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Carbon monoxide inhalation increases microparticles causing vascular and CNS dysfunction

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Abstract

We hypothesized that circulating microparticles (MPs) play a role in pro-inflammatory effects associated with carbon monoxide (CO) inhalation. Mice exposed for 1 h to 100 ppm CO or more exhibit increases in circulating MPs derived from a variety of vascular cells as well as neutrophil activation. Tissue injury was quantified as 2000 kDa dextran leakage from vessels and as neutrophil sequestration in the brain and skeletal muscle; and central nervous system nerve dysfunction was documented as broadening of the neurohypophysial action potential (AP). Indices of injury occurred following exposures to 1000 ppm for 1 h or to 1000 ppm for 40 min followed by 3000 ppm for 20 min. MPs were implicated in causing injuries because infusing the surfactant MP lytic agent, polyethylene glycol telomere B (PEGtB) abrogated elevations in MPs, vascular leak, neutrophil sequestration and AP prolongation. These manifestations of tissue injury also did not occur in mice lacking myeloperoxidase. Vascular leakage and AP prolongation were produced in naïve mice infused with MPs that had been obtained from CO poisoned mice, but this did not occur with MPs obtained from control mice. We conclude that CO poisoning triggers elevations of MPs that activate neutrophils which subsequently cause tissue injuries.

Keywords

Neutrophil activation; Vascular leak; Neurohypophysis; Action potential; Leukocytes; Platelets

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Introduction

This investigation centered on our hypothesis that circulating microparticles (MPs) play a role in the pro-inflammatory effects of carbon monoxide (CO). Few chemical agents cause as much human suffering as does CO. Elevations in environmental CO concentrations contribute to daily variations in hospital admissions and aside from these patients, nearly 50,000 individuals/year are treated for acute CO poisoning in the USA (Cook et al., 1995; Hampson, 1998; Moolenaar et al., 1995; Raub et al., 2000). Mortality and morbidity risks of CO poisoning are related to cardiac and neurological injuries (Choi, 1983; Ginsberg et al., 1974; Mathieu et al., 1996; Raphael et al., 1989; Scheinkestel et al., 1999; Thom et al., 1995; Weaver et al., 2002).

Pathophysiological mechanisms of CO extend beyond the well recognized hypoxic stress from carboxyhemoglobin (COHb) and current knowledge cannot explain the breadth of injuries (Agency E.P., 2010). CO toxicity includes hypoxic and oxidative stresses and long-term neurological morbidity involves an immunological response to modified proteins (Brown et al., 1990; Iheagwara et al., 2007; Ischiropoulos et al., 1996; Piantadosi, 2008; Thom, 1990, 1992; Thom et al., 1995, 1999a, 2004, 2006). Patients briefly exposed even to low concentrations of CO can exhibit intravascular neutrophil activation and elevations in circulating inflammatory proteins (Thom et al., 2006, 2010). One pathway for CO-mediated intravascular neutrophil activation involves interactions with platelet membranes by unclear mechanisms (Thom et al., 2006).

Blood-borne MPs are 0.1 to 1 μm diameter membrane vesicles shed from the surface of cells by what appear to be well regulated processes (Enjeti et al., 2007). MPs are characterized by their expression of antigenic markers from parent cells. As MPs bud from these cells negatively charged phosphatidylserine residues are exposed, which often leads to secondary binding of annexin V. MPs have been implicated to play roles in a number of pathological processes (Enjeti et al., 2008; Hugel et al., 2005; Sanborn et al., 2012; Thom et al., 2011). MPs can directly stimulate release of pro-inflammatory cytokines and platelet-derived MPs stimulate leukocyte activation and aggregation (Jy et al., 1995; Lo et al., 2006; Mesri and Altieri, 1999). Annexin V-positive platelet MPs exhibit pro-coagulant activity (Connor et al., 2010).

There are no studies showing that CO alters circulating MPs, but both cigarette smoke and air pollution have been reported to increase endothelial and platelet-derived MPs (Emmerichs et al., 2011; Frampton et al., 2012; Gordon et al., 2011; Heiss et al., 2008). Exposure to CO could generate MPs by all three known production mechanisms: oxidative stress, apoptosis and cell activation/ Ca^{++} influx (Enjeti et al., 2008; Hugel et al., 2005; Piantadosi, 2008; Pirro et al., 2008). CO causes oxidative stress related to xanthine oxidase activity and mitochondrial free radical production, lipid peroxidation and neutrophil activation (Brown et al., 1990; Piantadosi, 2008; Thom, 1990, 1992). CO also causes apoptosis (Piantadosi et al., 1997; Vince et al., 2009), and exposure will stimulate large-conductance Ca^{++} -activated K^{+} (BK) channels causing cell hyperpolarization (Wang and Wu, 1997; Wang et al., 1997a, 1997b). Demonstrating a role for MPs in pathological processes has been a challenge. Recently, an intravascular injection of the surfactant

polyethylene glycol telomere-B (PEGtB) was shown to emulsify MPs (Thom et al., 2011). This intervention can abrogate injuries in a murine model of decompression sickness where MP generation was shown to play a central role (Thom et al., 2011; Yang et al., 2012).

