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Systemic vasoprotection by inhaled carbon monoxide is mediated through prolonged alterations in monocyte/macrophage function.

Andrew Leake, MD^{1,2}, Karim Salem, MD^{1,2}, Michael C. Madigan, MD^{1,2}, Ghee Rye Lee, BS², Ankur Shukla, MD^{1,2}, Guiying Hong, MD^{1,2}, Brian S. Zuckerbraun, MD^{1,2}, Edith Tzeng, MD^{1,2}

¹Surgery Services, Department of Veterans Affairs Medical Center, Pittsburgh PA;

²Department of Surgery, University of Pittsburgh, Pittsburgh PA.

Abstract

Carbon monoxide (CO) is anti-inflammatory and protective in models of disease. Its actions in vitro are short-lived but are sustained in vivo. We hypothesize that systemic CO can mediate prolonged phenotype changes in vivo, with a focus on macrophages (Mφs). Mφs isolated from CO treated rats responded to lipopolysaccharide (LPS) with increased IL6, IL10 and iNOS expression but decreased TNF. Conditioned media (CM) collected from peritoneal Mφs isolated from CO treated rats stimulated endothelial cell (EC) proliferation versus CM from Mφs from air treated rats. This effect was mediated by Mφ released VEGF and HMGB1. Inhaled CO reduced LPS induced Mφ M1 inflammatory phenotype for up to 5 days. Mitochondrial oxygen consumption in LPS treated Mφs from CO treated mice was preserved compared to LPS treated Mφs from control mice. Finally, transient reduction of inflammatory cells at the time of inhaled CO treatment eliminated the vasoprotective effect of CO in a rodent carotid injury model. Thus, inhaled CO induces a prolonged mixed phenotype change in Mφs, and potentially other inflammatory cells, that contribute to vasoprotection. These findings demonstrate the ability of inhaled CO to modify Mφs in a sustained manner to mediate its therapeutic actions, supporting the translational potential of inhaled CO.

Keywords

carbon monoxide; macrophage; inflammation; intimal hyperplasia

Corresponding Authors: Edith Tzeng, MD, Chief of Vascular Surgery, VA Pittsburgh Healthcare System, Professor, Division of Vascular Surgery, Department of Surgery, University of Pittsburgh, South Tower, Rm 351.6, 200 Lothrop Street, Pittsburgh, PA 15213, Fax: 412-647-0289, Phone: 412-802-3025, tzeng@upmc.edu; Brian S. Zuckerbraun, MD, Professor of Surgery, Department of Surgery, University of Pittsburgh, NW653 MUH, 3459 Fifth Avenue, Pittsburgh, PA 15213, Phone: 412-647-8799.

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Introduction

Carbon monoxide (CO) is an environmental toxin that causes asphyxiation due to its greater affinity for hemoglobin (Hgb) than oxygen¹. In the 1950's, CO was recognized to be produced endogenously by heme oxygenases (HO) during heme catabolism². The inducible HO-1 isoform is a stress protein that confers cytoprotection through anti-inflammatory actions and these properties have been linked to its end-products of CO³, bilirubin and biliverdin⁴. HO-1 mediated cytoprotection can be reproduced with exogenous CO and inhibited by scavenging CO with Hgb⁵ or tin protoporphyrin (SnPP)³, defining a physiologically important role for CO.

A great interest developed in CO as a potential therapy for inflammatory disorders because of the ease and effectiveness of inhalational delivery. Inhaled CO significantly attenuates local and systemic inflammatory responses in animal models of acute and chronic injury^{6–10}. In the vasculature, inhaled CO at 250 parts per million (PPM) administered for 1hr prior to carotid angioplasty markedly reduced intimal hyperplasia (IH) in rats. A similar benefit was seen in pigs where perioperative inhaled CO reduced IH by 55%⁷. Lower sustained doses of CO protected against allograft vasculopathy in mice experiencing chronic rejection⁶. Inhaled CO is also protective in models of acute lung injury⁸, ischemia/reperfusion^{9, 10}, and sepsis¹¹. While CO delivered by CO releasing molecules (CORMs) seems to be effective as well^{12–14}, systemic administration of CO may potentially have greater efficacy through diverse actions on the systemic inflammatory response.

The mechanisms of by which CO mediates its anti-inflammatory actions are still not fully understood. Pathways involving hypoxia inducible factor-1 α (HIF1 α)¹⁵, HO-1¹⁶ and p38 MAPK^{6, 9, 17} are activated by CO in different cell types including macrophages (M ϕ s) and endothelial cells (ECs) in vitro¹⁷. CO has also been shown to maintain mitochondrial respiration and enhance reactive oxygen species (ROS) generation for molecular signaling as mechanisms of cytoprotection^{18, 19}. However, the majority of the mechanistic studies have been conducted in vitro where the cellular effects and anti-inflammatory changes are observed only during CO exposure. In striking contrast, the prolonged effects of brief exposures to inhaled CO on attenuating inflammation in vivo suggest unique mechanisms of action. Based on these observations, we hypothesize that CO induces persistent phenotypic alterations of inflammatory cells, specifically monocytes/M ϕ s, that mediate the cytoprotective actions of CO.

Methods

Cell culture and pharmacologic reagents:

Rat aortic smooth muscle cells (RASMC) were cultured from Sprague Dawley rat (Harlan; Indianapolis, IN, USA) aorta²⁰ and maintained in DMEM+10% FBS (ThermoFisher Scientific; Waltham, MA, USA) in a 5% CO₂ and 95% air incubator. Human umbilical vein ECs (HUVEC) were maintained in EBM2 media (Lonza; Basel, Switzerland).

Antibodies for Western blot analysis were from Abcam (Cambridge, MA, USA; inducible NO synthase or iNOS, VEGF, HMGB1, STAT, P-STAT3, I κ B) or Cell Signaling (Danvers,

MA, USA; endothelial NOS, P-eNOS). Antibodies for flow cytometry were from AbD Serotec (Atlanta, GA, USA; F4/80 and CD206) and Abcam (CD86 and MHCII).

