Delayed inhaled carbon monoxide mediates the regression of established neointimal lesions

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Objective: **Intimal hyperplasia (IH) contributes to the failure of vascular interventions. While many investigational therapies inhibit the development of IH in animal models, few of these potential therapies can reverse established lesions. Inhaled carbon monoxide (CO) dramatically inhibits IH in both rats and pigs when given perioperatively. It also prevented the development of pulmonary arterial hypertension in rodents. Interestingly, CO could reverse pulmonary artery structural changes and right heart hemodynamic changes when administered after the establishment of pulmonary hypertension. Thus, we hypothesize that inhaled CO may mediate the regression of established neointimal lesions.**

Methods: **Rats underwent carotid artery balloon angioplasty injury. Carotid arteries were collected at 2 and 4 weeks after injury for morphometric analysis of the neointima. Another group was treated with inhaled CO (250 parts per million) for 1 hour daily from week 2 until week 4. Additional rats were sacrificed 3 days after initiating CO treatment, and the carotid arteries were examined for apoptosis by terminal deoxynucleotidyl transferase dUTP nick end-labeling, proliferation by Ki67 staining, and autophagy by microtubule-associated protein light chain 3 I/II staining.**

Results: **At 2 weeks following injury, sizable neointimal lesions had developed (intimal/media = 0.92 ± 0.22). By 4 weeks, lesion size remained stable (0.80 ± 0.09). Delayed inhaled CO treatment greatly reduced neointimal lesion size vs the 2- and 4-week control mice (0.38 ± 0.05; $P < .05$). Arteries from the CO-treated rats exhibited significantly reduced apoptosis compared with control vessels (3.18% ± 1.94% vs 16.26% ± 5.91%; $P = .036$). Proliferation was also dramatically reduced in the CO-treated animals (2.98 ± 1.55 vs 10.37 ± 2.80; $P = .036$). No difference in autophagy between control and CO-treated rats was detected.**

Conclusions: **Delayed administration of inhaled CO reduced established neointimal lesion size. This effect was mediated by the antiproliferative effect of CO on medial and intimal smooth muscle cells without increases in arterial wall apoptosis or autophagy. Future studies will examine additional time points to determine if there is temporal variation in the rates of apoptosis and autophagy. (J Vasc Surg 2015;61:1026-33.)**

Clinical Relevance: **Intimal hyperplasia (IH) and restenosis limit the patency and efficacy of vascular interventions and bypasses. The treatment for this process focuses on prevention and on retreatment once the lesions have developed. No therapy has been shown to remodel these neointimal lesions once they have developed. Carbon monoxide is an inhaled therapy that has been shown to be vasoprotective in inhibiting IH. It has also been shown to reverse the arterial remodeling in pulmonary arterial hypertension that resembles IH. Thus, this study examined the ability of inhaled carbon monoxide to reverse established neointimal lesions and preserve vascular patency.**

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This material is based upon work supported in part by the Department of Veterans Affairs, Veterans Health Administration (VA Merit Grant BX000635), Office of Biomedical Laboratory Research and Development (E.T.), and through funding from NIH T32 HL098036 (E.T.).

Author conflict of interest: none.

The contents of this article do not represent the views of the Department of Veterans Affairs or the United States Government.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214

Published by Elsevier Inc. on behalf of the Society for Vascular Surgery.

http://dx.doi.org/10.1016/j.jvs.2013.11.072

Restenosis resulting from intimal hyperplasia (IH) occurs following cardiovascular surgery or endovascular interventions and remains a significant cause of stent or graft failure.[1] It increases the cost of health care because of the need for ongoing surveillance to detect the lesions and surgery or endoluminal interventions to treat the recurrent stenosis.[2] Drug-eluting stents for the coronary circulation have reduced the incidence of restenosis[3] but have inherent problems with delayed re-endothelialization and stent thrombosis.[3-6] In the peripheral circulation, effective drug-eluting stents are still being designed and tested for these more complex and extensively diseased arteries.[1,7-9] However, once restenosis has occurred, treatment options only include reintervention. To date, few investigational therapies have been developed that can mediate the regression of an established vascular lesion.[10,11]

We have previously reported that inhaled carbon monoxide (CO) can inhibit the development of IH in a rat carotid artery injury model[12] as well as in a pig iliac artery angioplasty model.[13] The effectiveness of inhaled CO was quite dramatic, with the beneficial effects being achieved with a single, 1-hour exposure pre-angioplasty in the rats and in the perioperative period in the pigs. Similarly, vein grafts stored in CO-saturated solution prior to implantation exhibited reduced evidence of warm ischemia-induced injury and reduced neointima formation.[14] The mechanism by which CO inhibits IH was through the upregulation of endothelial nitric oxide synthase and p38 signaling.[12] It can also upregulate the expression of heme oxygenase 1, the enzyme responsible for endogenous CO production, as another protective mechanism.[15] In other studies, CO was found to have profound anti-inflammatory effects on monocytes, antiproliferative and proapoptotic effects on vascular smooth muscle cells (SMCs), and prosurvival and proproliferative actions on endothelial cells.[16]

