonstrated that acute CO poisoning triggers an immunologic response that causes functional neurologic deficits (33). Adduct formation between products of lipid peroxidation and myelin basic protein (MBP) causes alterations in the three-dimensional structure and charge pattern of MBP, which initiate an adaptive immunologic response. In this article, we show that neutrophils and platelets are required for CO-mediated charge alterations in MBP. We have directly linked the alterations in MBP with MPO by examining responses in knockout mice lacking MPO. From these data, we conclude that platelet–neutrophil interactions and neutrophil degranulation are major components of CO toxicity.

METHODS

Patients

Analyses were performed on citrate-anticoagulated blood samples obtained from 50 consecutive patients undergoing evaluation for suspected acute CO poisoning. A total of 27 females and 23 males with an average age of 38.5 ± 2.9 (SE) yr (range, 22 mo to 86 yr) were evaluated. These were individuals suspected of suffering CO poisoning either due to faulty home-heating systems, internal combustion engines, or smoke
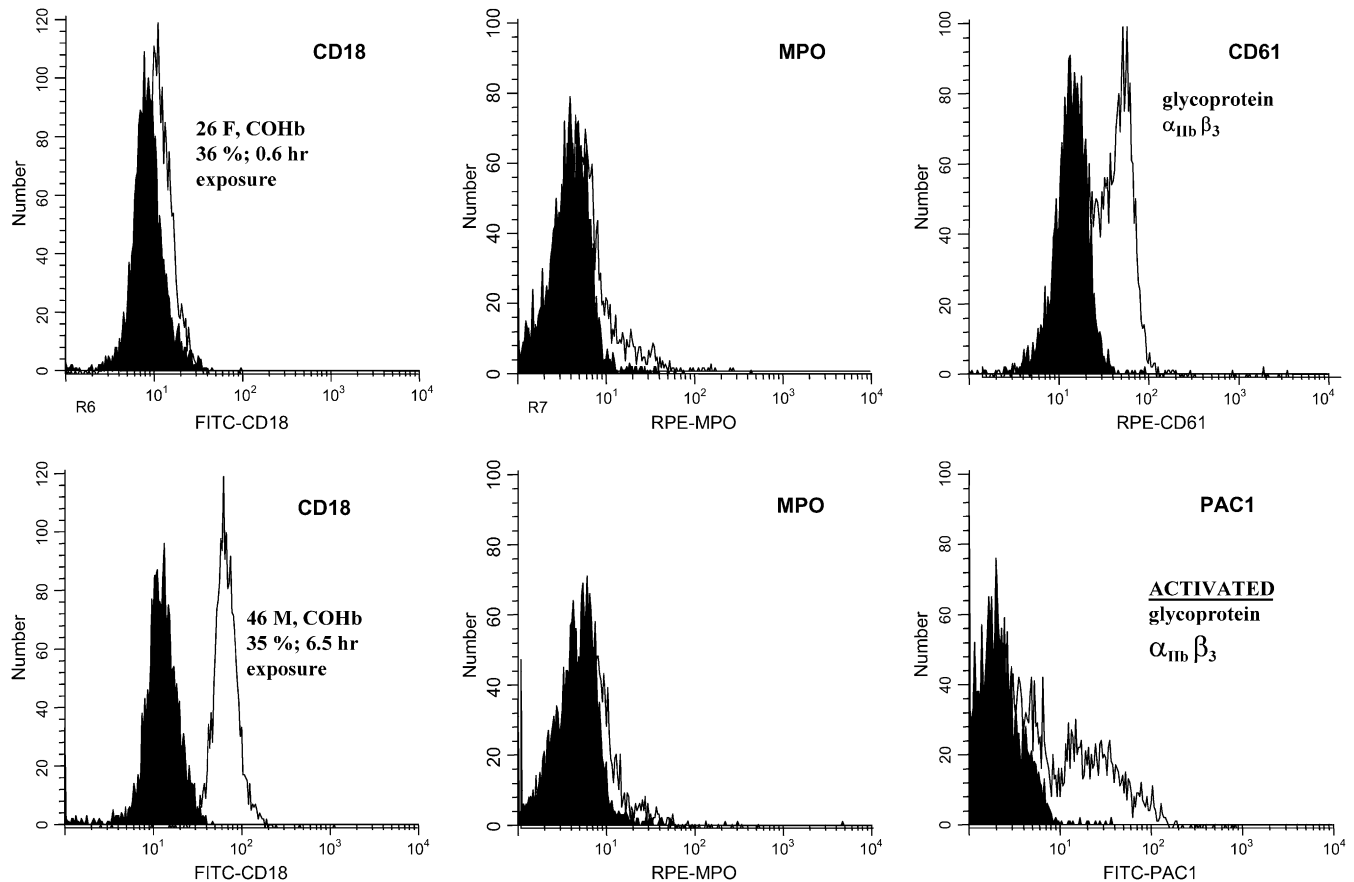

Figure 1. Representative neutrophil histograms for human control subjects and CO-poisoned patients. The *top three panels* show results for cells from a control person (*black curves*) and results for cells obtained from a 26-yr-old woman who presented to the emergency department after exposure for approximately 0.6 h to smoke in a house fire. Her presenting COHb level was 36%, and she suffered no loss of consciousness. A small shift in surface expression of CD18 and MPO was observed, and there was a moderate presence of platelets attached to neutrophils based on neutrophil surface expression of CD61, the β_3 component of the platelet glycoprotein $\alpha_{IIb}\beta_3$ integrin. The *lower three panels* show results for a different control person from the one in the *upper panels*, and surface expression for neutrophils from a 46-yr-old man exposed to CO from a faulty home heater for approximately 6.5 h. His COHb level on admission was 35% and he had suffered transient loss of consciousness. His neutrophils exhibited a substantial increase in surface CD18 expression, mild presence of myeloperoxidase (MPO), and activated platelet glycoprotein $\alpha_{IIb}\beta_3$ integrin. FITC = fluorescein isothiocyanate; RPE = R-phycoerythrin.

from house fires. Patients were considered to have been exposed to environmental CO if they had a COHb level above 1% and they did not smoke or above 10% if they smoked cigarettes. Table E1 of the online supplement outlines details for these individuals. Among the group, CO exposure was confirmed in all but eight patients.