Plasma experiments:

Whole blood was collected from anesthetized rats by cardiac puncture into heparinized syringes at one or 24hrs after inhaled CO treatment. Plasma was collected by centrifugation, diluted 1:2 by volume with DMEM, and proliferation of growth arrested RASMC was measured with ^3H -thymidine²¹.

Peritoneal M ϕ isolation:

All procedures conformed to the Guide for the Care and Use of Laboratory Animals and the policies of the Institutional Animal Care and Use Committee of the University of Pittsburgh and the Veterans Healthcare Administration (Protocols IS00003674 and 02960, respectively). Rats and C57Bl6 mice (Jackson; Bar Harbor, ME, USA) were treated with inhaled CO (250 PPM) for 1hr⁶ followed by room air (RA). Control animals remained in RA. To collect peritoneal M ϕ s, rats or mice were anesthetized with isoflurane. DMEM (10mL for rats, 3mL for mice) was instilled into the peritoneum and then collected and centrifuged (1,000 RPM for 5min). The pellet was resuspended with Tris-buffered ammonium chloride, centrifuged, and resuspended with DMEM+10% FBS. Equal cell numbers were plated. After 1hr, nonadherent cells were removed and the adherent M ϕ s were maintained in DMEM+10% FBS for experiments. Cells were cultured in a 5% CO₂ and 95% air incubator for these experiments.

Conditioned medium (CM) experiments:

CM was collected from peritoneal M ϕ s isolated from CO treated rats (CO-CM and CO-M ϕ) or air treated rats (air-CM and air-M ϕ) after 16hrs of culture for Western blot. In some experiments, M ϕ were cultured with or without LPS (100ng/mL), as an inflammatory stimulus, and the resultant CM were tested for NO using the Griess reaction²⁰ or TNF α , IL10 and IL6 using ELISA (Abcam, Cambridge; MA, USA). Air-M ϕ s were also treated with CO in vitro⁶ (CO 250 PPM, 5% CO₂, 21% O₂, balanced with nitrogen) to evaluate the direct effect of CO on the cells or under hypoxic conditions (5% CO₂, 1% O₂, balanced with nitrogen) in some experiments. In some experiments, M ϕ s were treated with SnPP or CoPP (25 μ mol/L; Frontier Scientific; Logan, UT, USA) to inhibit or induce HO-1, respectively.

In functional assays, CM was diluted with EBM-2 (3:2 ratio; Lonza) and added to HUVECs. VEGF inhibitor (Axitinib, 20nmol/L; Tocris, Bristol, UK), iNOS inhibitor (L-NAME, 100 μ mol/L; Sigma, St. Louis, MO, USA), anti-HMGB1 blocking antibody (20 μ g/mL; 2g7, gift from Dr. K. Tracey)²², or nonspecific IgG (Santa Cruz; Dallas, TX, USA) was added to HUVECs. Proliferation was measured with ^3H -thymidine incorporation²¹. In vitro angiogenesis was measured by quantifying HUVEC box formation on MatrigelTM (BD Biosciences; San Jose, CA, USA) after 11hrs of culture. Data from CM experiments were normalized to relative protein levels of the cells that generated the CM, as measured by Pierce BCA assay (ThermoFisher Scientific; Waltham, MA).

Western blot analysis:

For Western blot analysis²⁰, unconcentrated CM was separated on 10% SDS-PAGE gel and probed for VEGF and HMGB1. Band densitometry was normalized to protein levels of the source M ϕ s and expressed relative to levels in air-CM. HUVEC lysates were analyzed for eNOS and phosphorylated eNOS (P-eNOS) while M ϕ lysates were analyzed for inducible NO synthase (iNOS), I κ B, STAT3, P-STAT3 and GAPDH. M ϕ were treated with the NF κ B inhibitor pyrrolidine dithiocarbamate (50 μ mol/L; PDTC, Abcam) or STAT3 inhibitor S3I-201(100 μ mol/L; Santa Cruz) to determine the effect these transcriptional factors on VEGF and HMGB1 release.

Phagocytosis and ROS production:

M ϕ s were incubated with fluorescein labeled E. coli bioparticles (K-12 strain; ThermoFisher Scientific) and E. coli uptake was quantified per manufacturer's instructions. ROS production in the M ϕ s was quantified using the OxiSelect™ *In Vitro* ROS/RNS Assay kit (Cell Biolabs, Inc.; San Diego, CA, USA).

Mitochondrial respiration:

Peritoneal M ϕ s were harvested from mice 24hrs after treatment with air or CO. M ϕ s were treated with LPS (10ng/mL) for 24hr, washed, and placed in respiration buffer (120mM KCl, 25mM sucrose, 10mM HEPES, 1mM EGTA, 1mM KH₂PO₄, 5mM MgCl₂). Mitochondrial function was determined by placing M ϕ s in XF24 cell culture plates (Seahorse Biosciences; N. Billerica, MA, USA) in a final volume of 250 μ L. Oxygen consumption rate (OCR) was measured at baseline, with oligomycin (1 μ g/ml) to block oxygen consumption, or with FCCP (1 μ M; Abcam) to uncouple electron transport¹⁸. Rotenone/actinomycin A were used to inhibit mitochondrial respiration. Extracellular acidification rate (CAR) was also measured.

Flow Cytometry:

Peritoneal M ϕ s were collected from control mice and from mice at 1hr to 5days after inhaled CO exposure and were treated with/without LPS for 24hrs and analyzed by flow cytometry²³. Cells were stained with F4/80 to identify M ϕ s. CD206 was used to identify M2 (anti-inflammatory) M ϕ s while CD86 and MHCII were used for M1 (inflammatory) M ϕ s. OneComp eBeads (eBioscience; San Diego, CA, USA) were used for compensation. Cells were analyzed on a LSR II system (BD Biosciences; San Jose, CA, USA) and mean fluorescence intensity (MFI) was quantified using FlowJo software (Ashland, OR, USA).

