Nitric Oxide–Dependent Bone Marrow Progenitor Mobilization by Carbon Monoxide Enhances Endothelial Repair After Vascular Injury

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Background—Carbon monoxide (CO) has emerged as a vascular homeostatic molecule that prevents balloon angioplasty–induced stenosis via antiproliferative effects on vascular smooth muscle cells. The effects of CO on reendothelialization have not been evaluated.

Methods and Results—Exposure to CO has diametrically opposite effects on endothelial cell (EC) and vascular smooth muscle cell proliferation in rodent models of carotid injury. In contrast to its effect of blocking vascular smooth muscle cell growth, CO administered as a gas or as a CO-releasing molecule enhances proliferation and motility of ECs in vitro by >50% versus air controls, and in vivo, it accelerates reendothelialization of the denuded artery by day 4 after injury versus day 6 in air-treated animals. CO enhanced EC proliferation via rapid activation of RhoA (Ras homolog gene family, member A), followed by downstream phosphorylation of Akt, endothelial nitric oxide (NO) synthase phosphorylation, and a 60% increase in NO generation by ECs. CO drives cell cycle progression through phosphorylation of retinoblastoma, which is dependent in part on endothelial NO synthase–generated NO. Similarly, endothelial repair in vivo requires NO-dependent mobilization of bone marrow–derived EC progenitors, and CO yielded a 4-fold increase in the number of mobilized green fluorescent protein–Tie2–positive endothelial progenitor cells versus controls, with a corresponding accelerated deposition of differentiated green fluorescent protein–Tie2–positive ECs at the site of injury. CO was ineffective in augmenting EC repair and the ensuing development of intimal hyperplasia in eNOS−/− mice.

Conclusions—Collectively, the present data demonstrate that CO accelerates EC proliferation and vessel repair in a manner dependent on NO generation and enhanced recruitment of bone marrow–derived endothelial progenitor cells. (Circulation. 2010;121:537-548.)

Key Words: angioplasty; balloon; endothelium; nitric oxide synthase; signal transduction; stenosis

Proliferation of vascular smooth muscle cells (VSMCs) and their acquisition of a proinflammatory phenotype are central events in the pathogenesis of vascular lesions, including vein occlusion, postangioplasty restenosis, and transplant arteriosclerosis.1–3 Restenosis rates at 1 year approach 30% without stents versus 5% in patients who receive stents. More than 95% of percutaneous coronary interventions involve stenting.4–6 Although stents hold great promise, there continues to be a need for advances in current therapies. Drug-eluting stents are now used as novel drug-delivery devices, including rapamicin/sirolimus-coated devices that further reduce stenosis by interfering with VSMC proliferation.7,8 Drug-eluting stents, however, are complicated by in-stent thrombosis that results from delayed endothelialization if antiplatelet therapies are stopped. Appropriate reendothelialization of a stent or denuded vessel becomes crucial for effective vascular homeostasis. In the present study, we hypothesized that carbon monoxide (CO) administered as an inhaled gas or via a CO-releasing molecule would provide vascular protection by facilitating endothelial cell (EC) repair and preventing intimal hyperplasia. We demonstrated previously that a 1-hour exposure to CO at low, nontoxic concentrations before injury, with no further treatment, prevented the development of intimal hyperplasia caused by balloon angioplasty via direct effects on VSMC proliferation.9 Growth arrest in these cells occurred via a pathway that sequentially involved cGMP, p38 mitogen-activated protein kinase, and p21. Interference at any point in this cascade resulted in abrogation of the effects of

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CO. CO, like nitric oxide (NO), is pleiotropic in its effects, modulating cellular behavior and physiology in diverse ways depending on the cell type, circumstance, and model being evaluated. The end result, however, is that CO functions to reestablish vascular stability.

We hypothesized that the endothelium and more specifically the ECs that are physically injured through denudation trauma during the balloon procedure are a target by which CO would exert beneficial effects. Heme oxygenase-1 (HO-1), the cytoprotective enzyme responsible for the generation of endogenous CO, was shown to regulate EC proliferation in vitro; however, the role and the mechanism of action of HO-1 and CO in EC repair after trauma have not been evaluated. HO-1-deficient mice show an exaggerated response to vessel injury. We reasoned that CO, in addition to having an antiapoptotic role in ECs, would enhance proliferation and migration of ECs and promote vessel repair by facilitating mobilization of endothelial progenitor cells (EPCs). The knowledge that NO promotes the survival and proliferation of ECs and mobilization of EPCs prompted us to investigate the hypothesis that endothelial NO synthase (eNOS) may play a role in the effects of CO on ECs.

