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LOCAL ADMINISTRATION OF CARBON MONOXIDE INHIBITS NEOINTIMA FORMATION IN BALLOON INJURED RAT CAROTID ARTERIES

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Abstract - Recent studies indicate that systemic induction of heme oxygenase-1 (HO-1), which oxidatively degrades heme into iron, biliverdin, and carbon monoxide (CO), or adenoviral-mediated gene transfer of HO-1 inhibits neointima formation after experimental vascular injury. In the present study, we investigated whether the acute, local administration of the HO-1 product, CO, regulates the arterial remodeling response following injury. Immediately after balloon injury of rat carotid arteries, a saturated solution of CO or nitrogen (N_2), or phosphate buffered saline (PBS) was incubated luminally within the injured vessels for 30 min. Two weeks after injury, arteries exposed to CO exhibited significantly reduced neointimal area, neointimal area/medial wall area ratio, neointimal thickness, and medial wall area compared to arteries exposed to N_2 or PBS. Arteries exposed to CO also demonstrated significantly reduced DNA synthesis in the medial wall two days after injury as suggested by proliferating cell nuclear antigen immunostaining, and this was associated with a decrease in the protein expression of the G_1 cyclins, cyclin E and A, and transforming growth factor-beta1. These results indicate that the acute, local delivery of CO blocks the pathophysiological remodeling response to vascular injury, and identifies CO as a potentially important therapeutic agent in the treatment of vasculoproliferative disease.

Key words: Carbon monoxide, balloon injury, neointima, smooth muscle proliferation, cyclins, transforming growth factor-beta1

INTRODUCTION

Proliferation of vascular smooth muscle cells (SMCs) is an essential feature of the vascular response to injury and the major pathophysiologic mechanism responsible for the failure of interventional therapeutic approaches to treat occlusive vascular disease, including transplant vasculopathy, vein bypass graft failure, and post-angioplasty restenosis (9,23). Recent work indicates that heme oxygenase-1 (HO-1), which catalyzes the degradation of heme into equimolar amounts of iron, biliverdin and carbon monoxide (CO), exerts a significant inhibitory effect on SMC proliferation and on the pathologic remodeling response following balloon angioplasty (see 8). Inhibition of HO-1 activity potentiates the mitogenic action of several growth factors whereas overexpression of HO-1 limits vascular SMC proliferation (6,16,17,21,25,30). In addition, vascular SMCs obtained from HO-1 null mice exhibit elevated rates of growth relative to cells from wild-type animals (6). Several animal studies have also demonstrated that induction of HO-1 by hemin blocks injury-induced neointima formation (1,25,27). Furthermore, inhibition of HO-1 activity or deletion of the HO-1 gene promotes intimal thickening following balloon injury (6,25). More recently, localized adenovirus-mediated HO-1 gene delivery immediately following arterial injury has been demonstrated to attenuate neointima formation in rat carotid and pig femoral arteries (6,26). The reduction in neointima development is dependent on HO-1 activity and is associated with a pronounced decrease in SMC proliferation (6,26). Finally, HO-1 may also regulate the vascular response to injury in humans. In this respect, a microsatellite polymorphism in the human HO-1 gene that is linked to decreased inducibility is associated with augmented restenosis in patients undergoing percutaneous transluminal angioplasty in femoropopliteal arteries and with angiographic restenosis

Abbreviations: **CO**: carbon monoxide; **HO-1**: heme oxygenase-1; **N_2**: nitrogen; **PCNA**: proliferating cell nuclear antigen; **SMCs**: smooth muscle cells

and adverse cardiac events after coronary stenting (2,10).

The antiproliferative action of HO-1 is likely mediated via the release of CO. The CO scavenger, hemoglobin, enhances the proliferative response of cultured vascular SMCs to various mitogens (21,25). Moreover, exogenous administration of CO inhibits vascular SMC growth and DNA synthesis and arrests SMCs in the G_0/G_1 phase of the cell cycle by interfering with specific components of the cell cycle machinery, including the expression of p21 and cyclin A (16,20,21). Given its inhibitory effect on vascular SMC growth, the present study examined whether acute, local administration of CO regulates the pathophysiologic remodeling response to arterial injury.

MATERIALS AND METHODS

Materials

Monoclonal antibodies against cyclin D1, cyclin E, cyclin A, p21, p27, and beta-actin, and horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA); a monoclonal antibody against transforming growth factor-beta1 was from Oncogene Research Products (San Diego, CA, USA); CO gas was from Matheson Tri Gas (Houston, TX, USA); a monoclonal antibody against proliferating cell nuclear antigen (PCNA) and all other reagents were from Sigma Chem. (St. Louis, MO, USA).