Transient M ϕ depletion:

Clodronate liposomes (www.clodronateliposomes.com, Amsterdam, Netherlands) were used to deplete monocyte/M ϕ s²⁴. Briefly, 2mL of clodronate liposome (5mg/mL) or control PBS liposomes were injected IP into rats. Blood collected 3 days after injection showed about 60% reduction in monocytes/M ϕ s in the clodronate treated rats. Circulating monocyte numbers were improved by day 7. Thus, rats were treated with inhaled CO on post-injection day 3, followed by carotid artery balloon injury 1hr after CO treatment as described⁶.

Rat carotid artery balloon injury model and tissue analysis:

Male Sprague-Dawley rats (350–450g) were anesthetized with sodium pentobarbital (45mg/kg IP; Butler Schein, Columbus, OH, USA) and inhaled isoflurane (Butler Schein). The left common carotid artery was exposed and injured with a 2-French Fogarty catheter (Edwards Life Sciences; Irvine, CA, USA) inflated to 2ATM for 5min⁶. Animals were euthanized 2wks post-injury. Carotid arteries were perfusion fixed with 2% paraformaldehyde (pH 7.4), excised, and fixed for 1hr at 4°C in 2% paraformaldehyde and cryoprotected in 30% sucrose at 4°C. Vessels were frozen and sectioned into 7µm cryosections. Images were acquired under auto-fluorescence with an Olympus Provis microscope. Vessel wall morphometry was measured in 8 semi-serial sections at the site of maximal injury using ImageJ (NIH; Bethesda, MD, USA). Mφs were identified by staining for CD68 (ab125212; Abcam) and quantified with the Nikon Elements Software (Melville, NY, USA).

Statistical Analysis:

Data are presented as mean±SEM. Statistical analysis was performed using the Student's t-test or ANOVA using the SigmaStat 13.0 software (Systat Software, Inc.). Significance was determined at $P < 0.05$. All pairwise multiple comparisons were performed using the Holm-Sidak method. Normality was tested using the Shapiro-Wilk test. In non-normally distributed data, comparison was performed with the Kruskal-Wallis One Way Analysis of Variance on Ranks with pairwise multiple comparisons using the Tukey test.

Results

Inhaled CO altered plasma composition:

SMCs were treated with plasma collected from rats after CO inhalation or control rats maintained in room air. Plasma collected 1hr after CO inhalation (CO-1h) modestly reduced SMC proliferation by 20% compared to plasma from control rats (air) (Fig. 1A). Plasma collected 24 or 48hrs after CO inhalation (CO-24h or CO-48h) inhibited proliferation by over 40%, demonstrating that CO inhalation altered plasma composition to promote antiproliferative effects on SMC.

Effect of systemic CO treatment on Mφ inflammatory responses:

Monocytes/Mφs play a key role in inflammatory processes. Numerous studies demonstrated that CO inhibits LPS induced TNFα, IL6, and NO production in Mφs in vitro and systemically in vivo^{17, 25}. To evaluate the effects of inhaled CO on Mφ function, the responsiveness of peritoneal Mφ from CO treated (CO-Mφs) and control rats (air-Mφs) to LPS was tested. Plated Mφs isolated from peritoneal washings were confirmed by FACs to be >95% Mφs (data not shown). Baseline TNFα, IL6, and IL10 levels were minimal in CO-Mφ and air-Mφ (Fig. 1B–D). LPS stimulated air-Mφs to produce high levels of TNFα and IL6 while CO-Mφs responded with reduced TNFα but increased IL6 release (Fig. 1B,C). IL10, an anti-inflammatory cytokine, was significantly increased in LPS treated CO-Mφs versus air-Mφs (Fig. 1D). This altered responsiveness of CO-Mφs to LPS was sustained given that LPS was not applied until >4hrs after CO exposure. Similarly, Mφs isolated from

rats 1 and 2 days after CO treatment showed a persistent enhancement of LPS induced IL6 production (1.80 ± 0.07 and 1.42 ± 0.15 -fold increase vs. air-M ϕ s, respectively; N=4 exp; $P < 0.05$).

Another important inflammatory molecule is NO derived from iNOS. Unstimulated M ϕ s do not express iNOS. CO treatment in vivo or in vitro did not induce iNOS. LPS induced iNOS expression and NO production in air-M ϕ (Fig. 1E,F). In vitro CO treatment of M ϕ s suppressed LPS stimulated iNOS (Fig. 1F). In contrast, LPS treated CO-M ϕ s produced enhanced levels of NO and iNOS (Fig. 1E,F), further reinforcing the unique changes in M ϕ phenotype in response to inhaled CO versus direct CO exposure in vitro. LPS treatment of CO-M ϕ s collected 1 day after CO treatment yielded a persistent increase in iNOS expression compared to LPS treated air-M ϕ despite the remote nature of the CO treatment (4.30 ± 0.844 vs. 2.05 ± 0.36 -fold increase, respectively; N=4 exp.; $P = .019$). In CO-M ϕ s isolated 2 days following inhaled CO, there was still a trend toward increased iNOS expression after LPS treatment compared to air-M ϕ s (143% vs. air-M ϕ +LPS; $P = .062$, N=4 exp.).

Inhaled CO enhances M ϕ phagocytosis and ROS production:

Other alterations in CO-M ϕ behavior were also detected. CO-M ϕ s exhibited a ~1.6-fold increase in *E. coli* phagocytosis compared to air-M ϕ s and to M ϕ s treated with CO in vitro (Fig. 2A). CO-M ϕ s also exhibited significantly increased baseline ROS production compared to air-M ϕ s (1.47 ± 0.17 -fold increase; N=3/exp, repeated 5 times; $P = .022$).