In the present study, we describe the effects of both CO and a CO-releasing molecule (CORM) on the augmentation of EC proliferation and migration and EPC mobilization to the injured site in well-established rodent models of vascular trauma. We show that CO accelerates reendothelialization of the injured vessel, and we identify eNOS and NO as essential for the CO effect. In ECs, activation of Akt, eNOS, and retinoblastoma (Rb) protein dominate to accelerate cell cycle progression and EC migration. The diametrically opposite effects of CO on proliferation of ECs and VSMCs are clear evidence of the pleiotropic homeostatic effects of CO, in this case preventing the development of the intimal lesion by acting through disparate modes of action on these 2 vascular cell types.

Methods
Cell Culture and Pharmacological Reagents
Rat primary aortic ECs (RAECs) were purchased from VEC Technologies (Rensselaer, NY) and maintained in MCDB-131 Complete medium with antibiotics and growth factors (VEC Technologies) on 0.2% gelatin-coated plates. Cells were used between passages 3 and 10. Bone marrow–derived EPCs from Tie2–green fluorescent protein (GFP) mice (Tie2 receptor is expressed in endothelial lineage cells) were cultured in Endothelial Basal Medium (EBM-2) supplemented with EGM-2 BulletKit (Clonetics-Lonza, Rockland, ME) as described previously. Cells were exposed to 250 ppm CO, 5% CO2, 21% O2, 10 mmol/L sodium nitroprusside (NEN, Boston, Mass) for 24 hours with or without CO as indicated.

NO Generation
The effect of CO on proliferation of RAECs was determined with a nonradioactive bromodeoxyuridine-based cell-proliferation assay (per the manufacturer’s guidelines; Roche, Basel, Switzerland). Thymidine incorporation was measured in growth-arrested VSMCs and ECs stimulated to proliferate with 10% FBS in the presence of 5 μCi/mL of 3H-thymidine (NEN, Boston, Mass) for 24 hours with or without CO as indicated.

Cell Motility and Migration
Cell motility and migration are described in the Methods section of the online-only Data Supplement.

RhoA Activation
Activity of the small GTPase RhoA was determined by use of an EZ-Detect Rho Activation Kit (Pierce, Rockford, Ill) according to the manufacturer’s protocol.

Immunoblotting and Phalloidin
Details concerning immunoblotting and the use of phalloidin are provided in the online-only Data Supplement.

Fluorescence
RAECs were loaded with the NO-selective fluorophore 4-amino-5methylaminon-2',7'-difuorescein diacetate (DAF-FM; Molecular Probes, Carlsbad, Calif) 20 minutes before exposure to air or CO (250 ppm) for 5 to 60 minutes. Cells were fixed, and fluorescence was assessed by flow cytometry with excitation/emission of 495/515 nm at various time points.

Chemiluminescence
Human umbilical vein ECs were plated onto 24-well plates and treated for 1 hour in the presence and absence of CO gas (250 ppm), and NO was measured in cell supernatants with a Sievers chemiluminescence NO analyzer as described previously.

Carotid Artery Injury Model in Rats and Mice
Male Sprague Dawley rats (weight 250 to 300 g) were purchased from Harlan Laboratories (Indianapolis, Ind), and mice were purchased from Jackson Laboratories (Bar Harbor, Me). Balloon angioplasty and wire injury were performed as described previously. Injury of the vessel wall and subsequent pathological analysis were accomplished in a manner that was blinded to the treatment group.
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and was performed by trained scientists. Male C57BL/6 (Jackson Laboratories, Bar Harbor, ME), eNOS−/− mice (7 to 8 weeks old), and Tie2-GFP mice were purchased from Jackson Laboratories. Mice were treated with CO (250 ppm for 1 hour), ALF421 (10 mg/kg IP 1 hour before and 1 hour after), or iCORM. Water or air was used as a control. All animals were housed in accordance with the guidelines of the American Association for Laboratory Animal Science. The carotid injury and bone marrow transplantation protocols were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Immunostaining and Cell Population Histogrammetric Analysis

Vessels were harvested 1, 3, and 5 days after angioplasty. Rat leukocyte and EC populations were detected with anti-rat macrophage (CD68, ED1), intercellular adhesion molecule-1 (CD54; 1A29), myeloperoxidase, and CD31 antibodies. Mouse vessels were stained with hematoxylin and eosin, Sca1+, and CD31 as described previously.22 Eight to 10 images were captured from each injured carotid and analyzed as detailed above. Intima/media ratio was measured as described previously.9 RAECs were seeded on microscopic slides and treated with or without CO for 24 hours. Cell staining with P-histone H3 antibodies was applied as described previously.22

Bone Marrow Transplantation and Generation of Tie2-GFP Chimeras

Wild-type C57BL/6J (Jackson Laboratories) mice were lethally irradiated (12 Gy). On the same day, mice were injected with 5 × 106 bone marrow cells from Tie2-GFP mice (as above). Reconstitution of bone marrow was determined by flow cytometry 1 month after transplantation. Tie2-GFP chimeras were used for the experiments as described above. The average percent reconstitution was determined to be between 90% and 95%, with 0.9% to 12% Tie2-GFP-positive cells in the marrow 4 to 6 weeks after transplantation.

