**Ex vivo** carbon monoxide delivery inhibits intimal hyperplasia in arterialized vein grafts

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**Aims**
Veins are still the best conduits available for arterial bypass surgery. When these arterialized vein grafts fail, it is often due to the development of intimal hyperplasia (IH). We investigated the feasibility and efficacy of the ex vivo pre-treatment of vein grafts with soluble carbon monoxide (CO) in the inhibition of IH.

**Methods and results**
The inferior vena cava was excised from donor rats and placed as an interposition graft into the abdominal aorta of syngeneic rats. Prior to implantation, vein grafts were stored in cold Lactated Ringer (LR) solution with or without CO saturation (bubbling of 100% CO) for 2 h. Three and 6 weeks following grafting, vein grafts treated with cold LR for 2 h developed IH, whereas grafts implanted immediately after harvest demonstrated significantly less IH. Treatment in CO-saturated LR significantly inhibited IH and reduced vascular endothelial cell (VEC) apoptosis. Electron microscopy revealed improved VEC integrity with less platelet/white blood cell aggregation in CO-treated grafts. The effects of CO in preventing IH were associated with activation of hypoxia inducible factor-1α (HIF-1α) and an increase in vascular endothelial growth factor (VEGF) expression at 3–6 h after grafting. Treatment with a HIF-1α inhibitor completely abrogated the induction of VEGF by CO and reversed the protective effects of CO on prevention of IH.

**Conclusion**
Ex vivo treatment of vein grafts in CO-saturated LR preserved VEC integrity perioperatively and significantly reduced neointima formation. These effects appear to be mediated through the activation of the HIF1α/VEGF pathway.

**Keywords**
Carbon monoxide • Intimal hyperplasia • Vein graft • Vascular endothelial growth factor • Hypoxia inducible factor

1. **Introduction**
Autologous vein grafts are the most common and best-suited conduit for the reconstruction of arterial occlusive disease in the heart, peripheral circulation, mesenteric vessels, and in haemodialysis access.1–3 The patency of these vein bypass grafts surpasses that achieved with prosthetic conduits but is still hampered by the development of intimal hyperplasia (IH) in about 50% of the cases.4 This proliferative process that involves smooth muscle cells and inflammatory cells results in progressive stenosis and ultimately occlusion. Sites that are prone to IH are the anastomoses as well as lysed valves.5,6

Although the pathogenesis involved in formation and progression of neointimal lesions is not fully elucidated, events that incite and contribute to IH occur at the time of surgery when vascular endothelial cells (VECs) are injured by ischaemia during the harvesting process and then reperfusion when the vessel is implanted.7,8 VECs are also injured by the mechanical forces during the transition from venous to arterial flow dynamics and from surgical handling.9 All these events activate inflammation and promote coagulation that stimulate both smooth muscle cell migration and proliferation. The ability to preserve VEC integrity perioperatively and promote vascular healing will significantly impact on the development of IH and improve patient outcome.
Carbon monoxide (CO), while traditionally viewed as a toxic environmental gas, is endogenously synthesized during haem degradation. It has been recognized to function as a regulatory molecule that possesses a wide range of protective properties. In the vasculature, inhaled CO significantly inhibited injury-induced IH in rodents and pigs. At the cellular level, CO has been shown to protect VECs through the modulation of leucocyte–VEC interactions and the suppression of TNF-α or anoxia-reoxygenation-induced VEC apoptosis. More recently, we have reported that inhaled CO can induce the expression of vascular endothelial growth factor (VEGF), a multifunctional growth factor which induces endothelial cell growth, differentiation, and regeneration, and protect kidney grafts from cold IR injury. Taken together, we hypothesize that CO may mediate VEC protection and inhibit IH through VEGF induction. The current study was undertaken to explore this potential novel mechanism of CO-mediated vasoprotection using ex vivo CO delivery to vein grafts. This approach of ex vivo CO treatment was used because it represents a clinically feasible approach for clinical CO administration.