Inhaled CO activates NF κ B and STAT3 in M ϕ s:

CO-M ϕ s showed altered transcriptional signaling with increased NF κ B activation, as indicated by a >50% decrease in I κ B levels 2hrs after inhaled CO treatment vs air-M ϕ s (0.47 ± 0.10 -fold; N=4 exp.; $P < .001$) (Fig. 2B). There was a mild increase in P-STAT3 in CO-M ϕ at baseline but greatly enhanced activation following LPS treatment vs air-M ϕ s (39.58 ± 10.15 -fold increase; N=4 exp; $P = .022$) (Fig. 2B). Thus, inhaled CO induced a mixed inflammatory/anti-inflammatory M ϕ phenotype that is potentially mediated by enhanced NF κ B and STAT3 activation.

Inhaled CO altered mitochondrial oxygen consumption in M ϕ s:

CO has been reported to modulate M ϕ function through the regulation of mitochondrial oxygen transport¹⁸. We examined the effect of inhaled CO on M ϕ s mitochondrial function (Fig. 2C–E). At baseline, air-M ϕ and CO-M ϕ exhibit similar oxygen consumption rates (OCR) (Fig. 2C). Following LPS administration, CO-M ϕ exhibit increased OCR without increased extracellular acidification rate (CAR), a measure of anaerobic metabolism, while air-M ϕ exhibited increased CAR (Fig. 2C–E). Uncoupling of electron transport with FCCP increased OCR in control M ϕ and in LPS treated CO-M ϕ but not in LPS treated air-M ϕ (Fig. 2E). These findings indicate that CO-M ϕ s had preserved mitochondrial function.

CO-M ϕ s exhibit an altered secretory phenotype:

To characterize changes in M ϕ secretion induced by inhaled CO, conditioned culture media were collected from CO-M ϕ s and air-M ϕ s, yielding CO-conditioned medium (CO-CM) and air-CM, respectively. CO-CM reduced SMC proliferation by nearly 40% (0.64 ± 0.07 -fold vs

air-CM; N=3/group, 3 exp, $P<.001$). In contrast, CO-CM increased HUVEC proliferation by 1.54 ± 0.11 -fold over cells treated with regular culture medium or air-CM (Fig. 3A; N=3/group, 3 exp, $P=.01$). Boiled CO-CM lost the ability to modulate SMC or EC proliferation, indicating that CO-M ϕ s release a denaturable protein factor(s). CO-CM nearly doubled HUVEC box formation in Matrigel, a measure of angiogenesis, compared with air-CM (N=3/group, 3 exp, $P<.001$; Fig. 3B,C).

CO-M ϕ s release VEGF and HMGB1:

We evaluated the CM for the presence of the pro-endothelial factors VEGF and the endogenous danger signal, high mobility group Box 1 (HMGB1). We previously reported that HMGB1 stimulates EC proliferation and angiogenesis²⁶. By Western blot, unconcentrated CO-CM contained increased VEGF and HMGB1 compared to air-CM (Fig. 4A). When M ϕ s were harvested 1 or 2 days after CO treatment, HMGB1 release remained increased (1.44 ± 0.08 and 1.47 ± 0.09 fold, respectively; N=4; $P<.002$ vs. air-CM). VEGF release was still increased at day 1 (1.12 ± 0.03 fold; N=4; $P<.006$) but was baseline at day 2. Treatment with the STAT3 inhibitor S3I-201 or the NF κ B inhibitor PDTC did not alter VEGF levels in CO-CM (data not shown). However, both S3I-201 and PDTC significantly reduced HMGB1 release from CO-M ϕ s (Figure 4B,C). These findings indicate that inhaled CO activates NF κ B and STAT3 in M ϕ which mediate the release of HMGB1.

Inhaled CO induced secretion of VEGF and HMGB1 mediates pro-endothelial function:

To determine if the secreted VEGF and HMGB1 mediate the pro-endothelial functions of CO-CM, HUVECs were treated with CO-CM or air-CM with/without VEGF inhibitor (Axitinib) (Fig. 4D) or HMGB1 blocking antibody (Fig. 4E). Axitinib reduced HUVEC proliferation by 17% in air-CM treated cells but it completely reversed the proliferative effect of CO-CM (Figure 4D). Axitinib slightly reduced in vitro angiogenesis in air-CM treated HUVECs but completely reversed CO-CM mediated angiogenesis (Fig. 4F). Similarly, anti-HMGB1 slightly reduced HUVEC proliferation and tubing in air-CM treated cells but significantly reduced both in CO-CM treated cells (Fig. 4E,G). Anti-HMGB1 significantly reduced tubing in both air-CM and CO-CM treated cells but had a greater inhibitory effect in CO-CM treated cells (Fig. 4G). Nonspecific IgG did not alter HUVEC responses to CO-CM or air-CM. Thus, CO-M ϕ s released VEGF and HMGB1 both contribute to the pro-endothelial actions of inhaled CO.

Another important pro-endothelial regulator in ECs is eNOS. eNOS expression was similar in HUVECs treated with CO-CM or air-CM (data not shown). However, P-eNOS, the active form of eNOS, was upregulated in CO-CM treated cells (1.56 ± 0.14 -fold increase vs. air-CM, N=7; $P=.002$) (Fig. 5A). Treatment of HUVECs with the NOS inhibitor L-NAME reversed CO-CM mediated proliferation (Fig. 5B). Axitinib (Fig. 5C,D) and anti-HMGB1 (Fig. 5E,F) reduced P-eNOS levels in both air-CM and CO-CM treated HUVECs but with a greater effect in CO-CM treated cells, suggesting that the VEGF and HMGB1 released by CO-M ϕ s mediate pro-endothelial functions in ECs through eNOS activation.