Animals and Reagents

Wistar male rats were obtained from Harlan Laboratories (Indianapolis, IN). House mice (*Mus musculus*) of the C57B6J strain were either wild type or lacked a functional MPO gene (MPO$^{-/-}$). Wild-type mice were purchased from Jackson Laboratories (Bar Harbor, ME) and knockout mice were a generous gift from Dr. Charles Jennette, University of North Carolina at Chapel Hill.

Animal Manipulations

CO poisoning was performed for 1 h according to published protocol in a 7-L Plexiglas chamber (34). Neutropenia and thrombocytopenia were achieved by injecting antiserum as previously described (35). Some rats were injected intraperitoneally with 40 mg/kg L-nitroarginine methyl ester (L-NAME) 30 min before CO poisoning, or with 125 µg tirofiban intravenously plus 500 µg tirofiban intraperitoneally immediately before CO poisoning. At 90 min after CO poisoning, animals were anesthetized, blood was obtained by cardiac puncture into heparinized syringes, and brains perfused with phosphate-buffered saline (PBS) containing 100 U/ml heparin until the effluent was clear.

Tissue Preparation for Chemical Assays

Brain tissue was processed for Western blotting and for column chromatography as described previously (33, 36).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections after perfusion/fixation of anesthetized rats following published methods (33, 35–37).

Analysis of Blood

Within 5 min after obtaining blood from rats and mice, or 30 min for humans, 0.5 ml was combined with an equal volume of 0.5% paraformaldehyde in PBS, and stored at 4°C until being stained with antibodies for flow cytometry. Remaining blood was centrifuged at $250 \times g$ for 10 min and supernatant plasma was frozen at –20°C. Plasma MPO concentration for rats and the first eight patients was assessed by Western blots. Thereafter, plasma from patients was analyzed using a commercial MPO ELISA. MPO content of rat neutrophils was assessed by lysing cells and measuring MPO activity as the elevation in light absorption at 460 nm/min/1 $\times 10^6$ neutrophils in solutions containing ortho-diansidine following published methods (37).

Flow Cytometry

Formalin-fixed blood samples from rats, mice, and humans were processed in a similar fashion following the method of Li and colleagues (38) with minor modifications. Blood samples were incubated with saturating concentrations of antibodies for 30 min at room temperature, and with a cell-permeable anthraquinone, DRAQ5, to stain cell DNA. Neutrophils were identified by DRAQ5 staining, forward and side laser light scattering (*see* the online supplement). Studies were always coupled with analyses of control cells, either cells from air-breathing animals or from a normal healthy human volunteer.

Statistics

Statistical significance was determined by analysis of variance followed by the Dunn's test (SigmaSTAT; Systat Inc., Richmond, CA).

RESULTS

Intravascular Neutrophil Activation Occurs in Humans with CO Poisoning

Blood samples were obtained from 50 consecutive patients evaluated for suspected acute CO poisoning. Over the course of the trial, the number and type of studies were expanded as more

was learned from the animal models. Therefore, not all tests were performed on blood samples obtained from every patient.

Expression of the CD18 epitope of β_2 integrins was assessed by flow cytometry as a measure of neutrophil activation on all blood samples. Several examples of flow cytometric results are shown in Figure 1. The pattern and magnitude of CO exposure varied among the patients, and when CD18 expression level was plotted versus either the admission COHb level or an estimated exposure index calculated as COHb multiplied by duration of
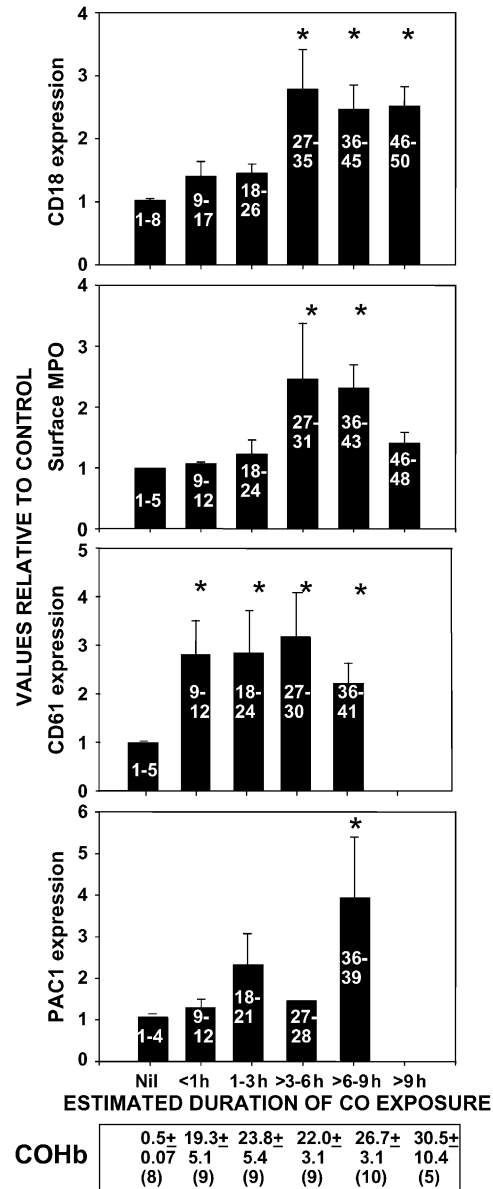