Colony Outgrowth Assay

Colony outgrowth assay was performed as described previously.23 C57B6 mice were exposed to CO (250 ppm) or air for 1 hour, and blood samples were collected. Mononuclear cells were freshly isolated with Ficoll gradient (20 minutes, 500 g) and seeded on the 0.2% gelatin-coated plates in EGM endothelial growth medium supplemented with an EGM Bullet Kit (Clonetics Lonza, San Diego, Calif). Colonies were counted and photographed 10 days after isolation of mononuclear cells.

Flow Cytometry

To assess the percentage of GFP-positive ECs that were mobilized to the circulation after CO/air treatment, blood samples were harvested from animals 12 hours after wire injury. Red blood cells were lysed at room temperature for 5 minutes with erythrocyte lysis buffer, followed by washing with PBS. Cells were fixed with 2% paraformaldehyde and blocked with 1% BSA in PBS followed by staining with antibody against GFP (Invitrogen, Carlsbad, Calif) for 1 hour at room temperature. Secondary antibody conjugated with fluorescein was applied for 1 hour at room temperature. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The chimeric mice were housed in the Beth Israel Deaconess Medical Center facility according to Institutional Animal Care and Use Committee–approved protocols. The average percent reconstitution was determined to be 0.9% to 1.2% GFP positive cells in the marrow 4 to 6 weeks after transplantation.

Statistical Analyses

In all of the in vitro experiments, the significance of difference was determined with 1- or 2-way ANOVA (with post hoc Tukey test) as described in the figure legends (SPSS Inc, Chicago, Ill). A nonparametric Wilcoxon test was used where indicated. Statistical analyses were applied to all of the independent experiments, with significance accepted at P < 0.05.

Results

CO Augments EC Proliferation

To test the effects of CO on EC proliferation, primary RAECs were exposed to CO (250 ppm), and [3H]thymidine or bromode-
CO Increases EC Motility
Using live cell time-lapse microscopy, we next evaluated the effects of CO exposure on EC motility. Using the well-described “scratch” model, we disrupted an endothelial monolayer to simulate EC denudation, to mimic which occurs during angioplasty trauma, and exposed the cells to media saturated with air or 250 ppm CO. In those cells exposed to CO, we observed a 2-fold greater motility rate than in air controls (Figure 2A). These data demonstrate that CO not only increased proliferation of ECs (Figure 1) but also increased their motility. Immunostaining for F-actin showed that ECs exposed to CO had a greater propensity for cytoskeletal organization that correlated with motility measurements (Figures 2B and 2C [air] versus Figures 2D and 2E [CO]). CO-treated cells showed a highly organized actin distribution, with stress fibers forming dense peripheral and polarized bands at the leading edge, which was otherwise irregular and punctate in air-treated cells.

CO Activates RhoA and Akt Kinase in ECs, Which Regulates CO-Induced Proliferation
We next attempted to elucidate the signaling mechanisms that were influenced by CO to control EC proliferation. Owing to the high diffusivity of CO, we hypothesized that CO would elicit a rapid effect on the cell initiated at the cell membrane. We assessed expression of the small GTPase RhoA, which is important in cell growth and cytoskeletal organization and which is an initiator of downstream signaling events. Exposure of ECs to CO resulted in a strong and rapid time-dependent activation of RhoA (Figure 3A). Downstream of RhoA are the mitogen-activated protein kinases, as well as Akt, both of which have been shown to be modulated by CO. We first evaluated p38 activation, which was decreased modestly (data not shown), in direct contrast to the effects of CO observed in VSMCs. Akt has been shown by others to be activated by CO, albeit in an inconsistent fashion depending on the cellular model. One report demonstrated in human ECs that CO inhibits Akt activation, whereas another report showed that CO increases Akt in heart tissue from rats undergoing ischemia/reperfusion injury. We therefore wanted to assess the effects in our model. In RAECs exposed to CO, we observed a time-dependent induction of Akt phosphorylation (Figure 3B; online-only Data Supplement Figure IIB). CO induces proliferation of ECs through activation of RhoA and Akt (Figure 3C). The effect of CO on Akt activation was abrogated in the presence of dominant-negative mutant RhoA (data not shown). Using a selective and well-characterized pharmacological inhibitor of PI3K (LY290024), we evaluated the role of PI3K-dependent Akt on CO-induced proliferation of ECs. Blockade of PI3K led to a partial inhibition of the effects of CO on phosphorylation of Rb (Figure 3D) and EC proliferation (Figure 3E). To strengthen our observation that Akt was linked to enhanced EC proliferation by CO, we transiently transfected RAECs with a dominant-negative mutant Akt expression vector (Figure 3F). When both pharmacological and genetic methodologies were used to block Akt, the effects of CO on augmentation of proliferation were lost in cells without functional
Akt signaling (Figures 3D through 3F) versus controls. Finally, we examined the role of RhoA on CO-induced proliferation and observed a partial reversal of the CO effects in RAECs transduced with a dominant-negative mutant RhoA (Figure 3G).