HO-1 upregulation in CO-M ϕ mediates HMGB1 release:

CO-M ϕ showed a baseline 3.65 ± 0.55 -fold upregulation of HO-1 protein compared to air-M ϕ ($P = .003$, Fig. 6A), demonstrating that inhaled CO stimulates HO-1 production. This is similar to other studies that report CO induced HO-1 expression in different cell types^{16, 26, 27}. HO-1 remained upregulated in CO-M ϕ s collected from rats 1 and 2 days after CO treatment (2.05 ± 0.36 and 1.84 ± 0.19 -fold vs air-M ϕ s, respectively; $N = 4-5$; $P < .05$). This HO-1 upregulation was reversed by STAT3 inhibition but not NF κ B inhibition (Fig. 6B,C). To determine if HO-1 mediated VEGF and HMGB1 secretion, M ϕ s were treated with SnPP to inhibit HO-1. SnPP reduced HMGB1 release by CO-M ϕ while VEGF was not affected (Fig. 6D,E). Air-M ϕ s were treated with CoPP to induce HO-1 expression (Fig. 6D,E). CoPP did not increase HMGB1 or VEGF release, suggesting that HO-1 is required but not sufficient to stimulate HMGB1 release in response to inhaled CO.

Inhaled CO effects on M ϕ s are not reproduced by hypoxia:

One potential mechanism of action of inhaled CO may be through the induction of hypoxia due to the ability of CO to bind Hgb with greater affinity than O₂. To evaluate this, M ϕ s from air treated rats were cultured in a CO chamber or a hypoxia chamber. M ϕ s from CO treated rats were cultured under standard conditions. CO-M ϕ s released significant levels of HMGB1 into the CM but air-M ϕ s exposed to ex vivo CO or hypoxia showed no evidence of HMGB1 release (Fig. 6F). LPS treated CO-M ϕ s showed increased NO production compared to air-M ϕ s treated with LPS. In contrast, air-M ϕ exposed to ex vivo CO or hypoxia responded to LPS with significantly reduced NO production (Fig. 6G). These findings indicate that the effects of inhaled CO are not mediated through the induction of hypoxia in the M ϕ s.

Effects of inhaled CO are not sex specific:

Male rats were used in the majority of the experiments. To confirm that the effect of inhaled CO is not specific to male rats/cells, we performed a limited analysis using M ϕ s from air or CO treated female rats. HO-1 expression was upregulated by 3.1-fold in CO-M ϕ s vs air-M ϕ s ($N = 3$ exp., $P = .003$) from female rats. CO-M ϕ s also demonstrated a 1.76-fold increase in HMGB1 release vs air-M ϕ s ($P = .004$). These findings were similar to that obtained with male M ϕ s (see Fig. 4A and 6A) and support that the effect of inhaled CO is not sex specific and is generalizable.

CO induces a prolonged anti-inflammatory phenotypic shift in M ϕ s:

Peritoneal M ϕ s were collected from mice at different time points (1hr, 1d, 3d, 5d) following inhaled CO or from control mice. Unstimulated CO-M ϕ s and air-M ϕ s had a similar proportion of M1 (CD86 and MHCII) and M2 (CD206) cells at all time-points (Fig. 7A-C). Following LPS stimulation, the number of CD86 positive cells was significantly reduced in CO-M ϕ s compared to air-M ϕ s and this difference persisted for up to 5 days after inhaled CO treatment (Fig. 7A). There was no change in MHCII expression in air-M ϕ s or CO-M ϕ s following LPS treatment (Fig. 7B). CD206 expression was increased by LPS in air-M ϕ s and CO-M ϕ s (Fig. 7C). However, CO-M ϕ s exhibited significant upregulated CD206 at the 1-day time point (Fig. 7C). These findings support a prolonged phenotypic shift in M ϕ s following

inhaled CO treatment with increased M2 phenotype at 1-day and inhibited M1 phenotype for up to 5 days.

Vasoprotective effects of inhaled CO are reversed by M ϕ depletion:

We previously reported that a brief treatment with inhaled CO dramatically inhibited vascular injury induced IH in rats⁶ and pigs⁷. Because of the changes in M ϕ phenotype induced by inhaled CO, we hypothesized that the vasoprotection provided by inhaled CO is mediated, at least in part, by M ϕ s. To test this, we transiently reduced monocyte/M ϕ with clodronate liposomes²⁴. Control rats were treated with empty liposomes. Clodronate reduced the absolute monocyte count by 57% compared to empty liposome treated rats by day 4 (115 ± 22 vs 265 ± 26 , respectively; $N=4-5$; $P=.005$) (cell count data expressed as $N \times 10^3/\mu\text{L}$). There was evidence of recovery at day 7 (147 ± 24 vs 230 ± 41 , $P=\text{NS}$). Clodronate can also affect other inflammatory cells. However, neutrophils (1145 ± 398 clodronate vs 784 ± 108 empty liposome; $P=\text{NS}$) and lymphocytes (4420 ± 643 clodronate vs. 6374 ± 661 empty liposome; $P=\text{NS}$) were not significantly reduced by the clodronate liposome at day 4. At the estimated nadir of the M ϕ count, rats were treated with inhaled CO followed by carotid artery balloon injury. At 2wks after balloon injury, CO treated rats exhibited a ~50% reduction in IH compared with air treated rats (Fig. 8A,B), similar to our prior report in naïve rats⁶. In air treated rats, there was no difference in IH formation between empty liposome and clodronate treated rats, indicating that transient inflammatory cell reduction did not impact neointima formation. However, the vasoprotective effects of inhaled CO were abolished in the clodronate treated rats (Fig. 8B). To confirm that the monocytes/M ϕ s did reconstitute to participate in the injury response, carotid arteries were examined at day 4 after arterial injury (7 days after clodronate) and abundant CD68 immunostaining was detected in the perivascular tissues (Fig. 8C). There was no difference in the number of CD68 positive cells between CO and air treated groups regardless of M ϕ depletion status (Fig. 8D).