As a component of this study we sought out a physiological index for CNS nerve dysfunction. Given that some data suggest that intravascular and/or perivascular events are associated with CO-mediated injuries, we hypothesized that neurohypophysial nerve terminal conductance; that is, membrane voltage changes during action potential (AP) propagation in the posterior pituitary, could be used to objectively evaluate neurological function following CO inhalation. The neurohypophysis has an incomplete blood brain barrier (Ganong, 2000). Moreover, there is a close association between neurohypophysial nerve terminals and the perivascular space; only a thin glial sheath separates most neuronal processes and nerve terminals from brain capillary endothelium (Hatton et al., 1984; Reaves et al., 1982). There is precedence for considering that perivascular neurological insults can be detected at the neurohypophysis. Morphological changes occur in the neurohypophysial microvasculature following anatomically distant focal cerebral ischemia; changes thought to occur due to bulk transport of vasoactive substances (Frontczak-Baniewicz, 2001).

This report describes elevations in circulating MPs from a variety of cells when mice are exposed to a range of CO concentrations. PEGtB treatment, which decreased circulating MPs, diminished neutrophil activation, vascular permeability and also CO-induced neurohypophysial AP broadening. Injecting MPs into naïve mice that are obtained from CO poisoned mice, but not from control mice, will recapitulate CO-mediated tissue changes.

Methods and materials

Materials

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Annexin binding buffer and the following antibodies were purchased from BD Pharmingen (San Jose, CA): FITC (fluorescein isothiocyanate) conjugated anti-Annexin V, FITC-conjugated anti-mouse myeloperoxidase (MPO), R-phycoerythrin (RPE) conjugated anti-human CD142, PerCP/Cy5.5 conjugated anti-mouse CD41, PerCP/Cy5.5 conjugated anti-mouse CD14, allophycocyanin (APC) conjugated anti-mouse glycoporphin A (Ter119, CD235), APC conjugated anti-mouse CD31, and APC conjugated anti-mouse vWF. R-PE-conjugated anti-mouse CD41 was purchased from e-Biosciences (San Diego, CA), PerCP Cy5.5-conjugated anti-human CD66b from Biolegend (San Diego, CA) and Alexa 647-conjugated anti-mouse CD18 from Serotec (Raleigh, NC).

Animals

All aspects of this study were reviewed and approved by the University of Pennsylvania institutional IACUC. Mice (*Mus musculus*) C57BL/6J were purchased (Jackson Laboratories, Bar Harbor, ME), fed a standard rodent diet and water *ad libitum*, and housed in the University animal facility. A colony of C57BL/6J MPO-KO mice, initially purchased from Jackson Laboratories, was maintained in the vivarium. Mice were left to breathe room air (control) or subjected to 1 h exposures to CO following our published procedures (Thom

et al., 1999a, 2004). In brief, exposures were carried out in a 7-liter Plexiglas chamber where CO mixed with air was flowed at ~8 l/min. Mice breathed 50, 100 or 1000 parts per million (ppm) CO for 60 min or else 1000 ppm for 40 min, then 3000 ppm for up to 20 min, until they lost consciousness, and then they were removed to breathe room air and regain consciousness. Where indicated mice received 0.3% solution (wt/vol) of polyethylene glycol telomere B (PEGtB, marketed under the name Zonyl by Sigma-Aldrich) *via* the dorsal tail vein at 0.7 μ l/g mouse just before exposure to CO and control mice in these studies received comparable volumes of sterile saline. At the times specified in Results, mice were anesthetized [intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg)], skin was prepared by swabbing with Betadine and blood was obtained into heparinized syringes by aortic puncture. Blood for MPs and neutrophil activation was immediately combined with commercial fixative 100 μ l/ml Caltag Reagent A fixation medium (Invitrogen Inc., Carlsbad, CA) before further work was done. A flow diagram showing the general division of experimental approaches and goals for the animals used in this study is shown in Fig. 1. Wild type and MPO-KO mice were used to first evaluate MPs and neutrophil activation responses in mice exposed to a range of CO concentrations, then the most severe exposures were used to investigate pathophysiological responses and finally, the role of MPs in CO pathophysiology was investigated by looking at a method for lysing MPs *in vivo* and also injecting MPs into naïve mice.

Standard procedures for MP acquisition and processing

All reagents and solutions used for MP analysis were sterile and filtered (0.2 μ m filter). When MP samples were prepared for injections into naïve mice, heparinized blood was centrifuged for 5 min at 1500 *g*. To diminish *ex vivo* MP aggregation this supernatant was made 0.2 M EDTA by diluting it 2:5 using a 0.5 M solution of tetrasodium EDTA prepared in phosphate buffered saline (PBS). The supernatant was then centrifuged at 15,000 *g* for 30 min to pellet the few remaining platelets and cell debris. Supernatant was parceled among centrifuge tubes at a ratio of 250 μ l + 4 mlPBS and centrifuged at 100,000 *g* for 60 min (typically 3–4 tubes/experiment were used). MPs, found in a loose pellet at the bottom of the centrifuge tubes, are recovered by first removing the overlying 4 ml supernatant and then resuspending the MPs in the remaining 250 μ l of fluid as previously described (Yang et al., 2012). The 4 ml supernatant as well as MPs in the resuspended pellet were used in separate experiments for injection *via* a tail vein into naïve mice.