**CO Increases Expression of eNOS and NO Generation in RAECs**

We have shown in previous work that there is an interrelation between the gases CO and NO, particularly in the vasculature. We hypothesized that 1 mechanism by which CO induces enhanced proliferation after injury involves effects on eNOS activation and NO generation given the relatively high expression of eNOS in ECs. RAECs exposed to CO gas or a CORM showed a time-dependent increase in phosphorylation of eNOS and NO generation in ECs as measured by immunoblotting, DAF fluorescence, and chemiluminescence (Figures 4A through 4E). Interestingly, CO gas showed slightly slower kinetics than the CORM, which we speculate may be due to the kinetics of gas exposure versus direct delivery of a CO releaser into the culture media. To validate the importance of this activation, we administered the selective NO synthase inhibitor N⁴-nitro-L-arginine methyl ester and evaluated proliferation by bromodeoxyuridine incorporation and phosphorylation of Rb. In the presence of NO high expression of eNOS in ECs. RAECs exposed to CO gas or a CORM showed a time-dependent increase in phosphorylation of eNOS and NO generation in ECs as measured by immunoblotting, DAF fluorescence, and chemiluminescence (Figures 4A through 4E). Interestingly, CO gas showed slightly slower kinetics than the CORM, which we speculate may be due to the kinetics of gas exposure versus direct delivery of a CO releaser into the culture media. To validate the importance of this activation, we administered the selective NO synthase inhibitor N⁴-nitro-L-arginine methyl ester and evaluated proliferation by bromodeoxyuridine incorporation and phosphorylation of Rb. In the presence of NO
blockade, CO was unable to impart proliferative effects, returning growth patterns to those of air controls (Figure 4F) and preventing increases in phosphorylated Akt and phosphorylated Rb (data not shown). These data support the interrelation of these gases in vitro in the regulation of proliferation of RAECs and suggest that CO not only increases eNOS phosphorylation but also influences its activity to generate NO and importantly to drive activation of Akt and Rb. The relation between NO, Akt, and Rb in ECs has been described in the literature in other models but to date has not been evaluated with CO in ECs. We describe here that CO clearly triggers activation of this pathway. NO has been shown to impart prosurvival effects in ECs. We conclude that by imparting prosurvival benefits to the EC, NO is critical in allowing CO to act via a RhoA–Akt–Rb cascade to augment proliferation.

CO Augments Reendothelialization After Balloon Angioplasty in Rats and Wire Trauma in Mice

CO can limit vascular occlusion, an effect driven primarily by reduced intimal thickening over the course of weeks. To date, the effects of CO treatment on early events that occur after injury within the first 3 to 5 days have not been evaluated. We exposed rats to either air or CO for 1 hour before angioplasty, as described previously, and evaluated the effects on reendothelialization after balloon trauma. Importantly, the animals were not exposed to CO again. We harvested vessels at 1, 3, and 5 days after injury and stained sections for CD31 and intercellular adhesion molecule, markers specific for ECs that are readily observed in uninjured vessels (Figure 5A). In animals exposed to air, the EC monolayer was absent at 1, 3, and 5 days (Figure 5B) after angioplasty but was fully restored by 7 days. In contrast, animals exposed to 1 hour of CO showed a complete restoration of the EC monolayer by 5 days (Figure 5C; 5 of 6 animals in the CO group versus 0 of 6 animals in the air group, \( P < 0.03 \)). In the same vessels, we evaluated the inflammatory response and observed increased macrophages (online-only Data Supplement Figure IIIA) and neutrophils (data not shown) infiltrating the lesion at day 3 to 5 after angioplasty, both of which were inhibited by CO. In vitro, both CO and CORM treatment effectively inhibited
transmembrane migration of U937 monocytes (online-only Data Supplement Figures IIIB and IIIC). We also evaluated the effects of CO on EC restoration in the murine model of wire trauma, a well-accepted surrogate for angioplasty. As was observed in rats, CO enhanced repair of the endothelium in mice, restoring the EC monolayer by day 4 (Figure 6A), whereas air controls showed little to no EC presence until days 5 and 6 after injury (data not shown). We also demonstrated that administration of the CORM ALF421 before and just after wire injury accelerated reendothelialization in the carotid artery, similar to CO gas (Figure 6B). The carboxyhemoglobin levels achieved with CORM were 11% versus 15% with 250 ppm for 1 hour. A separate cohort of mice were also treated with iCORM, which failed to enhance EC repair after injury (Figure 6B).