2. Methods

2.1 Reagents

The hypoxia inducible factor (HIF)-1α inhibitor YC-1 (3-{5-[(hydroxymethyl-2′ furyl]-1-benzyl indazole) was purchased from A.G. Scientific Inc. (San Diego, CA, USA) and dissolved in dimethyl sulfoxide to make a stock solution. The final concentration of YC-1 was 10 μM in Lactated Ringer (LR).

2.2 CO-saturated LR solution

LR was vigorously bubbled with compressed 100% CO gas (PRAXAIR, Danbury, CT, USA) for >5 min at 4°C under sterile conditions in the fume hood. CO is quickly saturated in LR and reaches equilibrium within 5 min. Longer CO exposure does not further increase CO concentrations in the solution. After equilibration, the solution contained approximately 1200 μM/L of CO as quantified with TRI lyser (Taiyo, Osaka, Japan). This CO-saturated LR was stored in a tightly capped tube without a gas layer.

2.3 Animals

Inbred male LEW (RT-Ti) rats weighing 200–250 g (Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were maintained in a laminar flow animal facility at the University of Pittsburgh on a standard diet and water supplied ad libitum. All procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh (protocol #0708120) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.4 Vein grafting

All surgical procedures were performed under sterile conditions using iso-flurane (inhalation) general anaesthesia. After donor animals were heparinized (300 units/rat), a 20 mm long segment of inferior vena cava was excised and gently irrigated with 3 mL of cold-iced LR. Vein grafts were kept in a bath of LR at 4°C for either <10 min or 2 h and transplanted into anaesthetized recipients by end-to-side anastomoses to the abdominal aorta as an interposition graft. Only grafts patent at harvest were used for the analysis and thrombosed grafts (<10%) were excluded from the studies. Vein grafts to be used for molecule analysis were harvested after perfusion with LR in situ and immediately harvested and snap-frozen in liquid nitrogen. Vein grafts to be used for morphologic analysis were perfused with LR followed by 10% formalin or 2% paraformaldehyde in phosphate-buffered saline (PBS) in situ. The grafts were then harvested and fixed for 1 h with formalin or paraformaldehyde and then cryoprotected in 2.3 M sucrose overnight and frozen in OCT (Optimal Cold Temperature; Sakura Finetek, Inc., Torrance, CA, USA) in liquid nitrogen-cooled isopentane.

2.5 Experimental groups

The following treatment groups were used in this study: (i) control naive vein harvested from unoperated normal LEW animals, (ii) control vein grafts without (<10 min) cold ischaemia in LR, (iii) vein grafts stored for 2 h in LR, (iv) vein grafts stored in LR containing the HIF-1α inhibitor YC-1 (10 μM) for 2 h, (v) vein grafts stored in CO-saturated LR for 2 h, and (vi) vein grafts stored in CO-saturated LR containing YC-1 (10 μM). Veins were implanted after these treatments. The recipient rats were sacrificed at 3 h, 6 h, 3 weeks, and 6 weeks after vein grafting.

2.6 Histomorphometric analysis

The slides were stained with haematoxylin/eosin, modified Masson’s trichrome and Verhoeff’s elastic tissue stain (Rowley Biochemical Institute, Danvers, MA, USA), and α-smooth muscle actin (α-SMA, DAKO, Carpinteria, CA, USA). Images of the cross-section of the vessels were photographed with a Zeiss microscope (Axioskop) and placed on a colour scanner (EPSON Smart Panel). After the digitized images were obtained, areas were measured by computer planimetry with NIH Image (v1.62) software and expressed as percent area of intima/area of intima plus media in each cross-section.