Figure 2. Expression of selected proteins on neutrophils from suspected CO-exposed patients relative to control cells run on the same day. The "nil" group indicates results using neutrophils from patients in whom CO poisoning was not confirmed. Other results are categorized according to the estimated duration of CO poisoning that a patient suffered. Surface CD18 was assessed using cells from all 50 consecutive patients. MPO was measured on the surface of neutrophils from 32 patients, CD61 on cells from 25 patients, and PAC1 on cells from 17 patients. Data are mean ± SE; *p < 0.05, analysis of variance (ANOVA). The numbers within each *bar* refer to individual patients described in Table E1. The mean COHb (± SE) for each group (n = total number of patients in each group) is shown at the *bottom* of the figure.

TABLE 1. PLASMA MYELOPEROXIDASE VALUES IN PATIENTS

	Nil	< 1 h	1–3 h	> 3–6 h	> 6–9 h	> 9 h
MPO, ng/ml	15.0 ± 2.0	117.2 ± 65.4	86.7 ± 38.8	23.5 ± 6.2	57.7 ± 22.5	$119.4 \pm 16.4*$
Patient numbers	1–8	9–17	18–26	27–35	36–45	46–50

Definition of abbreviation: MPO = myeloperoxidase.

Values are mean $\pm$ SE. Patient numbers refer to the list of patients in Table E1.

* $p < 0.05$, analysis of variance.

CO exposure, only a poor correlation was noted (data not shown). When samples were grouped and analyzed simply according to the duration of exposure, a significant elevation in CD18 expression was noted for patients exposed to CO for longer than 3 h (Figure 2). The COHb for patients was assessed as part of standard clinical evaluations using blood obtained on admission to the emergency department. The mean values for each group are listed at the bottom of Figure 2 and individual values for each patient are shown in Table E1.

When neutrophils degranulate, some of the liberated MPO can adhere to the cell surface. Subtle elevations of surface-bound MPO were present in many samples, as shown in Figure 1. A significant elevation was identified in two groups (Figure 2). Neutrophil–platelet aggregates were assessed in the neutrophil gate as surface expression of CD61, a component of the platelet glycoprotein $\alpha_{IIb}\beta_3$ integrin. An elevation in platelet–neutrophil aggregates was evident and it varied little among the samples, no matter how short the estimated CO exposures or the magnitude of COHb at admission (Figure 2). We also looked for the presence of an epitope on the surface of neutrophils that is recognized by the PAC1 antibody. PAC1 binds only the activated form of platelet glycoprotein $\alpha_{IIb}\beta_3$ integrin. Figure 1 shows there was negligible presence of PAC1 binding on cells from a control person, but substantial elevation on cells from a patient who experienced CO poisoning estimated to span 6.5 h. A significant elevation of PAC1 was observed on neutrophils from patients exposed to CO for over 6 h (Figure 2).

Plasma MPO concentration was elevated fivefold in CO-poisoned patients. The mean concentration was 75.7 ± 17.9 (SE; n = 42, p < 0.05, t test) versus 15.0 ± 2.0 (n = 10) ng/ml in control subjects and those in the "nil" group. When analyzed across groups, the plasma MPO value was highly variable, and reached statistical significance in only the group exposed to CO for over 9 h (Table 1).

Intravascular Neutrophil Activation Occurs in Rats Due to CO Poisoning

Animal studies were conducted concurrent with the clinical investigations. Flow cytometric evaluations were performed on neutrophils obtained from air-breathing and CO-poisoned rats. Typical histograms are shown in Figure 3. Elevations in expression of CD18, MPO, and surface-bound platelets occurred after CO poisoning (Figure 4). Neutrophils were also studied from rats that had been rendered thrombocytopenic or treated before CO poisoning with the nitric oxide synthase (NOS) inhibitor L-NAME or tirofiban to block platelet $\alpha_{IIb}\beta_3$ integrin–dependent adhesion. These interventions inhibited the CO-mediated increases in expression of CD18 and MPO and neutrophil–platelet aggregation (Figure 4).

Evidence that neutrophils had degranulated in the bloodstream was assessed by measuring MPO activity in cells. MPO activity was 0.85 ± 0.08 ($A_{460\,nm}$/min/1×10^6 neutrophils, SE; n = 5) for control cells and 0.52 ± 0.04 (SE; n = 5; p < 0.05, t test) for cells taken from CO-poisoned rats. For interpretation of these findings, it is important to know that CO does not impede MPO activity (19).

Plasma MPO concentration was elevated in CO-poisoned rats but not in those rendered thrombocytopenic or those pretreated with tirofiban or L-NAME. Figure 5 shows a typical Western blot and the quantitative changes in band density using the heavy chain subunit (60 kD). Results were very similar when light chain (12 kD) band densities were analyzed (data not shown).