Flow cytometry analysis of blood-borne MPs and neutrophils

Flow cytometry was performed with a 10-color FACSCanto (Becton Dickinson, San Jose, CA) using standard acquisition software. Analysis of neutrophils and MPs was performed as previously described (Thom et al., 2011; Yang et al., 2012). Neutrophils were taken as cells expressing CD66b and activation as elevations in surface staining for myeloperoxidase (MPO) and the CD18 component of β_2 integrins. Interactions with platelet membranes were identified by evaluating the platelet-specific CD41 protein on CD66b-positive cells based on standard procedures involving omission of the negative control staining, the fluorescence-minus-one (FMO) control test (Balasubramaniam et al., 2007; Tung et al., 2004).

Vascular permeability and neutrophil sequestration

Mice were anesthetized, exsanguinated, and injected with lysine fixable rhodamine– dextran (2×10^6 Da) and then with colloidal silica following published methods (Thom et al., 2011; Yang et al., 2012). Endothelium enriched fractions were obtained for analysis from the brain, leg skeletal muscle and heart; values were normalized to a value obtained with a control mouse included in each experiment because of variability in rhodamine–dextran manufacturing. Neutrophil sequestration was evaluated by performing Western blots on endothelium-enriched tissue homogenates, probing for CD66b and normalizing band density to β -actin bands on the same blots.

Neurohypophysis action potential

Details of the neurohypophysis preparation and the associated apparatus have been reported previously (Kosterin et al., 2005; Salzberg et al., 1983) The neurointermediate lobe (comprising neurohypophysis and pars intermedia) was obtained from mice killed by exsanguination after anesthesia and pinned in a manner so that the infundibular stalk lies clasped between a pair of platinum– iridium electrodes. After the preparation has been bathed in oxygenated mouse Ringer's solution (inmM: 154 NaCl, 5.6 KCl, 1MgCl₂, 2.2 CaCl₂, 10 glucose, 20 HEPES, adjusted to pH 7.4 with NaOH) containing 20 μ MJPW 3031 (di-2-ANEPPDHQ), a fast-response potentiometric styryl dye (Molecular Probes/Life Technologies, Inc., Grand Island, NY) for 15 min (Obaid et al., 2004), brief balanced bipolar shocks (100–200 V) lasting between 300 and 500 μ s are delivered through a stimulus isolator, and the resulting changes in the extrinsic fluorescence of the stained tissue are recorded by a single large-area silicon photodiode (PV-444, Perkin- Elmer Optoelectronics, Vaudreuil, Canada) which is positioned in the image plane of an epifluorescence microscope (UEM, Carl Zeiss, N.Y.). The photo-current is then converted into a voltage signal. The resulting optical record is a high fidelity representation of the shape of the AP in the neurohypophysial nerve terminals (Salzberg et al., 1983). From 4 to 12 individual AP images were analyzed from each mouse. To quantify results across different experiments the mean AP width at the half maximal height was calculated.

Statistical analysis

We used Sigmapstat software (Systat, Point Richmond, CA) for the statistical analysis. Data were analyzed by ANOVA followed by theHolm–Sidak test. Single comparisons were performed using Student's t-tests.

Results

CO exposure increases circulating microparticles

Alterations in the number of circulating MPs in mice exposed for 1 h to CO at various concentrations are shown in Fig. 2. Results from studies are reported as MP number relative to a control animal studied on the same day because antibody preparations and flow cytometer settings can influence the day-to-day counts. Across all experiments the mean MP number in air-only breathing control mice was 1709 ± 99 ($n = 64$)/ μ l plasma. Exposure to 50 ppm CO caused no significant elevations of MPs in mice killed immediately but at 90 min

post-COMPs were decreased to $42 \pm 10\%$ ($n = 3$, $p < 0.05$) of the control level. No increase was observed immediately after 100 ppm CO, but the number of particles was 3.8-fold higher 90 min later. Similar elevations were found immediately and also 90 min after exposure to 1000 ppm CO. We also investigated a model of severe CO poisoning known to cause inflammatory responses in brain and neurological dysfunction involving exposure to 1000 ppm CO for 40 min and then to 3000 ppm CO for 20 min (Han et al., 2007; Thom et al., 2004). A five-fold elevation of MPs was found in mice killed immediately after this pattern of exposure and the elevation persisted at 90 min post-exposure.

Neutrophil activation

Activated neutrophils have been shown to play a role in brain injury following the severe (1000/3000 ppm) CO poisoning model, but effects from lower concentrations of CO have not been investigated (Thom et al., 2006). Cell surface expression of the carcinoembryonic antigen gene family member 6 (CD66b) was used as a relatively specific marker for neutrophils. Neutrophil activation following exposures to 100 ppm CO or more was discerned as CD66b expressing cells having increased surface staining for MPO and the CD18 component of β_2 integrins (Fig. 3). Because interactions with platelet-derived components have been reported following CO poisoning, we also probed for the platelet specific CD41 component of the $\alpha_2\beta_3$ integrin on the neutrophil surface. Results are shown as the fold-elevation over control in proportion of CD66b-positive cells exhibiting these surface markers and, as an alternative approach to assess cell activation; the mean fluorescence of MPO, CD18 and CD41 was also evaluated. Increases over control were identified following exposure to CO at all concentrations tested.