CO Requires NO to Enhance Reendothelialization

Our in vitro studies showing that CO increased NO generation in part through phosphorylation of eNOS prompted us to evaluate whether CO would enhance repair in the absence of eNOS in vivo. CO was unable to enhance reendothelialization in eNos<sup>−/−</sup> mice at 4 days, as opposed to results in CO-exposed wild-type mice (Figure 6C). To begin to assess a link between reendothelialization and development of intimal hyperplasia, we also evaluated the ability of CO to block the development of intimal hyperplasia in eNos<sup>−/−</sup> versus wild-type mice in the presence of CO, having demonstrated previously that CO can inhibit intimal hyperplasia in response to wire trauma. We validated that again here and additionally demonstrated that CO was unable to prevent intimal hyperplasia in the absence of NO (Figures 6D and 6E). We observed similar effects in rats in which NO was blocked by the administration of N<sup>ω</sup>-nitro-L-arginine methyl ester with or without CO (data not shown). Although this could be interpreted as NO also being absent in VSMCs in these animals, we showed previously that neither eNOS nor inducible NO synthase was important in the ability of CO to inhibit growth of VSMCs.

CO Increases Progenitor Cell Recruitment Into the Circulation and the Injured Vessel

Reendothelialization of injured vessels is thought to be driven in part by the influx of undifferentiated progenitor cells. We therefore tested the hypothesis that CO targeted and enhanced the influx of progenitor cells into the denuded site after injury via expansion of the bone marrow pool of ECs and mobilization of EPCs. Immunostaining of vessels from wire-injured mice showed that CO-exposed animals had a significantly higher number of sca1-positive cells at the site of injury than did air-treated controls (online-only Data Supplement Figure IV). Interestingly, the sca1-positive cells did not express CD34 at this time point, and we speculate that this phenotypic change occurs rapidly, particularly with CO and likely in the adventitia. We speculate that the enhanced recruitment is responsible in part for the more rapid infiltration and repair. Assessment of outgrowth colonies showed that CO enhanced the number of colonies by 6- to 8-fold versus control mice (P<0.001). We next exposed progenitor cells to CO in culture and observed that CO induced...
differentiation of progenitors into EPCs/ECs. Bone marrow progenitor cells purified from Tie2-GFP mice were exposed to CO for 3 days in culture. In these cells, Tie2, which is a specific EC promoter, is linked to GFP; therefore, GFP can be used as a surrogate marker for mature ECs (Figure 7A). Air-treated cells showed limited expression of GFP by day 3 in contrast to CO-treated progenitors, which showed a 2- to 3-fold increase in GFP expression versus air-treated cells (Figures 7B through 7D). As a control for specificity of EC proliferation, we also differentiated progenitors into macrophages by treating them with macrophage colony-stimulating factor and observed no effects of CO on GFP after differentiation (Figure 7B). These data thus support a direct effect of CO in inducing progenitor cells to differentiate into ECs. To recapitulate in vivo our observations in vitro, we generated Tie2-GFP chimeric mice and analyzed EPC mobilization into the blood after wire injury at 12 hours (Figures 8A and 8B). We selected this time point because we observed a strong recruitment of sca1-positive cells to the injured vessel after CO exposure at 12 hours (online-only Data Supplement Figure IV). Vessel injury alone induced recruitment of ~5% GFP-positive EPCs to the circulation (Figures 8A and 8B); however, pretreatment with CO before vessel injury induced a
further 2- to 5-fold enhancement of GFP-positive EPC mobilization into the blood (Figures 8A and 8B). Enhanced mobilization of bone marrow–derived progenitors after CO exposure for 1 hour was further confirmed by the performance of a colony outgrowth assay. Blood mononuclear cells were collected from mice exposed to air or CO and then cultured in EC media for 10 days after isolation. CO significantly increased colony numbers over air-treated cells, and in some of the wells, we observed tubule formation in response to CO (online-only Data Supplement Figure V). Finally, we assessed whether GFP-positive EPCs in the Tie2-GFP chimeric mice contributed to reendothelialization of the vessel after CO treatment. We detected GFP-positive ECs in the injured vessels after CO treatment at day 4 (Figures 8C and 8D). We detected no GFP-positive cells in air-treated mice because EC repair was not present at day 4 (Figures 8C and 8D). Collectively, these data suggest that CO-enhanced EC repair occurs in part via enhanced mobilization and differentiation of EPCs.

**Discussion**

The denudation of the endothelium caused by physical balloon trauma, combined with a rapid increase in leukocyte infiltration, leads to increased smooth muscle cell proliferation and formation of the neointima. The loss of the endothelium is perhaps the initiating element associated with subsequent vaso-occlusion. The direct effects of CO to induce growth arrest of VSMCs and prevent intimal expansion in vivo are clear, driven primarily by a cGMP–p38-p21 signaling pathway that is NO independent.\(^9,31\)

We hypothesized that CO treatment must initiate additional mechanisms involved in repair of the vessel that contribute to inhibition of intimal expansion after trauma. Namely, CO would modulate the acute inflammatory response (ie, leukocyte infiltration), as well as target the endothelium to facilitate the regeneration/replacement of this barrier lamina. We used both the clinically relevant balloon-injury model in rats and the wire-injury trauma model in mice, which permitted mechanistic experiments to test the effects of CO on the early events after vessel trauma.