MPO Elevations in Rat Brains

Western blots were performed on brain homogenates from control and CO-poisoned rats. Only the brains from untreated CO-poisoned rats exhibited a significant elevation in MPO subunit band density, with a relative elevation above control of $2.54 \pm$
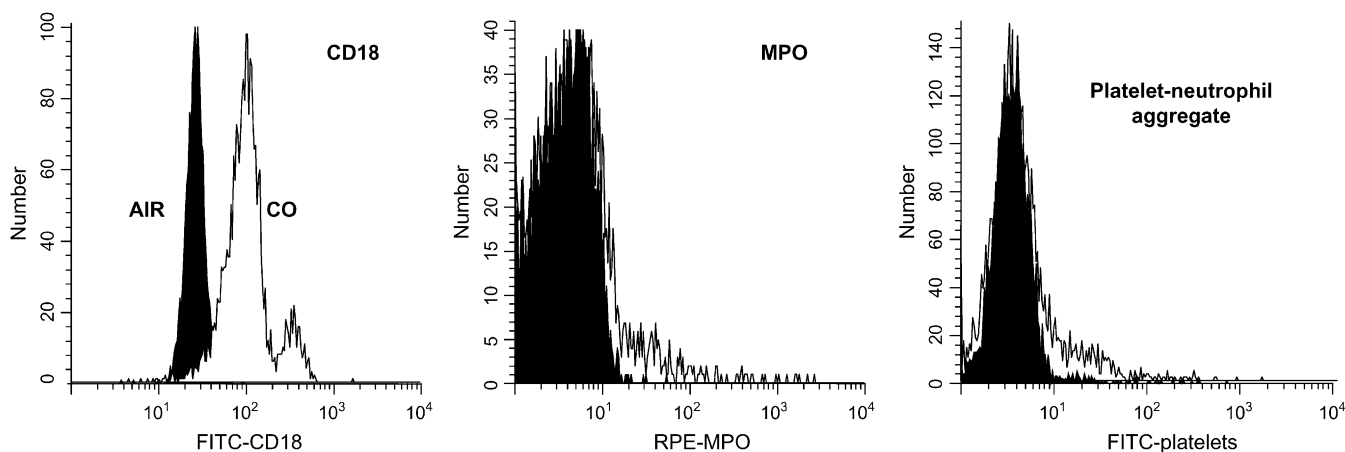

Figure 3. Representative histograms for expression of markers on the surface of rat neutrophils. *Panels* show results for cells from a control rat (*black curves*) and results for cells obtained from a rat poisoned with CO and killed 90 min later. After CO poisoning, the cells exhibited increases in surface expression of CD18 and MPO, and a mild elevation in platelets adherent to the cell surface.
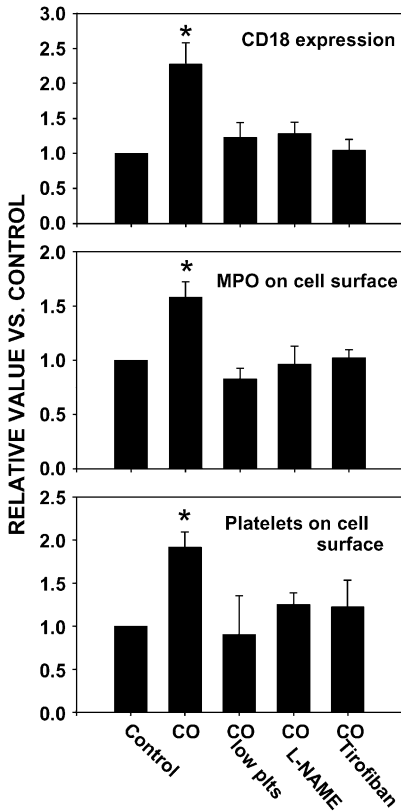
Figure 4 content (left side graphs):

RELATIVE VALUE VS. CONTROL

CD18 expression

MPO on cell surface

Platelets on cell surface

Control CO CO low plts CO L-NAME CO Tirofiban

Figure 4. Relative surface CD18 expression, surface MPO presence, and platelet–neutrophil aggregates for rat neutrophils. Data were obtained for cells from control rats, CO-poisoned untreated rats, those first injected with antiserum to render them thrombocytopenic, rats injected with L-nitroarginine methylester (L-NAME), or rats injected with tirofiban prior to CO poisoning (n = 4 rats in each group). Data are mean ± SE; *p < 0.05, ANOVA. plts = platelets.

	Control	CO	CO low platelets	L-NAME +CO	Tirofiban +CO
MPO						60 kDa
CD66						150 kDa
MPO/CD66	1.0±0.02	1.4±0.08*	1.0±0.10	1.1±0.07	0.9±0.07

Figure 6. Content of MPO and CD66 protein in brain homogenates from control and CO-poisoned rats. Data were obtained from control rats, untreated CO-poisoned rats, those first injected with antiserum to render them thrombocytopenic, or rats injected with L-NAME or tirofiban prior to CO poisoning. Western blots were prepared and probed with antibody to MPO and to the structural neutrophil CD66 protein (n = 4 rats in each group). Data are the ratio of band density (MPO/CD66), mean ± SE; *p < 0.05, ANOVA.

Immediately after CO poisoning, there was sparse evidence for *bona fide* neutrophil adherence but, by 90 min after CO poisoning, there was an extensive presence of neutrophils along the endothelial lining. This finding is consistent with previous studies (35, 40).

Western blots of brain homogenates were also analyzed for the three isoforms of NOS. There were no significant differences between brains from control rats and rats killed 90 min after CO poisoning (Table E2).

MBP Changes Due to CO

The chromatography elution pattern of brain MBP demonstrated the loss of multiple peaks after CO poisoning, as we have reported in previous trials (33, 36) (Figure 8). These changes did not occur in neutropenic or thrombocytopenic rats, or those treated with L-NAME or tirofiban.

Mouse CO Poisoning

Based on flow cytometry analyses, circulating neutrophils from wild-type CO-poisoned mice exhibited 1.84 ± 0.22 (SE; n = 6; p < 0.05, *t* test)–fold greater CD18 expression than did neutrophils from air-breathing wild-type mice. There was a 1.33 ± 0.11 (SE; n = 6; p < 0.05, *t* test)–fold greater expression of MPO present on the surface of neutrophils from CO-poisoned mice.