2.7 Cell death stain

Cryostat sections (5 μm) were placed on charged glass slides, washed three times with PBS, and then incubated in 1% Triton X-100 (Sigma, St Louis, MO, USA) in PBS for 20 min. The slides were incubated for 1 h at room temperature with primary antibody for CD31 (1:100, Serotec, Raleigh NC, USA), followed by incubation for 1 h with goat anti-mouse CY3 (1:1000, Jackson, West Grove, PA, USA) secondary antibody. After three rinses with PBS, sections were incubated with terminal deoxynucleotidyl transferase-mediated DUTP-biotin nick end labelling (TUNEL) reagent (Roche, Indianapolis IN, USA) for 30 min at 37°C. After incubation with 1 h with Streptavidin 488 (1:1000, Invitrogen, Carlsbad, CA, USA), nuclear stains were performed with Hoeschts dye (bisBenzimide, 1 mg/100 mL) for 30 s. Sections were mounted with Gelvatol and confocal microscopy was performed on an Olympus Fluoview 1000 confocal microscope.

2.8 Electron microscopy

The tissue for TEM was perfused in situ and fixed in 2.5% glutaraldehyde for 1 h, cut into ~1 mm oblong cubes for future specimen orientation and post-fixed in aqueous 1% OsO4, 1% K2Fe(CN)6, for 1 h. After PBS washes, the tissue was dehydrated through a graded series of 30–100% ethanol, 100% propylene oxide, and then infiltrated in 1:1 mixture of propylene oxide and 100% propylene glycol. The following treatment groups were used in this study: (i) control naive vein harvested from unoperated normal LEW animals, (ii) control vein grafts without (<10 min) cold ischaemia in LR, (iii) vein grafts stored for 2 h in LR, (iv) vein grafts stored in LR containing the HIF-1α inhibitor YC-1 (10 μM) for 2 h, (v) vein grafts stored in CO-saturated LR for 2 h, and (vi) vein grafts stored in CO-saturated LR containing YC-1 (10 μM). Veins were implanted after these treatments. The recipient rats were sacrificed at 3 h, 6 h, 3 weeks, and 6 weeks after vein grafting.

Taken together, we hypothesize that CO may mediate VEC protection and inhibit IH through VEGF induction. The current study was undertaken to explore this potential novel mechanism of CO-mediated vasoprotection using ex vivo CO delivery to vein grafts. This approach of ex vivo CO treatment was used because it represents a clinically feasible approach for clinical CO administration.
mounted to metal stubs, and sputter-coated with 3.5 nm coating of gold palladium. The samples were digitally imaged with a JEOL JEM-6335F scanning electron microscope (JEOL).

2.9 Total RNA extraction and SYBR green real-time RT–PCR

The mRNA levels for VEGF, intracellular adhesion molecule-1 (ICAM-1), tumour necrosis factor (TNF-α), and glyceraldehyde 3-phosphate dehydrogenase were quantified in duplicate samples using SYBR Green two-step, real-time RT–PCR, as previously described.19,24,25 PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA, USA). Each sample was analysed in duplicate using the conditions recommended by the manufacturer.

2.10 Western blot

Thirty micrograms of cytosolic proteins (for VEGF) and nuclear proteins (for HIF-1α) were electrophoresed on 6–15% acrylamide sodium dodecyl sulfate gels and transferred to nitrocellulose membranes (Sleicher & Schuell, Keene, NH, USA). The membranes were blocked with 5% non-fat dry milk in PBS-Tween (0.1%) for 1 h at room temperature. Membranes were incubated overnight with mouse monoclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), HIF-1α (Novus Biologicals, Littleton, CO, USA), rabbit anti-human polyclonal antibodies for β-actin (Sigma) or polyclonal rabbit Histone-1 (FL-219, Santa Cruz Biotechnology), followed by incubation with secondary antibody (anti-rabbit or mouse IgG, Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h. After repeated washings with PBS-Tween, membranes were developed with the SuperSignal detection systems (Pierce Chemical, Rockford, IL, USA).

2.11 Data analysis

The results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using unpaired Student’s t-test or analysis of variance where appropriate. A probability level of P < 0.05 was considered statistically significant.