The effects of CO in restoring homeostasis continue to implicate a critical role for NO. NO is a potent prosurvival factor in ECs, unlike VSMCs.\(^35,36\) We thus hypothesized that NOSIII/eNOS would be a likely target for CO in ECs to increase NO through direct binding to NO synthase or via specific signal transduction and would contribute to survival even if NO did not necessarily promote a proliferative response. Increased survival would then allow other signaling pathways, such as phosphorylation of Rb, to promote growth. The present data suggest that 1 mechanism by which CO promotes cell growth is through phosphorylation of eNOS and activation of Akt and Rb. The ability of CO to increase eNOS phosphorylation, however, may occur indirectly through an upstream potassium channel–mediated event that leads to activation of PI3K and Akt. Blockade of potassium channels resulted in a loss of the effects of CO on EC
proliferation (unpublished observation), and CO is known to function in part through activation of this channel.37

Cyclins and cyclin-dependent kinases induce hyperphosphorylation of Rb, liberating E2F and other transcription factors such as YY1 that play a pivotal role in the coordinated transactivation of cell cycle regulatory genes.24 We speculate that CO modulates growth and genome transcription at the level of histone and chromatin modification. CO-induced mitosis is blocked by the inhibitor of histone deacetylase, trichostatin (unpublished observation). The present results clearly demonstrate that exposure to CO in ECs leads to quick activation of the small G protein RhoA and accelerated entrance into the S phase, with increased phosphorylation of Rb that results in enhanced growth; however, we cannot exclude the possibility that CO activates other molecules involved in cytoskeletal organization and signaling. CO promotes migration of the ECs in the scratch assay. The exact mechanism by which CO modulates the cytoskeleton remains to be fully elucidated but likely involves the RhoA signaling machinery described herein, which is known to be involved in F-actin cytoskeleton reorganization and stress-fiber formation. The combination of the present observations in vitro and in vivo, including bone marrow progenitor cell recruitment to the site of injury, supports the concept that CO administered as a gas or a CORM fosters earlier reendothelialization and involves recruitment, differentiation, and motility of ECs in an effort to augment repair of the injured vessel, which ultimately contributes to less intimal hyperplasia. The CO-mediated benefit is sustained for more than 21 days despite the 1-time exposure of the animals to CO, which indicates that the process of vascular remodeling is determined in large measure early after acute injury. The kinetics of the events that lead to augmentation of repair are multifactorial and clearly reflect decreased inflammation, earlier EC deposition, and ultimately decreased hyperproliferation of VSMCs. In a model of pulmonary hypertension in rodents, we demonstrated that intermittent exposure to CO, initiated after the establishment of disease, results in reverse remodeling (ie, a return to original architecture and function).31 In these animals, CO induced ECs to generate NO, which ultimately led to restoration of normal artery and vessel size. In this instance, CO-induced NO arose from the ECs present in the vessels. In the data presented here, in which ECs were not present at the time of CO exposure, the origin of the ECs is likely circulating or recruited endothelial progenitors, given the present GFP data, or a significant contribution from the ECs immediately adjacent to the denuded lesion that proliferated and mobilized into the injured area, perhaps driven by an augmented chemokine gradient elicited by NO, such as stromal cell–derived factor (SDF). SDF has been demon-
strated to be involved in the ability of HO-1 to regulate angiogenesis.38 HO-1–deficient mice were unable to form capillary sprouts, which was reversed by administration of a CORM. The mechanism by which HO-1/C0 regulates angiogenesis is different from what we describe here and involves VASP (vasodilator-stimulated phosphoprotein) and PGE2 (prostaglandin E2) versus the Akt-eNOS-Rb pathway.12,38 Perhaps this speaks to the vast differences between the processes of neovascularization and EC repair. In this same vein, eNOS/NOSIII is essential for EPC mobilization.39 Neovascularization does not occur in mice lacking eNOS, which strongly supports the observations described here that CO requires NO to enhance reendothelialization of denuded vessels. From a therapeutic standpoint, these results, combined with our data showing that CO prevents neointima formation after trauma, combine 3 actions: (1) Antiinflammatory effects, (2) direct effects on VSMCs to block proliferation, and (3) proliferative actions on ECs that lead to rapid reendothelialization of the denuded EC. As such, exposure to CO results in greater efficacy in preventing neointima formation and stenosis after balloon angioplasty than other approaches aimed at the sole blockade of VSMC proliferation and stenosis after balloon angioplasty than other approaches aimed at the sole blockade of VSMC proliferation.40–42 One of the principal challenges with stent therapy is the inability of the stent to become endothelialized because the coatings also limit EC proliferation. The data presented here offer a potential therapeutic adjuvant involving treatment with CO gas or local delivery of a CORM to substitute or complement stent placement, perhaps even impregnating 1 of the emerging CORM molecules onto a stent. In principal, either mode of CO delivery would yield the same beneficial result. If the pulmonary data showing that exposure to CO can reverse intimal expansion without intervention hold true, the need for angioplasty and stents may be reduced, which would be particularly useful in the peripheral circulation, in which drug-eluting stents have not proven as efficacious, with restenosis rates approaching 30% at 1 year.