Discussion

CO has been shown in numerous models of local tissue injury and systemic inflammation to dramatically blunt inflammatory responses^{3, 6-13, 16, 19, 25}. We have previously reported that inhaled CO markedly reduced neointima formation following arterial injury in rats and pigs^{6, 7}. What is remarkable about these and other studies is that the protection afforded by CO is achieved with a short exposure to a modest and safe dose of CO, suggesting the ability of CO to create a prolonged anti-inflammatory milieu. In this report, we examined the in vivo mechanisms of CO action because these are most relevant to understanding its ability to attenuate injury and disease. Hgb in RBCs can serve as a circulating reservoir for CO for about 2–3 hrs after CO inhalation. Vanova et al.²⁸ showed that this may be even shorter at the tissue level. Despite the short half-life of CO in the blood and tissue, the anti-inflammatory effects persisted up to 12hrs in their cholestasis model. We also observed sustained effects of inhaled CO with the ability of plasma from CO treated rats to inhibit SMC proliferation up to 24hrs after CO inhalation. These findings start to reveal a mechanism for the marked protective effects of a brief treatment of inhaled CO in models of disease⁶.

The M ϕ is a key cell type involved in most inflammatory conditions²⁹, including atherosclerosis and IH^{30, 31}. There are numerous reports on the ability of CO to promote potent anti-inflammatory responses in immortalized as well as primary M ϕ s in vitro^{11, 12, 15, 17, 18}. We observed a striking difference in the behavior of M ϕ s exposed to systemic CO in vivo versus cells directly treated with CO. While no change in baseline cytokine production was detected in M ϕ s isolated from CO treated rats, these cells responded to LPS with a mixed inflammatory/anti-inflammatory output of cytokines in sharp contrast to a purely anti-inflammatory response in cells directly exposed to CO^{11, 12, 15, 17, 18}. This is best illustrated by LPS stimulated iNOS and IL6 production which is inhibited in M ϕ s exposed to CO in culture^{11, 32} while CO-M ϕ s exhibited enhanced iNOS expression and IL6 production. The upregulation of IL6 and iNOS may appear to contradict the anti-inflammatory and protective function of CO but both have been shown to play important roles in attenuating cardiovascular injury^{33, 34}. It can be postulated that inhaled CO may induce systemic hypoxia due to its greater binding affinity for Hgb which may then mediate the divergent M ϕ responses. However, culturing M ϕ s under hypoxic conditions did not reproduce the effects of inhaled CO but instead resembled the responses observed in M ϕ s exposed to ex vivo CO. The similarity in M ϕ s responses to ex vivo hypoxia and ex vivo CO may be explained by ability of hypoxia to induce HO-1 expression³⁵ and endogenous CO production. Inhaled CO induced changes in M ϕ behavior that were sustained for days while cells treated with CO in vitro only exhibited changes during CO exposure. The prolonged CO-induced alterations in M ϕ function is supported by the distribution of M1 (proinflammatory) and M2 (anti-inflammatory) phenotypes in response to LPS with an early increase in M2 responsiveness and a sustained down-regulation of M1 responses up to 5 days. These findings further highlight the distinct actions of systemically administered CO that cannot be overlooked when evaluating its translational potential. This is the first report that defines the dramatic differences between the direct and systemic effects of CO.

Weigel et al. reported the ability of inhaled CO or CORMs to accelerate reendothelialization of injured femoral arteries in mice²³ through endothelial progenitor cell mobilization. In our study, M ϕ s from rats treated with inhaled CO have significant pro-endothelial function. CO-CM increased HUVEC proliferation and angiogenic activity. VEGF is known to be up-regulated after CO exposure through increased HO-1 expression in many cell types including ECs³⁶, astrocytes³⁷, and cardiac myocytes³⁸. We demonstrated that M ϕ derived CO-CM was enriched for VEGF. In addition, HMGB1, which promotes EC proliferation and angiogenesis²⁶, was also increased in CO-CM. Inhibition of VEGF or HMGB1 blocked CO-CM effects on ECs. Downstream signals of VEGF and HMGB1 include eNOS and NO production that contributed to the pro-endothelial behavior. While CO has been shown to independently increase eNOS expression on ECs^{23, 39}, our findings suggest that VEGF and HMGB1 derived from M ϕ s may contribute to eNOS upregulation in vivo.

CO has been shown to signal through p38 MAPK, upregulation of HIF1 α , the induction of HO-1 in a feed forward mechanism, and alterations of mitochondrial respiration^{6–13, 15–19}. Following inhaled CO, we detected baseline activation of NF κ B and STAT3 in the M ϕ s. NF κ B activation regulated the release of HMGB1 by the CO-M ϕ s. The ability of intravenously administered CORMs to activate these transcriptional factors has been reported in myocardial ischemia/reperfusion⁴⁰ while CO activated NF κ B in cultured primary

hepatocytes where it served in an anti-apoptotic role⁴¹. There are also reports that CO inhibits these transcriptional factors⁴². The divergent effect is likely related to the activation state of the cells. Cells exposed to inflammatory stimuli tend to exhibit inhibition of NF κ B and STAT3 in response to CO⁴². Another important response in the CO-M ϕ s is HO-1 upregulation. The ability of CO to feedforward to increase HO-1 expression has been documented in the setting of injury in vivo and stressed cells in vitro. In the CO-M ϕ s, HO-1 expression was regulated by STAT3. Inhibition of HO-1 with SnPP reduced HMGB1 release from CO-M ϕ s but HO-1 activation by CoPP in isolation was not sufficient to stimulate HMGB1 release. From these data, we created Figure 9 to illustrate the pathways that converge to mediate the effects of inhaled CO on M ϕ behavior. What remains unclear is how systemically delivered CO initiates and sustains these inflammatory changes.