The cation-exchange chromatography elution pattern for MBP was markedly altered in wild-type mice due to CO poisoning

0.17 (SE; n = 4; p < 0.05) (Figure 6). To discern whether MPO elevations were due to neutrophils adherent to brain tissue versus from MPO that may have been released in the bloodstream and then deposited on brain vasculature, blots were also probed for CD66, which is a glycoprotein present in neutrophils and epithelial cells (39). CD66 band density was significantly increased in brains from the untreated CO-poisoned rats (relative elevation, 1.81 ± 0.33 [SE; p < 0.05], above control), but the MPO/CD66 ratio was also significantly elevated (*see* Figure 6).

Immunohistochemical Studies of Brain

Colocalization between perivascular MPO and nitrotyrosine could be clearly discerned in fluorescence micrographs (Figure 7).
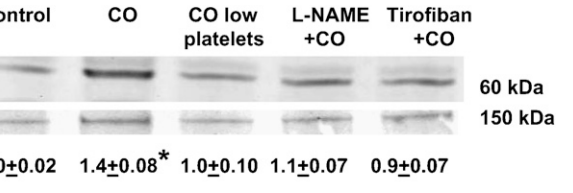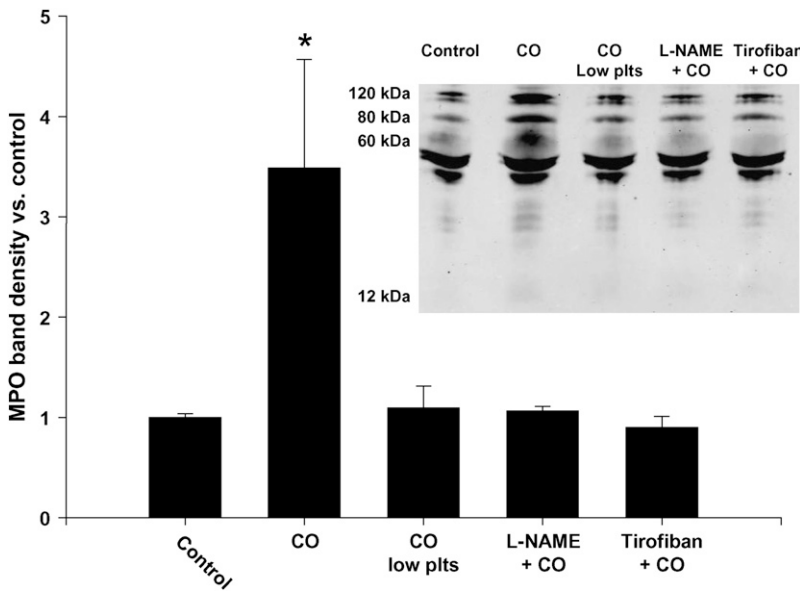

Figure 5. Plasma MPO content in rats. Data were obtained from control rats, untreated CO-poisoned rats, those first injected with antiserum to render them thrombocytopenic, or rats injected with L-NAME or tirofiban prior to CO poisoning. The figure shows a typical Western blot and quantitative evaluations of the 60-kD band density using 50 μg of plasma protein from rats (n = 4 rats in each group). Data are mean ± SE; *p < 0.05, ANOVA.
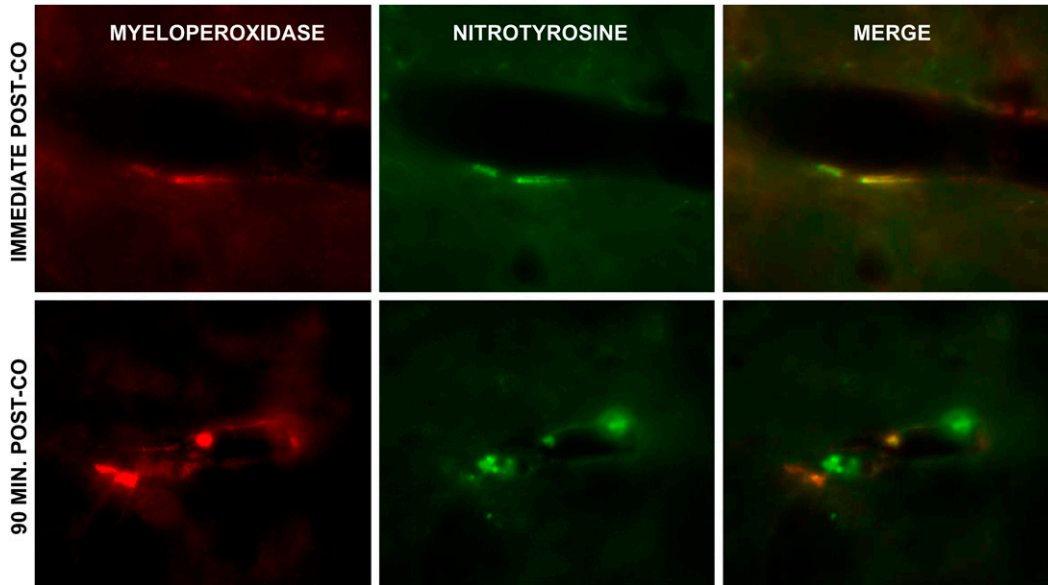
Figure 7. Immunohistochemical images of brain sections from rats killed immediately or at 90 min after CO poisoning. Dual staining was performed with an anti-MPO antibody counterstained with Cy3 and an anti–nitrotyrosine antibody counterstained with FITC. Image processing allowed both stains to be overlaid and *yellow* demonstrated exact colocalization of staining.