In conclusion, we demonstrate a novel function of CO in promoting reendothelialization that likely is the result of enhanced proliferation, recruitment, and migration of neighboring ECs. Additional experiments are under way to address both the detailed mechanism and whether chronic delivery of CO would be needed to prevent long-term restenosis and reduce the need for reintervention. Of course, with longer-term CO exposure, it will be necessary to carefully accrue additional safety data. Accelerated reendothelialization adds to the clinical vascular protective benefit achieved by short exposures to CO before angioplasty. The sooner reendothelialization has occurred in the vessel, the sooner the entry of circulating monocytes and T cells will be blocked, thereby limiting inflammation and subsequent vaso-occlusion.43 Finally, these data identify, delineate, and add to the growing database of the interrelation between the 2 gas molecules NO and CO, which act in tandem to reestablish homeostasis, in vascular proliferative diseases. With careful clinical testing, CO may prove to be a novel therapeutic agent in the treatment of numerous vascular disease syndromes.

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References

Carbon monoxide (CO) is recognized as a potent therapeutic molecule at low, nontoxic doses. CO is in phase II clinical trials to improve kidney function after transplant of a kidney allograft. Inhaled CO is known to block intimal hyperplasia. We demonstrate that CO prevents intimal expansion in mice in part by early and enhanced preconditioning. Collectively, our data support the use of CO as a therapeutic modality in the treatment of vascular proliferative disorders.
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**Supplemental Material**

**Supplementary Figure Legends**

**Supplementary Figure 1. The effect of CORM ALF 421 and hypoxia on the proliferation of RAEC.**

**A.** BrdU incorporation was used to assess the proliferation rate of CORM ALF 421 (1-20 µM) or vehicle treated RAEC cells measured at 24 hours. Data are representative of 2 independent experiments performed in triplicate. Two-way ANOVA, p<0.0001. CORM vs control. Tukey post-hoc; Control vs 1µM, p=0.505; vs 5µM, p=0.079; vs 10µM, **p<0.001; vs 20µM, **p<0.0001.

**B.** BrdU incorporation testing proliferation of RAEC cells exposed to normoxia (21%O₂) and hypoxia (1% O₂). The data are representative of 2 independent experiments in triplicate. One-way ANOVA *p<0.001 vs normoxia.

**Supplementary Figure 2.** Densitometric analysis of CO-treated RAEC at 4 hr for (A) P-Rb; one-way ANOVA, *p=0.003. and (B) P-Akt, one-way ANOVA, *p=0.04 and 10 min for (C) P-eNOS, one-way ANOVA, *p=0.001. Data represent mean ± SD of values obtained from 3 separate blots comparing CO vs Air.

**Supplementary Figure 3. CO blocks the migration of macrophages in vitro and in vivo.**

**A.** Immunostaining for the macrophage marker ED-1 of carotid arteries from air and CO-treated animals 4 days after injury. Note that CO blocks inflammatory cell influx into the injured vessel. Images are representative of 6-8 fields from 4-5 animals/group. Magnification is 40x. Arrows indicate positive staining. Scale bar=50 µm.

**B.** Transwell
migration of U937 monocytes treated with Air or CO for 24 hours. Data are representative of 2 independent experiments preformed in triplicate. One-way ANOVA **p<0.001.

C. Effect of CORM ALF 421 (10-50 µM) treatment on U937 macrophage migration in Boyden chambers. Cells were treated for 24 hours and the amount of cells that migrated to the lower chambers were counted. Data represent mean ± SD from 3 independent experiments (n=3/group). Two-way ANOVA; **p<0.001 CORM vs Control (C). Tukey post-hoc; Air vs 10µM, *p=0.04; vs 20µM, **p=0.009; vs 50µM, **p<0.0001.

Supplementary Figure 4. Carbon monoxide induced sca-1+ progenitor recruitment to the injured vessel in mice. Mice were treated with air or CO as described previously. Carotid artery segments were harvested 12 hrs after injury and sectioned and stained for the presence of sca1+ and CD31. Vessels from naïve, untreated mice are shown as control. Note that CO increased the number of sca1+ cells in the artery at 12 hr, which was not present in air-treated mice. Representative images are from 4-6 animals/group. Arrows indicate positive staining. Magnification is 20x, Scale bar=50 µm.