3. Results

3.1 Ex vivo carbon monoxide of vein grafts reduced neointimal formation

The effect of ex vivo CO treatment on vein graft IH was examined 3 weeks after the bypass procedure. The vein grafts were analysed by morphometric analysis and assessed for αSMA and collagen deposition. The vein grafts stored for 2 h in cold LR developed significant neointimal lesions consisting predominantly of αSMA-positive smooth
muscle (Figure 1). Vein grafts that were not exposed to cold ischaemia and immediately implanted after they were harvested developed minimal IH (Figure 1). Vein grafts treated in CO-saturated LR and 2 h of cold ischaemia demonstrated significantly less IH. To test our hypothesis that the actions of CO may be mediated by the HIF-1α, vein grafts were incubated in CO-saturated LR and the HIF-1α inhibitor YC-1. The neointimal lesions in grafts treated with YC-1 alone were similar to that identified in the control vein grafts. However, YC-1 significantly reversed the inhibitory effect of CO against neointima formation.

3.2 CO reduced vein graft apoptosis

The early vascular injury response and subsequent endothelial apoptosis are critical for stimulating IH.26 Apoptotic cells, as identified by TUNEL staining, were distributed throughout the vessel wall in the control grafts 6 h after bypass. Immunostaining revealed the colocalization of TUNEL and CD31, the pan-endothelial cell marker, suggesting apoptosis was occurring in the VECs as well as in the medial cells. In CO treated vein grafts, very few TUNEL-positive cells were identified at the 6 h time point. In contrast, HIF-1α inhibition reversed the anti-apoptotic effects of CO with TUNEL-positive cells dispersed throughout media and the adventitia (Figure 2).

3.3 CO reduced endothelial denudation with local cell aggregation

To further evaluate the changes in the vein graft endothelium, the grafts were harvested 3 h after implantation and examined using SEM and TEM. SEM revealed significant endothelial denudation with the deposition of platelets, microthrombi, and leucocytes in control vein grafts stored in LR (Figure 3A). By TEM, necrotic and detached endothelial cells were detected in the control vein grafts (Figure 3B). In CO-treated grafts, VECs were well-preserved with distinct junctions with few adherent platelets and leucocytes on the luminal surface (Figure 3C). By TEM, the VECs on the CO-treated grafts exhibited preserved integrity of cytoplasmic organelles and nuclei (Figure 3D). These potent protective effects of CO were significantly reversed by HIF-1α inhibition. The SEM examination of the vein grafts treated with CO and YC-1 revealed findings similar to that observed in control vein grafts with numerous platelet/white blood cell aggregates and detached VEC (Figure 3E and F).

3.4 CO reduced ICAM-1 expression in the grafts

Leucocyte adhesion and transmigration is dependent on the expression of adhesion receptors on the endothelium. One of the predominant adhesion molecules in the vascular injury response is ICAM-1.27 ICAM-1 expression in the vein grafts was examined with RT–PCR (Figure 4). ICAM-1 expression was increased in control vein grafts 3 h after bypass grafting. In CO-treated grafts, ICAM-1 mRNA expression was significantly reduced. This inhibition of ICAM-1 expression by CO was inhibited by concurrent treatment of the vein graft with HIF-1α inhibition with YC-1.

3.5 Inhibition of HIF-1α reversed CO-induced up-regulation of VEGF

Western blot analysis of nuclear proteins isolated from the vein grafts revealed that CO treatment induced strong HIF-1α expression, whereas control grafts and those treated with YC-1 had low level expression 3 h after grafting (Figure 5A). CO treatment also significantly increased VEGF mRNA levels by PCR in the vein grafts. YC-1 also inhibited CO-mediated upregulation of VEGF expression (Figure 5B). Western blot analysis of cytoplasmic protein preparations revealed a similar elevation of VEGF protein in CO-treated grafts 6 h after implantation while control vein grafts exhibited only low levels of VEGF expression. YC-1 inhibited CO-stimulated VEGF protein expression (Figure 5C). These data suggest that ex vivo CO treatment of vein grafts activates HIF-1α which then induces VEGF expression.