Increased vascular permeability and role for MPs and neutrophils

To investigate whether MPs may play a role in tissue damage after CO, studies focused on exposures to 1000 ppm CO. Vascular permeability was quantified as lysine-fixable rhodamine-labeled 2000 kDa dextran in tissue extracts (see Methods and materials). Vascular permeability was significantly elevated in the brain and skeletal muscle immediately after 1000 ppm CO exposure (Table 1). Interestingly, elevated rhodamine content was not identified in the heart (values were 1.20 ± 0.25 fold *versus* control, $n = 5$, NS), nor were values significantly different in tissues of animals killed 90 min post-CO (data not shown).

Infusing PEGtB surfactant into air-breathing mice (no CO exposure) reduced circulating MPs by 32% at 1 h post-injection. The MP counts were 0.68 ± 0.12 ($n = 7$, $p < 0.05$ *versus* control) fold compared to values found in matched control mice studied on the same day. When PEGtB was administered just before 1000 ppm CO for 1 h, vascular leak was not observed in mice killed immediately after the exposure (Table 1). MP counts in these mice were significantly lower than in untreated CO-exposed mice and not elevated over control. MPs were just 0.99 ± 0.07 (SE, $n = 5$, NS *vs* control) fold different from sterile saline-injected, control (no CO exposure) mice. PEGtB-treated, CO-exposed mice exhibit less evidence of neutrophil activation than untreated CO-exposed mice shown in Fig. 3 but there was persistent activation based on surface presence of MPO and CD41 (Table 2).

To further explore whether neutrophils may play a role in vascular leak after 1000 ppm CO a series of studies was conducted in knock-out (KO) mice lacking myeloperoxidase (MPO). CO poisoned MPO-KO mice exhibited a similar magnitude of MP elevations as wild type mice. Immediately after 1000 ppm CO circulating MPs were 2.8 ± 0.42 (SE, $n = 5$, $p < 0.05$) fold elevated over air-exposed, control MPO-KO mice and 90 min after CO exposure 2.2 ± 0.83 ($n = 5$, $p < 0.05$) fold-elevated. Despite the MP elevation, however, dextran permeability in the brain and muscle did not increase after exposure to 1000 ppm CO (Table 1).

Neutrophil sequestration

We next looked for neutrophil sequestration in tissues by probing for CD66b on Western blots of endothelium-enriched tissue homogenates (see Methods and materials). A representative Western blot is shown in Fig. 4. As shown in Table 3, there were significant elevations of CD66b relative to β -actin in the brain and muscle and, notably, this was not evident in mice infused with PEGtB prior to CO poisoning or in CO-exposed MPO-KO mice.

Changes in MP sub-types

We were also interested in assessing whether the variety of MP subtypes differed due to varying CO exposures and PEGtB treatment. Fig. 5 displays proportions of MPs analyzed based on surface detection of annexin V and co-expression of proteins found on a variety of vascular cells. The fraction of each subtype was multiplied by the total number of circulating annexin V-positive particles in each mouse to arrive at a proportion present in the circulation, and data are expressed relative to the appropriate control animals (wild type or MPO-KO). Significant elevations were found for virtually all sub-types in mice exposed to 1000 ppm CO and to the 1000/3000 CO model. The gross MP pattern in MPO KO mice was similar to that in wild type, CO-exposed mice but no statistically significant changes *versus* controls were identified. PEGtB injection prior to exposure to 1000 ppm CO altered subtype elevations, the only statistically significant elevation occurred for CD66b-expressing MPs. If PEGtB was administered immediately after exposure to 1000 ppm CO in mice that were killed 90 min later, only CD235- expressing MPs were found to be significantly different.

Neurohypophysial action potential (AP) broadening

AP shape in neurohypophysial nerve terminals was used as an index for CNS nerve damage following CO poisoning (Fig. 6). To quantify results across different experiments the data are expressed as AP width at the half maximal height using magnified images (Table 4). No alterations were noted immediately after exposure to 1000 ppm CO, but statistically significant broadening occurred 90 min later. Finding a delayed effect, in contrast to the vascular leakage change that was documented immediately but not 90 min after CO poisoning (Table 1), offered an opportunity to evaluate PEGtB as a treatment *versus* prophylaxis from tissue injury. Mice were exposed to 1000 ppm CO for 1 h, then injected with PEGtB and killed 90 min later. The number of MPs in these mice was reduced by 34% to 0.66 ± 0.03 ($n = 3$, $p < 0.05$) fold of the level in untreated CO-exposed mice and, as

shown in Table 4, PEGtB intervention diminished AP broadening. Neutrophil activation in these mice did not occur (Table 2).

We also measured action potential broadening in mice subjected to 1000 ppm CO for 40 min, then 3000 ppm for 20 min. Broadening was observed at 90 min but not immediately after CO poisoning (Table 4). PEGtB infusion following this pattern of CO poisoning also prevented AP broadening. Similarly, AP broadening was not observed in MPO KO mice after the 1000/3000 CO poisoning model.