(Figure 9). Although the pattern in air-breathing MPO-knockout mice was slightly different from that obtained from wild-type mice, the MBP pattern was unchanged due to CO poisoning.

DISCUSSION

Results from this investigation indicate that CO exposure triggers platelet–neutrophil aggregation and neutrophil activation in the bloodstream. A role for platelets in neutrophil activation due to CO poisoning is supported by findings that neutrophil activation was significantly reduced in thrombocytopenic rats and also those treated with tirofiban, a platelet glycoprotein $\alpha_{IIb}\beta_3$ inhibitor. Detection of activated glycoprotein $\alpha_{IIb}\beta_3$ on human neutrophils by the PAC1 antibody further supports involvement of platelets with neutrophil activation. Platelet–neutrophil aggregates were found in all groups of patients, even those exposed to CO for less than 1 h. Because neutrophil activation (CD18 expression) was only significantly increased among patients exposed to CO for over 3 h, we conclude that platelet–neutrophil aggregation precedes neutrophil activation.

The mechanism for platelet–neutrophil interactions is likely to be complex. The inhibitory effect of L-NAME in our trials indicates that $\cdot$NO synthesis is involved, and $\cdot$NO-derived oxidants are known to activate platelets (20, 21). In response to a
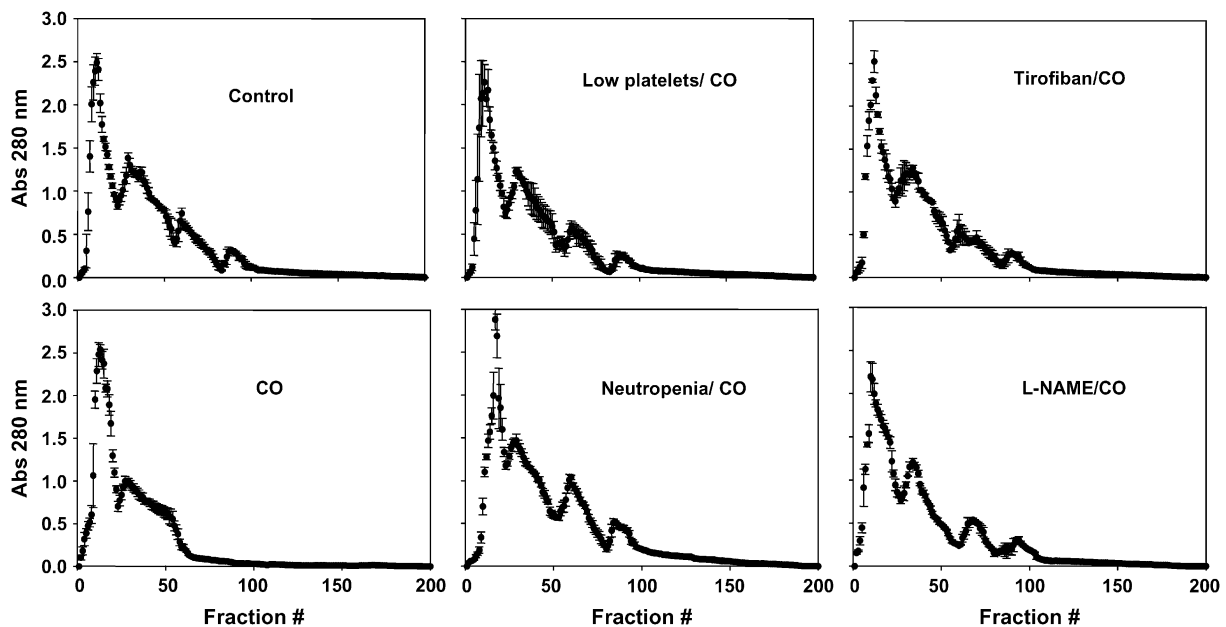

Figure 8. Column chromatography analysis. *Panels* show the elution patterns of myelin basic protein from cation exchange chromatography columns using acid-soluble brain material obtained from control rats, rats killed 90 min after CO poisoning, and CO-poisoned rats first injected with antiserum to render them thrombocytopenic or neutropenic, or rats injected with L-NAME or tirofiban. Higher fraction numbers indicate more positively charged proteins. *Data points* are mean $\pm$ SE; n = 4 rats in each group.
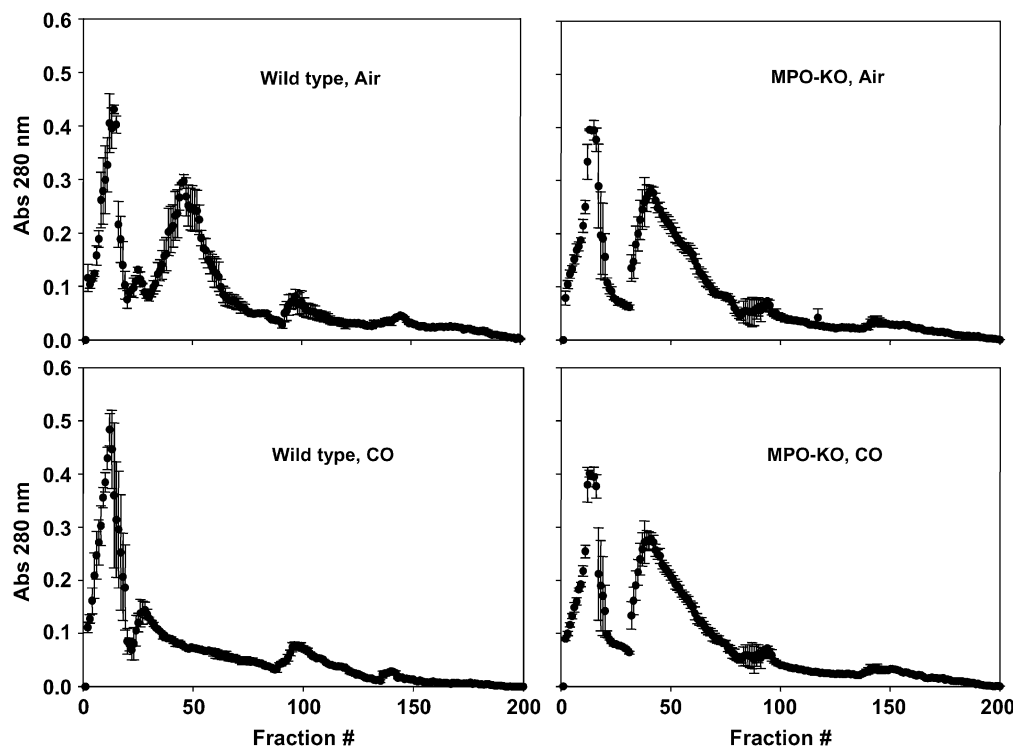
Figure 9. Column chromatography analysis of acid-soluble material from mouse brain. *Panels* show the protein elution patterns for wild-type and MPO-knockout (MPO-KO) mice breathing air (control) and at 90 min after CO poisoning. *Data points* are mean $\pm$ SE; n = 3 mice in each group.