3.6 CO inhibited IH in arterialized vein grafts in a prolonged fashion

To confirm that CO treatment did not simply delay the development of IH, vein grafts were also analysed 6 weeks after grafting. Similar to the 3 weeks time point, the vein grafts stored in cold LR for 2 h developed significant intimal thickening at 6 weeks. CO-treated vein grafts exhibited a persistent inhibition of IH at this time point (Figure 6A). Quantitative RT–PCR showed that TNF-α and ICAM-1 mRNA
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Figure 4 ICAM-1 mRNA expression. Real-time RT–PCR showed a five-fold increase in ICAM-1 mRNA levels in the control vein grafts compared with naive vein 3 h after implantation. CO-treated grafts had significantly reduced ICAM-1 mRNA expression. The inhibition of ICAM-1 expression in CO-treated grafts was abrogated by the HIF-1α inhibitor, YC-1 (*P < 0.05 vs. control vein grafts, **P < 0.05 vs. CO-treated grafts; n = 4–5).

Figure 5 HIF-1α expression in the vein grafts. (A) Western blot of nuclear proteins isolated from the vein grafts revealed visible HIF-1α expression 3 h after grafting. A western blot is shown with two individual experiments from four vein grafts. (B) VEGF expression in the grafts. CO significantly upregulated VEGF mRNA expression in the vein grafts 3 h after grafting. Histogram depicts mean ± SD. *P < 0.05 vs. control vein grafts, **P < 0.05 vs. CO-treated grafts CO, n = 4–5. (C) VEGF protein expression. Western blot analysis of cytoplasmic proteins revealed strong VEGF expression in both normal vein and CO-treated vein grafts. VEGF was reduced in control vein grafts. A representative blot is shown from three individual experiments using four different vein grafts for each group.

levels were elevated in the vein grafts at 6 weeks. While VEGF expression was no longer altered at 6 weeks post-implantation, CO-treated vein grafts exhibited persistent inhibition of TNF-α and ICAM-1 mRNA expression at this time point (Figure 6B).

4. Discussion

The longstanding limitation of bypass surgery, be it in the coronary or peripheral circulation, is the development of IH in the vein conduit leading to graft failure. Despite intense investigation, no innovation has been successful at reducing or preventing graft stenosis resulting from IH. In this study, we demonstrate that vein grafts treated with CO ex vivo developed significantly smaller neointimal lesions by 3 and 6 weeks post-bypass. The vasoprotective actions of CO have been previously reported by Otterbein et al.12 who showed that rats treated with inhaled CO at 250 parts per million for 1 h responded to subsequent angioplasty injury of the carotid artery with a greater than 75% reduction in neointima formation. Inhalational administration of CO, however, raises concern about potential systemic toxicity that results from the ability of CO to compete with oxygen for binding to haemoglobin. Our data reveal that ex vivo application of CO, a much safer method of CO delivery, may also be effective in blocking IH in vein bypass grafts, a process that is more resistant to other experimental therapies than simple arterial injury.

The ability to treat venous conduits with CO prior to implantation into patients makes the translation of this method of CO therapy to clinical application a very tangible and feasible goal. Although the biological mechanisms underlying IH formation in arterialized vein grafts have not been fully elucidated, VEC injury/loss early after grafting is clearly an important inciting event.1,28 Mechanical stretch and increased tensile stress resulting from the shift from venous to arterial flow dynamics contribute to VEC injury by direct trauma as well as by promoting alterations in cell behaviour that results in the generation of reactive oxygen species.8,29,30 Prolonged preservation and ischaemia following vein harvest and prior to implantation elicit a cascade of adverse responses that further increase IH in vein grafts.29,31 Since the vascular endothelium serves as a selective blood/tissue barrier, preventing the extravasation of solutes and macromolecules and the passage of leucocytes into surrounding tissues, preservation of VEC integrity is expected to reduce IH. The disruption of the endothelium with marked apoptosis was demonstrated by confocal microscopy and TEM in the vein grafts isolated 3 h after bypass. These findings illustrate the significant amount of endothelial injury and loss that occurs shortly after bypass completion and exposure to arterial flow.