MP injections into naïve mice

To directly assess the role of MPs in tissue injuries, particles were harvested from air-breathing control mice or mice first exposed to CO and they were injected into naïve mice. No significant vascular leakage was found in the brains of mice injected with MPs from air-breathing control mice, but a significant leak occurred when using MPs from mice exposed to CO (Table 1). There was substantial variability in the measurements of leakage from skeletal muscle; no significant leak occurred with control MPs but a significant leak was identified with the use of MPs from CO-exposed mice. In these studies the mean number of control MPs injected into each mouse was 7.09×10^5 ($\pm 12,287$, $n = 5$) and the mean number of CO-MPs was 6.99×10^5 ($\pm 25,385$, no significant (NS) difference).

The AP width in mice injected with MPs from control mice was 3.76 ± 0.19 ms ($n = 3$, NS *versus* control as in Table 4) whereas after injecting MPs from CO poisoned mice the AP width was 6.13 ± 0.36 ms ($p < 0.05$ *vs* control MP injection). The mean number of control MPs injected in these studies was 7.22×10^5 ($\pm 19,123$); the mean number of CO-MPs was 5.66×10^5 (± 3733 ; NS *versus* control).

There was a small percentage of plasma proteins injected along with MPs in these studies. To assess whether something other than MPs may be responsible for neutrophil sequestration or vascular leak in the brain and muscle, the plasma supernatant from CO-exposed mice, rather than the MPs obtained in the centrifugation pellet (see Methods and materials) was injected into naïve mice. No significant vascular leakage was identified (Table 1) and neutrophil sequestration expressed as CD66b/actin relative to same-day control for Western blots of tissue homogenates was $87 \pm 7.5\%$ ($n = 3$, NS) for the brain and 101.8 ± 17.2 ($n = 3$, NS) for skeletal muscle.

Discussion

The initial goal of this investigation was to determine whether exposures to CO cause elevations in circulating MPs. Results demonstrate that 1 hour exposures to CO at concentrations of 100 ppm or more will increase circulating MPs (Fig. 2) and these particles are derived from a variety of cell types (Fig. 5). Clearly the time-course for elevations differs with CO concentration; no elevation was observed immediately after 100 ppm CO whereas 1000 ppm CO and the 1000/3000 ppm CO patterns resulted in elevations detected immediately after the exposures. Exposures to 100 ppm CO and 1000 ppm CO elevated total MPs ~two to three-fold and the increases in various sub-types expressing vascular cell markers increased from 2 to 10 fold (Figs. 2 and 5). The discrepancy in fold-changes

between total MPs and specific sub-types relates to the intense sharing of surface markers that occurs in the circulation so MPs can express more than one protein marker (Thom et al., 2011, 2012, 2013). Because MPs can bear markers from many cell types, identifying a protein specific for one cell such as CD66b, a protein found predominantly on neutrophils, does not provide assurance that all CD66b bearing MPs actually were derived from neutrophils. Exposure to the 1000/3000 ppm CO pattern increased total MPs five-fold and most MP sub-types expressing vascular cell surface markers were increased 20 to 30-fold (Figs. 2 and 5).

There is a long history of viewing CO-mediated injuries as being directly related to hypoxic stress from COHb formation. In prior rodent studies we have reported the following mean COHb values for CO exposures: 50 ppm, 4.8%; 100 ppm, 10.6%; 1000 ppm, 49%; and 1000 ppm for 40 min/3000 ppm CO for 20 min, 54% (Thom et al., 1994, 1999a, 1999b). It is unlikely that hypoxic stress is the sole reason for responses identified in this study. Despite marked differences in COHb between 100 ppm and 1000 ppm CO or the 1000/3000 patterns, respective MP elevations were not statistically significantly different.

Pathways linked to MP generation include oxidative stress, cell activation/calcium influx and apoptosis (Enjeti et al., 2008; Hugel et al., 2005; Pirro et al., 2008). CO-mediated MP elevations could be related to any of these processes, or to alterations in MP clearance pathways. Mechanisms for MP generation relate to perturbations in membrane phospholipid turnover and actin cytoskeleton but many details are lacking and MP clearance pathways are also poorly understood (Morel et al., 2011). In this regard, the drop in MP number 90 min after some exposures, such as 50 ppm CO, may offer an opportunity to evaluate clearance pathways as this is the most likely explanation for this change.

In previous work, exposure to the 1000/3000 pattern was shown to trigger neutrophil interactions with platelet membranes that caused neutrophil activation leading to brain tissue oxidative stress and functional deficits (Han et al., 2007; Ischiropoulos et al., 1996; Thom, 1993; Thom et al., 2004, 2006). In this study CO exposure to just 50 ppm increased platelet-specific CD41 on neutrophils (geometric mean elevation, Fig. 3) and exposure to 100, 1000 ppm or the 1000/3000 pattern resulted in increases in platelet CD41 as well as MPO and CD18, more overt manifestations of neutrophil activation (Fig. 3).