Based on the M ϕ changes induced by inhaled CO, we hypothesize that M ϕ s mediate, at least in part, the anti-inflammatory and vasoprotective actions of inhaled CO. We utilized clodronate liposomes to transiently deplete M ϕ s at the time of CO therapy²⁴ to reduce M ϕ at the time of CO exposure but allowed them to recover to participate in the vascular injury response. This study design was essential because of the important contribution of M ϕ s to the development of IH³¹. Indeed, this transient depletion strategy did not impact IH. It has been reported in a model of acute pancreatitis that CORM-2 can inhibit monocyte recruitment to a site of injury¹⁴. Immunostaining at 4 days after balloon injury confirmed abundant monocyte/M ϕ staining in the carotid artery, demonstrating that CO did not alter M ϕ recruitment, similar to findings in the setting of peritonitis where inhaled CO minimally impacted M ϕ recruitment but increased M ϕ uptake of apoptotic polymorphonuclear leukocytes⁴³. While transient M ϕ depletion did not alter the vascular injury response, the reduction of monocytes/M ϕ s during CO treatment reversed CO mediated vasoprotection. This solidifies the pivotal role M ϕ s play in the in vivo mechanisms of CO mediated anti-inflammation. While M ϕ s have been recognized in many other studies to be an important target for CO actions in vivo, this is the first demonstration that CO's protective actions require M ϕ s. While we and others have reported that localized tissue treatment with CO gas or CORM is protective against vascular injury and ischemia reperfusion^{39, 40}, our current findings suggest that the systemic actions of inhaled CO may be more effective by targeting multiple facets of the injury response ranging from local cellular signaling, pro-endothelial effects, to modulation of systemic inflammatory pathways. One limitation of clodronate depletion is that it is not specific to the mononuclear cells. We observed an approximately 30% reduction in lymphocytes but not a significant change in neutrophils at 4 days after clodronate. While the impact was the greatest on monocytes with a 56% reduction, we cannot discount the potential contribution of other inflammatory cell populations. Future studies will examine the impact of CO on the other inflammatory cells.

Our results raise important questions. First, how does inhaled CO induce the baseline phenotypic changes in M ϕ s? Because these changes cannot be reproduced in vitro, systemic CO may signal through a central target that initiates a cascade of signals that terminate in the M ϕ s and potentially other inflammatory cells (Fig. 9). This would explain the divergent responses elicited from these cells in the setting of in vitro versus in vivo CO treatment. A second question is how does systemic CO mediate a sustained alteration of M ϕ responsiveness up to 5 days after exposure? Candidate pathways include epigenetic

modifications⁴⁴ and central signaling pathways^{44, 45} that mediate persistent changes in transcriptional responses to inflammatory perturbations. These questions are the focus of ongoing investigation.

From a translational perspective, our studies provide rationale for the development of inhaled CO as a therapy for a variety of inflammatory conditions. Systemic CO does not appear to change the ability of monocytes/Mφs to target the site of injury but the function of these cells at the site is greatly altered, favoring a healing phenotype through both inflammatory and anti-inflammatory pathways. The duration of these changes far exceeds the direct CO effects on cell signaling and are achievable with a brief and safe exposure to inhaled CO. These are extremely favorable properties and support inhaled CO as a therapy for vascular and other diseases.

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Abbreviations:

CO	Carbon monoxide
HO1	Heme oxygenase 1
HMGB1	High mobility group box 1
iNOS	Inducible nitric oxide synthase
IL6	Interleukin 6
LPS	Lipopolysaccharide
Mφ	Macrophage
NO	Nitric oxide
PPM	Parts per million
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial cell growth factor

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Highlights

- Systemic effects of inhaled carbon monoxide on macrophages cannot be reproduced by direct carbon monoxide exposure.
- The systemic effects of inhaled carbon monoxide on macrophage behavior persists for days.
- The vasoprotective effects of inhaled carbon monoxide are mediated through monocyte/macrophages.

Figure 1:

Inhaled CO induces changes in plasma and Mφs. (A) Plasma was collected from inhaled CO (250 PPM for 1hr) treated rats at 1, 24 or 48hrs after treatment and used to treat rat SMC. Plasma collected at 1hr inhibited SMC proliferation to a modest degree but the antiproliferative effects significantly increased with plasma collected 24 and 48hrs after CO treatment (N=6 exp). (B) Peritoneal Mφs were isolated 1hr after inhaled CO treatment and cultured. Nonadherent cells were removed and fresh media and LPS (100ng/ml) were added. LPS stimulated TNFα release (N>4 exp) in control Mφs. Mφs from CO treated rats responded to LPS with significantly reduced TNFα production. (C) LPS induced IL6 production was greatly enhanced in cells from CO treated rats compared to other groups. (D) IL10 was upregulated by LPS in Mφs from CO treated rats. (E) LPS induced NO production and (F) iNOS expression were increased in Mφs from CO treated rats compared to control rats. In contrast, Mφs exposed to CO in vitro had reduced LPS induced iNOS expression (F).

Figure 2:

Inhaled CO preserves Mφ function. (A) CO-Mφs demonstrated increased phagocytosis compared to air-Mφs and to cells treated with CO in vitro (N=5 exp). (B) CO-Mφs showed increased NFκB and STAT3 activation compared to air-Mφs (P<.001 and P=.06, respectively; N=4 exp). LPS further activated NFκB and STAT3 in CO-Mφs (P<.002 vs. CO-Mφs and P<.001 vs. air-Mφs+LPS for IκB; P=.003 vs. CO-Mφs and P=.022 vs. air-Mφs +LPS for P-STAT). (C) LPS treated CO-Mφs exhibited increased oxygen consumption but no increase in (D) anaerobic metabolism/glycolysis as compared to air-Mφs (N=3/treatment, 4 exp). (E) LPS treated CO-Mφs increased OCR in the setting of electron transport chain uncoupling with FCCP while LPS treated air-Mφs were unable (*P<.05 vs other groups).