The beneficial vascular effects of inhaled CO were also noted in the pulmonary vascular bed, where it inhibited the development of pulmonary artery hypertension (PAH).[10] Daily treatment with an hour of inhaled CO could prevent the pulmonary artery remodeling and the right ventricular remodeling induced by hypoxia or monocrotaline. Interestingly, Zuckerbraun et al demonstrated that inhaled CO administered for an hour daily after the establishment of PAH could reverse the pulmonary artery changes in the same models of PAH.[10] The pulmonary arterial changes in PAH are very similar to those observed in neointimal lesions with heavy SMC proliferation and hypertrophy. Based on these effects, we hypothesized that inhaled CO can mediate the regression of established neointimal lesions following angioplasty injury.

METHODS

Rat carotid artery balloon injury model. All animal procedures were performed using aseptic technique in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and as approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (IACUC protocol 1104675A-1) and the VA Pittsburgh Healthcare System (protocol 02960). Adult male Sprague-Dawley rats (350-400 g; Harlan, Indianapolis, Ind) were cared for as directed by the Department of Laboratory Animal Research at the University of Pittsburgh. Animals were anesthetized with intraperitoneal Nembutal (45 mg/kg) and supplemented with isoflurane as needed. Right carotid artery angioplasty injury was created as previously described[17] with a 2-F Fogarty catheter (Edwards Lifesciences, Irvine, Calif). Briefly, the catheter was inserted through the external carotid artery retrograde into the common carotid where it was inflated to 2 atm and held for 5 minutes as described.[17] The catheter was then removed, and the branch was ligated. Analgesia was provided with Buprenex (0.05 mg/kg) for 48 hours and as needed thereafter. Animals were recovered and housed under routine conditions for 2 weeks. At that time point, rats were divided into three separate groups. One group was sacrificed, and the carotid arteries were collected for histologic examination. A second group was housed for another 2 weeks, totaling 4 weeks from the day of carotid injury, at which time they were sacrificed for carotid artery collection. In the final group, rats were initiated on inhaled CO treatment at 250 parts per million (ppm) for 1 hour daily for the remaining 2 weeks. These rats were sacrificed at the 4-week time point for carotid artery collection.

Tissue processing and morphometric analysis. Rat carotid arteries were perfusion fixed in situ with phosphate-buffered saline (PBS) and 2% paraformaldehyde. They were excised and then fixed for 1 hour at 4°C in 2% paraformaldehyde, cryoprotected in 30% sucrose overnight, and then quick frozen with 2-methylbutane and stored at −80°C as described.[18] The arteries were embedded in OCT and then sectioned (5 μm) in a semiserial fashion along the length of the vessels and stained with hematoxylin and eosin. The center of injury was identified, and four sections on either side of this location were used to quantify intima and medial areas. Images were obtained using the Olympus Provis microscope (Olympus, Center Valley, Pa), and vessel areas were measured using Metamorph (Molecular Devices, Sunnyvale, Calif). Autofluorescence of the elastic lamina was used to identify the different regions of the arterial wall. Quantification was performed by calculating the areas within the external elastic lamina, the internal elastic lamina, and the inner edge of the intima. The area between the inner edge of the intima and the internal elastic lamina denoted the neointimal area. Data were presented at intimal area/media area (I/M) ratios.

Arterial wall proliferation. For evaluation for SMC proliferation, rats underwent carotid artery injury and recovered for 2 weeks. At this time, some rats were treated with inhaled CO for 1 hour daily while other rats remained on room air. Rats were sacrificed at 3 days after starting the CO treatment, and control animals were sacrificed at the same time point (a total of 17 days after carotid injury). Carotid arteries were perfusion-fixed and sectioned as described above. Tissue sections were incubated with 2% bovine serum albumin in PBS for 1 hour, followed by five washes with PBS + 0.5% bovine serum albumin. Ki67 staining was performed using mouse monoclonal anti-Ki67 (1:100; Abcam, Cambridge, Mass) followed by secondary antibody for 1 hour. Sections were counterstained with DAPI (Sigma-Aldrich, St. Louis, Mo) to identify cell nuclei. Quantification was performed by counting of Ki67-positive cells and dividing by total number of cells.

Positively stained cells in five random fields were imaged on a Fluoview 1000 confocal scanning microscope (Olympus). Alternatively, the entire sample was imaged at 20× magnification using a Nikon 90i Upright microscope (Nikon, Melville, NY). Imaging conditions were maintained at identical settings, with original gating performed within each antibody-labeling experiment with the negative control (no primary antibody). Quantification was performed by a blinded observer Metamorph. Three sections were analyzed for each animal with five to seven rats per treatment group.