After finding that MPs were elevated with CO exposures, effort was focused on addressing whether MPs could be linked to tissue injuries. These studies centered on responses to 1000 ppm CO. MPs were implicated in causing the abnormalities because they were abrogated by injecting PEGtB. PEGtB will lyse MPs in mice and dosing was established by studies of lysis in *ex vivo* trials (Thom et al., 2011). PEGtB was used in contrast emulsions during early clinical echocardiography trials and the same dose as we used in mice was well tolerated in humans (Beppu et al., 1997). PEGtB will diminish circulating MPs and prevent most injuries related to MPs in a model of decompression sickness (Thom et al., 2011). Finding that treatment reduces MPs in control, air breathing mice in the current study is consistent with this previous work. When mice were injected with PEGtB before exposure to 1000 ppm CO, MPs post-exposure were not increased; vascular leak and neutrophil sequestration were diminished (Tables 1–3). PEGtB had little effect on the proportions of

circulating MPs, as reflected in Fig. 5. Persistent elevations of CD66b- and CD31-bearing MPs suggest that under at least some circumstances, MPs may not be equally sensitive to emulsification by PEGtB. We have not observed this phenomenon with PEGtB in a prior study (Thom et al., 2011).

Vascular leak, assessed as perivascular accumulation of 2000 kDa dextran–rhodamine, appears to be an early manifestation of tissue injury that resolves within 90 min after CO exposure. Leakage of radio-labeled albumin (67 kDa) from vessels in skeletal muscle and lungs was shown to occur after just 50 ppm CO for 1 h, and to persist for 18 h (Thom et al., 1999a, 1999b). It is interesting that leakage from cardiac muscle could not be shown in this study. We have seen a heterogeneous pattern for vascular leak in other forms of inflammatory injuries (Thom et al., 2011). Whether differences in heart tissue are due to protective processes or to differences in how the perivascular structures respond to CO mediated stress will require further work.

Neutrophil sequestration in muscle and the brain was documented after exposure to 1000 ppm CO in wild type, but not MPO-KO mice (Table 3). We previously reported that MPO-KO mice subjected to the 1000/3000 ppm CO model do not exhibit the adaptive immunological changes in the CNS that cause functional deficits (Thom et al., 2006). We think it notable that MP number was significantly increased in MPO-KO mice exposed to CO, but these mice do not exhibit neutrophil sequestration, vascular leak (Table 1) or AP broadening (Table 4) after CO poisoning. Some of the MPO released from degranulating neutrophils can adhere to the cell surface and cause auto-activation (Lau et al., 2005) as well as contribute to vascular damage when deposited on the endothelial lining (Brennan et al., 2002). We conclude that neutrophil activation/degranulation following CO-poisoning involves interactions with MPs and MPO contributes to tissue injuries. This perspective is also supported by results due to PEGtB injection, where MP number is reduced and neutrophil activation, vascular leak and AP prolongation do not occur. Because the total number of circulating MPs was the same in MPO-KO and wild type CO-exposed mice, results suggest that neutrophil activation is a consequence rather than a cause for generation of MPs. This sequence differs from what has been reported for MP-related events in some other conditions (Thom et al., 2011).

We used broadening of the neurohypophysial AP as an indicator of functional neurological injury from CO. Changes in nerve AP have been used by a number of groups to document CNS neurological injury from CO poisoning (Davies et al., 2008; Fechter et al., 1987), although direct neurohypophysial injury is only rarely reported with clinical CO poisoning (Chang and Lin, 2001). We view AP broadening as a useful index of dysfunction rather than suggesting that neurohypophysial changes have direct clinical relevance. Changes 90 min post-CO provided an opportunity to assess whether PEGtB could be used as treatment following CO exposure. We interpret abrogation of the CO-mediated AP widening as evidence that MPs play a role in CNS injury.

Obviously, CO will cause a variety of insults in addition to elevating MPs. Therefore, to directly evaluate whether tissue injuries arise because of MPs we injected particles from CO-poisoned animals into naïve mice. Others have reported cell injury and vascular

alterations can be triggered using serum isolated from animals and humans exposed to diesel exhaust, ozone or nitrogen dioxide (Channell et al., 2012; Robertson et al., 2013). Changes occurred with serum dilutions as low as 2.5%, the same dilution that resulted from our MP isolation process (40% with addition of EDTA followed by 6.25% of PBS addition for the ultracentrifugation step — see Methods and materials). We did not find a vascular leak (Table 1) or neutrophil sequestration in tissues after injections of MP free dilute plasma samples from CO-poisoned mice. Introducing MPs from control mice also caused no statistically significant insults. Therefore, finding a vascular leak and AP widening with MPs from CO poisoned mice provides compelling evidence for MPs having a direct pathophysiological role in CO poisoning. Further, because equal numbers were injected as used in control studies, the results argue that intrinsic changes within MPs generated in response to CO, *versus* merely an increase in their number as shown in Fig. 1, trigger tissue injuries.

In conclusion, results from this study improve our understanding of CO pathophysiology and, as is true of most animal models, raise a variety of questions. Further work is necessary to determine the mechanism(s) for MP production and clearance. Results with the MPO-KO mice indicate that neutrophil activation occurs proximal to tissue damage but the mechanism for how MPs activate neutrophils remains unclear. Beyond the mechanistic issues our study also opens the way to consider alternatives to current clinical management of CO poisoning. That is, treatment directed against MPs represents a novel approach worthy of additional investigation.

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Fig. 1.

Schematic on animal use in this investigation. Mice were exposed to a range of CO concentrations to first evaluate MPs and neutrophil activation. Neutrophil sequestration, vascular leak and neurohypophysial AP prolongation were then assessed as indices of pathophysiology. To evaluate the role that MPs play in CO-mediated pathophysiology, studies were performed using PEGtB to lyse intravascular MPs and naïve mice were injected with MPs.