Figure 3:

M ϕ s from CO treated rats release pro-endothelial factors. Peritoneal M ϕ s isolated from CO-rats or air-rats were cultured and the conditioned media (CO-CM and air-CM) were used to treat HUVECs. (A) CO-CM increased HUVEC proliferation by 53% compared to air-CM (N=3 exp). (B) CO-CM stimulated HUVEC angiogenesis in Matrigel versus air-CM (N=9 exp). (C) Representative photomicrographs show endothelial tube formation in Matrigel in cells treated with air-CM or CO-CM.

Figure 4:

Inhaled CO induces Mφs secretion of VEGF and HMGB1. (A) Peritoneal Mφs collected 1hr after inhaled CO treatment were cultured overnight and CM collected. CO-CM had increased levels of VEGF and HMGB1 compared to air-CM (N=4 exp). (B,C) Inhibition of NFκB or STAT-3 reduced HMGB1 release by CO-Mφs. CO-CM stimulated HUVEC proliferation was reversed by (D) VEGF or (E) HMGB1 inhibition (N=6–7 exp). CO-CM mediated angiogenesis behavior was also reversed by (F) VEGF or (G) HMGB1 inhibition (N=4–6 exp). Nonspecific IgG did not alter the effects of CO-CM. Anti-HMGB1 significantly reduced angiogenesis in both air-CM and CO-CM treated HUVECs (*P 0.001 vs CO-CM and CO-CM+IgG; **P=.016 vs air-CM and air-CM+IgG groups).

Figure 5:

CO-CM stimulates eNOS phosphorylation in HUVECs. (A) Two representative Western blots for P-eNOS in HUVECs treated with CO-CM or air-CM showed greater P-eNOS levels in cells treated with CO-CM (1.56 ± 0.14 -fold increase vs. air-CM, $N=7$ exp; $P=.002$). (B) eNOS inhibition with L-NAME reversed CO-CM mediated HUVEC proliferation but did not impact proliferation in air-CM treated cells ($N=5$ exp). (C) A representative Western blot analysis and quantification (D) shows that VEGF inhibition with Axitinib reduced P-eNOS in HUVECs treated with air-CM and reversed the effects of CO-CM ($N=4$ exp). (E,F) Western blot shows that anti-HMGB1 similarly reversed CO-CM mediated increase in P-eNOS ($N=3$ exp).

Figure 6:

Effects of inhaled CO may be mediated by HO-1 expression in the peritoneal Mφs but cannot be reproduced by hypoxia. (A) Western blot analysis shows 3.65±0.55-fold increase in HO-1 compared to air-Mφs (P=.003; N=3 exp). (B, C) HO-1 up-regulation in CO- Mφs was reversed with STAT3 inhibitor S31-201 (N=3 exp) but not affected by NFκB inhibition. (D, E) Treatment of CO-Mφs with SnPP (50μmol/L) inhibited HMGB1 release but did not change VEGF release. Stimulation of HO-1 activity with CoPP in air-Mφs did not increase HMGB1 release (N=4 exp). (F) Western blot analysis of CM shows that peritoneal Mφs from rats treated with inhaled CO released HMGB1 but Mφs exposed to ex vivo CO or hypoxia (1% O₂) did not (representative of 3 exp.). (G) LPS treatment of Mφs from inhaled CO treated rats showed increased NO production by Griess reaction compared with Mφs from air treated rats (N=4). Air-Mφs treated with ex vivo CO or hypoxia responded to LPS

with reduced NO production (*P<0.001 vs. all other groups; **P<0.001 vs. Mφs from air treated rats; ***P<0.05 vs. Mφs treated with ex vivo CO).

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Figure 7:

Inhaled CO alters the inflammatory responsiveness of peritoneal Mφs for a prolonged period of time. Peritoneal Mφs were isolated from control mice or mice treated with inhaled CO for 1h and then returned to room air for 1hr to 5ds. Mφs were labeled with F4/80 and markers of M1 (CD86 and MHCII) or M2 (CD206) phenotype. At baseline, there was no difference in the expression of any of these markers between cells isolated from CO or air treated mice (A-C, black bars). (A) Following LPS (100ng/mL) treatment, Mφs from CO-mice showed a prolonged suppression of CD86 expression lasting to at least 5ds after CO exposure (* $P < .001$ vs. all other groups; ** $P < .05$ vs. air, CO 1hr, and CO 1–3d $\pm$ LPS; $N = 3-6$ mice/time point). (B) There was no change in MHCII expression following LPS treatment. (C) CD206 expression was transiently upregulated in cells isolated 1d after CO treatment (*** $P < .001$ vs. all other groups, $N = 3-6$ mice/group).

Figure 8:

Transient M ϕ depletion at the time of inhaled CO treatment reversed CO mediated vasoprotection. (A) Representative photomicrographs showing the neointima formation 2wks after rat carotid artery injury and 17days after transient M ϕ depletion by autofluorescence. (B) Quantification of neointima 2wks after carotid artery injury is expressed by intimal area/medial area (N=6–7 rats/group). (C) Representative immunofluorescence of carotid section from M ϕ depleted rat 7ds after depletion and 4ds after inhaled CO and carotid injury (blue=DAPI; red=CD68) showing significant M ϕ recruitment to the artery wall (top panel showing primary antibody delete, bottom panel showing CD68 staining). (D) Quantification of CD68 positive cells in the carotid artery sections of Air and CO treated rats with/without M ϕ depletion at 7ds following depletion and 4ds following balloon injury showed no difference between groups (N=4 rats/group; P=NS).

Figure 9:

Diagram of pathways involved in inhaled CO mediated changes in inflammatory function. Inhaled CO may activate central signaling pathways or epigenetic regulation to modify M ϕ behavior, leading to the activation of NF κ B and STAT3 and the induction of HO-1. These changes result in prolonged altered inflammatory responsiveness with reduced M1 populations. These pathways mediate the release of VEGF and HMGB1 that stimulate pro-endothelial actions independently as well as through eNOS activation.