CO has numerous protective properties. It has been shown to inhibit systemic inflammatory responses.10 In alveolar macrophages, it has been reported to mediate anti-inflammatory preconditioning through the induction of HIF-1α.32 The use of ex vivo methods of delivering CO to the vein grafts removes the influence of CO on inflammatory cells and isolates its vasoprotective actions to the vessel wall. While the safety of such ex vivo delivery is certainly greater, the potential existed that eliminating its systemic anti-inflammatory actions may limit its efficacy. However, this did not prove to be the case in our studies. Isolated treatment of the vein grafts with CO reduced IH significantly. Mechanistically, the protection offered by CO appeared to involve the VECs. The early apoptosis and cell necrosis observed in vein grafts shortly after implantation into the arterial circulation was markedly reduced in
CO-treated grafts. By reducing VEC injury or loss, CO likely reduced IH through earlier recovery of the endothelial barrier function. The ability of the HIF-1α inhibitor, YC-1, to reverse the protective actions of CO indicates that HIF-1α is involved in the downstream signalling for CO in vascular smooth muscle cells and the VECs. The significance of HIF-1α in this setting is further supported by the increased levels of HIF-1α in the vein grafts following CO treatment. HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits. It binds to upstream hypoxia response elements in genes encoding proteins that mediate adaptive responses to reduced oxygen availability such as VEGF and erythropoietin. 33 While HIF-1β subunits are constitutively expressed, HIF-1α subunits are regulated by oxygen-dependent enzymatic hydroxylation. 34 Although proline hydroxylation is blocked and HIF-1α degradation is inhibited in hypoxic conditions, HIF-1α is rapidly hydroxylated and degraded during normoxia. Recently, Chin et al. 32 showed that a transient burst of reactive oxygen species arising from the mitochondria in response to CO activates HIF-1α, leading to the upregulation of multiple genes responsible for angiogenesis and cytoprotection. Thus, multiple mechanisms may be involved in the effects of CO mediated by HIF-1α activation.

Previous observations illustrated that a single topical application of VEGF on vein grafts before implantation reduced IH and improved endothelial function in a rabbit bypass model. 35 Similarly, local delivery of VEGF accelerates re-endothelialization and attenuates IH in a rat carotid artery injury model. 36 This evidence thus suggests that local VEGF expression may reduce IH. However, VEGF also has been shown to have potent pro-inflammatory properties by promoting monocyte chemotaxis and the activation of adhesion molecules which may contribute to enhanced IH. 37,38

Our novel observations are that ex vivo CO treatment of vein grafts resulted in increased VEGF expression in the vein grafts in association with HIF-1α activation. In addition, YC-1 treatment of the grafts blocked CO-induced VEGF expression. While not conclusive, these findings suggest that CO-mediated inhibition of IH is dependent on HIF-1α upregulation and that the downstream effects may, in part, be mediated by VEGF. The absence of proinflammatory effects in our studies may be due to the local upregulation of VEGF. Further
studies are required to fully examine the role of VEGF in CO-mediated vasoprotection.

In conclusion, exposure of vein grafts to CO-saturated LR ex vivo resulted in preservation of VEC integrity perioperatively through the activation of HIF-1α and significantly reduced neointimal formation. These findings support the potential application of ex vivo CO treatment in bypass surgery which may assist in improving graft patency. Further studies are needed to understand the link between CO and VEGF in this process.

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