Fig. 2.

CO exposure increases circulating microparticles. Data show fold-elevations over control air-exposed mice of circulating annexin V positive MPs in mice exposed for 1 h to 50, 100, or 1000 ppm CO or to CO exposure split between 1000 ppm for 40 min and 3000 ppm for 20 min. Mice were killed immediately after exposures or 90 min later. Values are mean $\pm$ SE; * p < 0.05 versus control (ANOVA).

Fig. 3.

Neutrophil activation. Data show fold-elevations over control for cell surface presence of myeloperoxidase (MPO), CD18 and CD41 on neutrophils identified by expression of CD66b. Mice were exposed for 1 h to 50, 100, or 1000 ppm CO or to 1000 ppm

CO for 40 min and 3000 ppm for 20 min; and then killed immediately or 90 min after exposures. The bar graphs depict proportions as percent of CD66b positive cells expressing greater than 10 arbitrary fluorescence units. The numbers below each segment in the figure show fold-change in CD66b positive cell geometric mean fluorescence (GM) ± SE for each marker. The symbol (*) indicates $p < 0.05$ versus control.

Fig. 4.

Representative Western blots. Endothelium-enriched brain and skeletal muscle tissue homogenates were prepared from air-breathing mice (no CO exposure) injected with sterile PBS (control) or PEGtB (PEGtB only) at 0.7 μ l/g mouse and left to breathe air for 1 h, injected with PBS and exposed to 1000 ppm CO for 1 h (CO), or injected with PEGtB in PBS and killed immediately after exposure to 1000 ppm CO for 1 h (PEGtB-CO). Western blots were probed for CD66b and actin as described in Methods and materials. Data from replicate studies are displayed in Table 3.

Fig. 5.

MPs expressing surface markers for various antigens. Changes in MPs expressing a variety of surface markers after CO exposures as outlined in Fig. 1 are expressed as fold change *versus* air-breathing, control mice. CD41 is a platelet-specific marker, CD66b predominantly neutrophil-specific, CD31 is platelet-endothelial cell adhesion protein, CD14 is the common leukocyte antigen, and CD142 is tissue factor. CD235 is erythrocyte-specific glycophorin A and vWF indicates von Willebrand factor. WT indicates wild type mice and MPO KO indicates knock-out mice lacking myeloperoxidase. Data are mean $\pm$ SE. The (*) symbol indicates values significantly different from control ($p < 0.05$ ANOVA) and n = number of mice in each group. The first row indicates results from mice exposed to 1000 ppm CO for 1 h and killed either immediately (gray filled bars) or 90 min later (black filled bars). The second row shows MPs in mice injected with PEGtB and killed immediately after 1000 ppm CO for 1 h (gray filled bars) and mice poisoned with 1000 ppm CO for 1 h, then injected with PEGtB and killed 90 min later (black filled bars). The third row shows MP results from CO poisoned MPO-KO mice *versus* air breathing MPO-KO mice killed either immediately (gray) or 90 min (black) after exposures. Not shown are data that there were no significant

differences in MP sub-type proportions or total number between airbreathing MPO-KO and wild type mice. The fourth row shows MPs in mice exposed to 1000 ppm CO for 40 min and 3000 ppm CO for 20 min killed either immediately (gray) or 90 min (black) after exposures. Note the difference in ordinate scale for this group.

Fig. 6.

Neurohypophysial nerve terminal membrane voltage changes: A. The figure demonstrates the APs when a series of 4 shocks were applied to neurohypophysial preparations from an air-breathing, control mouse (black) and a mouse killed 90 min after exposure to 1000 ppm CO (red). B. Magnified images were used to measure AP width half-way between the times from its initiation to its maximum height (action potential half-width) to facilitate data comparisons across multiple experiments as summarized in Table 4. Ordinate scale is based on AP fluorescence signal divided by the initial fluorescence at start of study.

Table 1

Vascular leak due to CO. Data show fold-change in vascular leakage of 2000 kDa lysine-fixable rhodamine-dextran (fluorescence/mg protein) in homogenates from CO-exposed mice *versus* air-breathing, control mice studied on the same day. First row indicates leakage from mice killed immediately after being subjected to 1000 ppm CO for 1 h (labeled CO). PEGtB indicates mice had been injected with polyethylene glycol telomere B before exposure to 1000 ppm CO. Control mice for these studies were injected with a volume of sterile PBS comparable to PEGtB volume. PEG only indicates air-breathing mice (no CO exposure) injected with PEGtB and killed 1 h. MPO-KO indicates knock-out mice exposed to 1000 ppm CO and values in CO-exposed mice were compared against air-breathing MPO-KO mice (vascular leak was insignificantly different between air-breathing wild type and MPO-KO mice). IV control MPs indicates vascular leakage in mice injected with MPs obtained from air-breathing control mice and IV CO MPs is vascular leakage in mice injected with MPs obtained from mice killed immediately after exposure to 1000 ppm CO for 1 h. Control mice used to normalize data in the IV MP injection studies received 400 µl sterile PBS, the same volume as was used to suspend MPs for injection into the experimental mice (250 µl MP suspension + 150 µl PBS). The lowest row in the table labeled IV CO-plasma indicates vascular leakage in naïve mice injected with 150 µl PBS+250 µl of the plasma supernatant from CO-exposed mice that was ultra-centrifuged to obtain the MPs.