variety of insults, intravascular platelet–neutrophil interactions are mediated initially by platelet P-selectin binding to neutrophil PSGL-1 ligand, and then secondarily by a fibrinogen bridge between platelet glycoprotein $\alpha_{IIb}\beta_3$ and neutrophil β_2 integrins (27, 41–43). Our observation that tirofiban inhibits platelet–neutrophil aggregation suggests that a dynamic interaction occurs whereby initial contacts involving P-selectin lead to activation of platelet glycoprotein $\alpha_{IIb}\beta_3$. Such a process could be mediated by one or more products from closely adherent neutrophils. Chlopicki and colleagues (44) have shown that, when platelets are stimulated by a variety of physiologic stimuli, platelet–neutrophil adhesion involves an initial association mediated by P-selectin, as well as glycoprotein $\alpha_{IIb}\beta_3$, platelet activating factor, and neutrophil β_2 integrins. Platelet–neutrophil aggregates can also be formed by linkages between β_2 integrins and platelet glycoprotein 1α or junctional adhesion molecule-3 (45, 46).

The plasma MPO concentration was variably elevated in CO-poisoned patients. This is likely to be related to the magnitude of neutrophil activation and also clearance of MPO from the circulation. In response to mild stimuli, such as vigorous exercise, plasma MPO can be elevated for as long as 1 h (47). Plasma levels remain elevated for 1 to 5 d after major stresses such as cardiopulmonary bypass (48). Plasma MPO was elevated significantly in patients and animals, indicating that CO poisoning triggers intravascular neutrophil degranulation. In the rat model, approximately 40% of intracellular neutrophil MPO is liberated. Based on flow cytometry results in both animals and humans, some of the released MPO adheres to the surface of neutrophils. This may cause still more neutrophil activation because when MPO binds to β_2 integrins it causes "outside-in" neutrophil activation (49). A significant elevation in surface MPO was not observed in patients exposed to CO for over 9 h. Cells from only three patients were analyzed, so it is unknown whether this reflects an aspect of pathophysiology or merely statistical variability.

Previous studies have demonstrated that CO poisoning markedly elevates nitrotyrosine along vascular walls in brain (35, 37).

We found that the MPO concentration is increased in brains of CO-poisoned animals. Immunohistochemical investigations indicate that MPO colocalizes with nitrotyrosine, suggesting a causal relationship. We hypothesize that perivascular oxidative stress from MPO may trigger endothelial cell activation and adhesion molecule expression. By 90 min after CO poisoning, neutrophils are firmly adherent to the vascular wall (40). This was documented in the current trial by immunohistochemical images and CD66 elevation on Western blots.

We have shown that neurologic sequelae in the animal model result from an adaptive immunologic response to chemically altered MBP (33, 36). The intravascular events described in this study can be related to chemical changes in MBP because the charge pattern of MBP was preserved if CO-poisoned rats were neutropenic, thrombocytopenic, or treated with L-NAME or tirofiban. MPO is directly involved with brain oxidative stress because alterations in the MBP charge pattern were not observed in CO-poisoned MPO-knockout mice.

N-methyl D-aspartate (NMDA) neurons and neuronal NOS activity are required for development of neurologic sequelae in our animal model (50). We hypothesize that the $^{\cdot}$NO synthesized in brain in response to CO poisoning leads to higher nitrite levels and that the perivascular MPO focuses oxidative stress at the vascular lining. This may be the reason why the brain, as opposed to other organs in the body, appears particularly sensitive to CO poisoning. We have shown that thrombocytopenic and neutropenic rats exhibit lower NMDA neuronal activation due to CO poisoning (50). This suggests that early intravascular events (possibly perivascular deposition of MPO) are involved with neuronal activation, and this may give rise to a feedback loop of progressive free-radical production. Figure 10 illustrates the events that we believe occur and give rise to immune-mediated neurologic sequelae.