Supplementary Figure 5. Carbon monoxide induces recruitment of bone marrow cells to the circulation as measured by the colony outgrowth assay. A. Representative images of colony outgrowth (black arrows) 10 days after isolation of blood mononuclear cells from Air or CO (1h, 250ppm;) treated animals cultured in EC medium (n=4 mice/group). Upper Panels: Air-treated animals, Lower Panels: CO-treated animals. B. Quantitation of the outgrowth data represented as the number of colonies per field of
view (FOV) at 40x magnification. Data represent mean ± SD of 8-10 FOV from each animal/well in duplicate. One way ANOVA *p=0.007 vs air; Wilcoxon *p=0.031. Note bottom right panel showing a representative FOV showing tubule-like formation (white arrows) in a few FOV from cells harvested from animals treated with CO, which was not observed in air-treated animals.

**Supplementary Materials and Methods**

**Immunoblotting**

Cells were lysed by a freeze-thaw cycle in ice-cold lysis buffer (0.5% NP-40, 0.1% NaDOC, 0.1% SDS, 50mM Tris-HCl pH=7.5, 150mM NaCl, 1mM EDTA pH=8.0, 1mM NaF, in the presence of a protease inhibitor cocktail. Samples were centrifuged for 30 min at 14000g at 4°C and the supernatants were harvested. 20-40 µg of each protein sample were electrophoresed on NuPAGE 4-12% Bis-Tris Gel (Invitrogen, CA) followed by transfer to PVDF membrane. The membranes were then blocked with 5% non-fat dry milk, probed with appropriate primary antibodies, followed by HRP-conjugated secondary antibodies at a dilution of 1:5000. Bands were visualized using Super signal chemiluminescent substrate (Pierce, Rockford, IL) exposed to ECL Film (ISC BioExpress, Kaysville, UT).

**Source of antibodies**

The following antibodies were used: rabbit anti-P(Ser473)-Akt (Cell Signaling), rabbit anti-total Akt (Cell Signaling), rabbit anti-P (Ser780)-Rb (Cell Signaling), mouse anti-GAPDH (Calbiochem), rabbit anti-P-eNOS (Ser1177) and rabbit anti-total eNOS (Millipore Upstate), rabbit anti-Histone H3 (Cell Signaling), rat anti-mouse and mouse
anti-rat CD31 (BD Biosciences), anti-ICAM (BD Biosciences), mouse anti-rat mononuclear phagocyte (ED-1) (BD Pharmingen), rat anti-Ly-6A/E (Sca1) (BD Biosciences) and rabbit anti-GFP (Invitrogen, Molecular Probes).

Cell motility assay

RAEC were grown to confluency on gelatin-coated coverslips. Prior to imaging, a diametric scratch was made exposing a cell-free region approximately 25 to 30 µm wide. The coverslips were loaded into 37°C incubated closed chambers (FCS2®, Bioptechs, Inc, Butler, PA), aligning the scratch longitudinally with the flow of medium at 0.5 ml/hr delivered by a KDS 100 syringe pump, (KD Scientific). The coverslips were exposed to buffered medium (CO-saturated and non-saturated containing 5% CO2/air). The medium was prepared by bubbling with 250 ppm CO continuously for 30 min and then loaded into an airtight Hamilton syringe. The system was designed and tested to be gas impermeable). Differential interference contrast (DIC) images were taken every 20 min at 12 different positions along the scratch over 24 hr with a Nikon TE300 with 20X objectives; the experiments were done simultaneously. Cellular motility was analyzed with MetaMorph 6.2 (Universal Imaging Corp.), and measured as the average speed of the cells’ nuclei (n=120 cells) along their migratory pathway.

Monocyte migration. U937 monocytes were seeded in the upper chamber of migration Boyden chambers (8 µm, Transwell, Costar) in serum free medium. Serum containing medium was added to the lower chamber and served as the chemoattractant. Cells were treated with CO or 10-50 µM CORM ALF421 for 24 hours. The amount of cells, which migrated to the lower chambers were counted using a Neubauer hemocytometer.
**Phalloidin staining**

RAEC were cultured in MCD-B131 complete media (VEC Technology) on 22 mm x 22 mm coverslips in 6 well plates. 1 mm wide scratches of uniform size were then created, after which the cells were either placed in air or CO for 24 hr. Staining for actin using Alexa Fluor 488 phalloidin (Molecular Probes, Eugene, OR) was performed according to manufacturer’s directions. Briefly, the coverslips were fixed in 2% paraformaldehyde for 15 min at room temperature, washed with PBS and incubated with phalloidin for 15 min. Images were captured of randomized fields using a Zeiss Apotome fluorescent microscope.