	Brain	Skeletal muscle
CO (n = 5)	1.95 ± 0.20 *	1.26 ± 0.03 *
PEGtB, then CO (5)	1.06 ± 0.03	0.97 ± 0.04
PEG only (4)	1.09 ± 0.22	1.01 ± 0.19
MPO-KO (5)	0.94 ± 0.03	0.99 ± 0.06
IV control MPs (5)	0.91 ± 0.09	1.48 ± 0.17
IV CO-MPs (4)	1.23 ± 0.04 *	2.68 ± 0.61 *
IV CO-plasma (3)	0.89 ± 0.16	1.12 ± 0.26

Values are mean ± SE, n = number of mice studied in each group. The symbol (*) indicates $p < 0.05$ *versus* control (ANOVA).

Table 2

Neutrophil activation in mice injected with PEG. Data show mean $\pm$ SE fold-elevations over those found in cells from control air-breathing mice for cell surface markers as described in Fig. 3. Values in (%) columns indicate fold-change of CD66b positive cells expressing a mean fluorescence value 10 arbitrary units for each surface marker. Values in (GM) columns show fold change in CD66b positive cell geometric mean fluorescence. The first row of data labeled PEG shows values from air-breathing mice (no CO exposure) injected with PEG and killed 60 min later. The second row shows neutrophil activation in mice injected with PEG then exposed to 1000 ppm CO for 1 h and killed immediately after the exposure. The third row shows data from mice exposed to 1000 ppm CO for 1 h, then injected with PEG and killed 90 min later.

	MPO		CD18		CD41	
	%	GM	%	GM	%	GM
PEG (n = 7)	1.11 $\pm$ 0.07	1.01 $\pm$ 0.01	1.04 $\pm$ 0.03	0.96 $\pm$ 0.01	1.09 $\pm$ 0.05	1.09 $\pm$ 0.01
PEG/CO (n = 7)	1.39 $\pm$ 0.04 *	1.03 $\pm$ 0.02	1.12 $\pm$ 0.02	0.99 $\pm$ 0.03	1.22 $\pm$ 0.06 *	1.10 $\pm$ 0.06
CO/PEG (n = 4)	1.17 $\pm$ 0.02	1.04 $\pm$ 0.02	1.08 $\pm$ 0.05	1.02 $\pm$ 0.01	1.05 $\pm$ 0.02	1.03 $\pm$ 0.02

The number of replicate studies is shown in the left margin and the symbol (*) indicates p < 0.05 versus control (ANOVA).

Table 3

Neutrophil sequestration due to CO. Data show fold-change *versus* air-breathing, controls for CD66b from tissue Western blots normalized to β actin on the same blot for mice (labeled CO) injected with sterile saline IV at 0.7 μ l/g mouse and killed immediately after being subjected to 1000 ppm CO for 1 h. PEGtB, then CO indicates that mice had been injected with PEGtB just before exposure to 1000 ppm CO. PEGtB only indicates air-breathing mice (no CO exposure) injected with PEGtB and killed 1 h later. MPO KO indicates knock-out mice exposed to CO (these values were compared against KO mice exposed to just air).

	Brain	Skeletal muscle
CO (6)	2.62 $\pm$ 0.22 *	3.16 $\pm$ 0.39 *
PEGtB, then CO (6)	1.30 $\pm$ 0.15	1.44 $\pm$ 0.21
PEGtB only (4)	0.94 $\pm$ 0.13	1.04 $\pm$ 0.13
MPO-KO (6)	0.92 $\pm$ 0.01	0.91 $\pm$ 0.01

Values are mean $\pm$ SE and n = number of mice studied in each group. The symbol (*) indicates $p < 0.05$ *versus* control.

Table 4

Neurohypophysial action potential (AP) width. Values shown are the mean AP half-width in milliseconds as described in Methods and materials. Groups include wild type (WT) and MPO-KO air-breathing mice, those exposed to 1000 ppm CO for 1 h or exposed to 1000 ppm CO for 40 min followed by 3000 ppm CO for 20 min. Mice were killed immediately after CO exposures or 90 min later. When effects of PEG were to be studied in mice immediately after CO exposure the agent was injected prior to CO. When mice were to be studied 90 min after the CO exposure, they were injected with PEG immediately after CO exposure.

	WT, no interventions	WT + PEG injections	MPO KO
Air only	3.97 ± 0.14 (20)	3.63 ± 0.14 (3)	4.51 ± 0.20 (6)
1000 CO—Immed	4.14 ± 0.13 (7)	3.62 ± 0.08 (6)	ND
1000 CO—90 min	5.65 ± 0.26 (6)*	4.18 ± 0.21 (6)	ND
1000/3000—Immed	4.18 ± 0.26 (5)	ND	4.67 ± 0.56 (3)
1000/3000—90 min	5.56 ± 0.09 (5)*	3.58 ± 0.10	4.66 ± 0.39 (3)

Values are mean ± SE and n = number of mice studied in each group. The symbol (*) indicates $p < 0.05$ versus air-only control. ND indicates no experiments in this group were done.