Our results do not negate the pathophysiologic importance of hypoxic stress from CO, but they offer a possible explanation for some quizzical observations. Clinical imaging studies have shown that CO poisoning causes vascular injuries in brain.
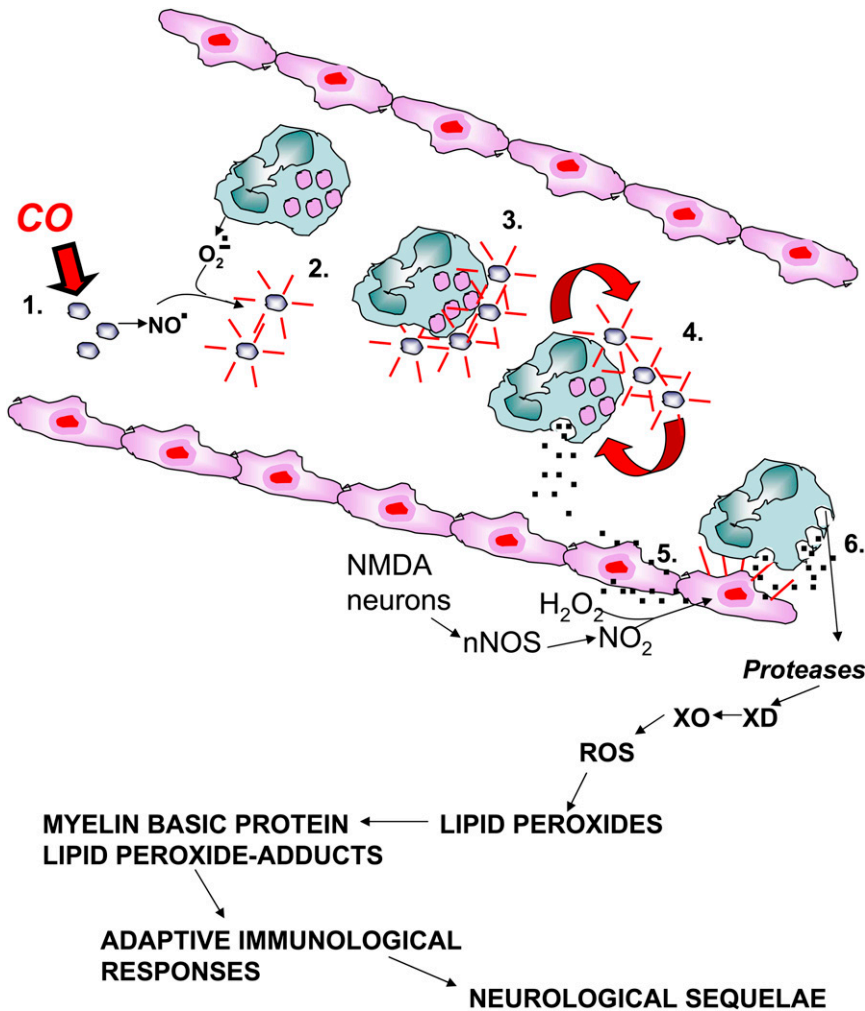
Figure 10. Schematic illustrating the proposed mechanism for immune-mediated neurologic sequelae. Events numbered *1* through *6* are described in the current study. Additional elements are based on results from published studies (20, 34–36, 38, 41, 51). *1*: CO binds to platelet hemoproteins and the competition with intraplatelet ·NO increases ·NO efflux (first reported in Reference 20). *2*: Platelet-derived ·NO reacts with neutrophil-derived superoxide (O_2^-), giving rise to reactive species that activate platelets and cause platelet–neutrophil aggregates (*Step 3*). *4*: Ongoing interactions involving reactive products and adhesion molecules cause firm aggregation and stimulate intravascular neutrophil degranulation. *5*: MPO is deposited along the vascular lining and some is transcytosed to the subendothelial matrix, where it reacts with nitrite generated by N-methyl D-aspartate (NMDA) neurons. Products from MPO-mediated reactions cause endothelial cell activation, facilitating firm neutrophil adhesion and further degranulation. *6*: Neutrophil-derived proteases react with endothelial cell xanthine dehydrogenase (XD), converting it to xanthine oxidase (XO). Reactive oxygen species (ROS) initiate lipid peroxidation and adducts interact with brain myelin basic protein. The altered myelin basic protein triggers an adaptive immunologic response that causes neurologic dysfunction. nNOS = neuronal nitric oxide synthase.

Atypical coupling between cerebral blood flow and neuronal O_2 demand and other acute vascular abnormalities have been found in CO victims (51–54). Our findings offer a possible pathophysiologic basis for these observations. Furthermore, recent clinical reports have described a high incidence of cardiac injuries in CO-poisoning patients with normal coronary arteries (55, 56). Platelet–neutrophil interactions may be the basis for many of the CO-mediated cardiac injures. Platelet–neutrophil interactions, reduced neutrophil MPO index (MPO/cell), and elevated intravascular MPO are linked to a heightened risk for acute coronary syndromes (57–59).

In this trial, we were not able to discretely assess whether intravascular neutrophil activation and degranulation were correlated with brain injury in patients. We performed telephone follow-up on all patients, but we did not perform detailed neuropsychologic examinations that are likely to be required to detect some forms of dysfunction (12–14). Only one patient reported difficulty with thinking and activities of daily living when contacted 6 wk after poisoning (*see* Table E1). We must note, however, that many patients in this investigation received hyperbaric oxygen therapy, which can reduce the incidence of some neurologic sequelae (10, 13, 14, 51). We have shown that hyperbaric oxygen inhibits neutrophil β_2 integrin adhesion in both the rat model and humans (60, 61). Recently, we demonstrated in the rat model that hyperbaric oxygen treatment inhibits the CO-induced structural alterations in MBP as well as the associated inflammatory response and learning abnormalities

(36). A prospective clinical study to correlate intravascular alterations with neurologic outcome is planned.

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