Rat carotid artery balloon angioplasty and CO delivery

Male Sprague Dawley rats (Harlan, Indianapolis, IN, USA) were anesthetized with a combination anesthetic (ketamine, xylazine, and acepromezine; 0.5 to 0.7 ml/kg IM, Vetmed Drugs, Houston, TX, USA) and the left common carotid artery injured, as previously described (26,27). Briefly, a Fogarty 2F embolectomy catheter (Baxter Healthcare, Irvine, CA, USA) was introduced into the external carotid branch through an arteriotomy incision and advanced to the aortic arch. The balloon was then inflated and withdrawn with rotation to the carotid bifurcation three times. Immediately after injury, a polyethylene catheter was introduced through the external carotid arteriotomy site. The injured lumen was washed with warmed phosphate buffered saline (PBS), and 50 µl of a CO-saturated solution (875 µM), was infused in the common carotid artery and incubated with the solution for 30 min. Control animals received a nitrogen (N_2)-saturated solution or PBS. The saturated gas solutions were prepared by bubbling room temperature PBS with CO or N_2 gas for 20 min (12). After treatment, solutions were removed from the lumen which was washed with PBS and the external carotid branch ligated, and the incision closed. After full recovery from anesthesia, animals were returned to the animal facility and provided standard rat chow and water ad libitum. At specified times, rats were euthanized by exsanguination, and vessels harvested. All experimental protocols complied with guidelines of the institutional animal care and use committee.

Morphometric analysis

Perfusion-fixed vessels were processed in graded alcohols and xylene, paraffin-embedded, and 5 mm sections stained with Verhoff's van Gieson solution, as previously described (26,27). Morphometric analyses were performed using Image-Pro Plus (Media Cybernetics, Silver Springs, MD, USA) and Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA, USA) software systems linked through a CCD color camera (Leaf Micolumina, Leaf Systems, Southborough, MA, USA) to a Zeiss Axioskop50 light microscope (Carl Ziess, Germany). Images were captured, and quantitative measurements made of perimeters and areas corresponding to the internal and external elastic laminae, and lumen. Data transformations provided data for neointimal and medial wall areas and vessel diameters.

PCNA immunohistochemistry

Immunostaining for proliferating cell nuclear antigen (PCNA) was performed as previously described (26). Perfusion-fixed, paraffin-embedded tissues were incubated with an anti-PCNA monoclonal antibody (1:25 dilution) followed by a biotinylated anti-mouse secondary antibody (1:100 dilution). Slides were treated with an avidin-biotin block and exposed to DAB black with nuclear fast red counterstain and analyzed under light microscopy. Data are represented as a PCNA labeling index (LI), defined as the percentage of total cells within a given area positive for PCNA staining.

Protein analysis

Protein expression was determined in freshly isolated carotid arteries that were cleaned of blood, fat, and connective tissue and immediately snap-frozen. Frozen vessels were ground to a fine powder in liquid N_2, solubilized in electrophoresis buffer [125 mM Tris (pH 6.8), 12.5% glycerol, and 2% SDS] boiled, and sonicated. Proteins (50 µg) were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked in PBS and non-fat milk (5%) and then incubated with antibodies directed against cyclin D1 (1:500 dilution), cyclin E (1:500 dilution), cyclin A (1:500 dilution), p21 (1:500 dilution), p27 (1:300 dilution), TGF-β1 (2 µg/ml), and β-actin (1:500 dilution) for one hour. Membranes were washed in PBS and incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2,000 dilution) or rabbit anti-goat antibody (1:2,500 dilution). After further washing in PBS, blots were incubated in commercial chemoluminescence reagents (Amersham Biosciences, Piscataway, NJ, USA) and exposed to photographic film. Relative protein levels were quantified by scanning densitometry (LKB 2222-020 Ultrascan XL laser densitometer, Bromma, Sweden).

Statistics

Results are expressed as the mean ± SEM. Statistical analysis was performed with the use of a Student's two-tailed *t* test and an analysis of variance when more than two treatment regimens were compared. A value of $p<0.05$ was considered statistically significant.

RESULTS

Representative photomicrographs of Verhoeff-van Gieson-stained balloon-injured carotid artery cross-sections are shown in Fig. 1A. Two weeks after injury, PBS-treated animals exhibit a significant and concentric neointima, and the medial wall is clearly defined by the internal and external elastic laminae. Similarly, a substantial neointima is observed in vessels exposed to a saturated solution of N_2 immediately after injury. In contrast, a markedly attenuated neointima is found along the luminal border of carotid arteries treated with a saturated solution of CO. Morphometric analyses indicated that neointimal area, neointimal/medial wall area ratio, neointimal thickness, and medial wall area were all significantly reduced in CO-treated vessels compared with PBS- or N_2-exposed vessels (Fig. 1B). Although a decrease in neointima formation is noted in N_2-treated animals relative to PBS-treated animals this was not significant (Fig. 1B). No significant differences were observed between the three groups of animals for

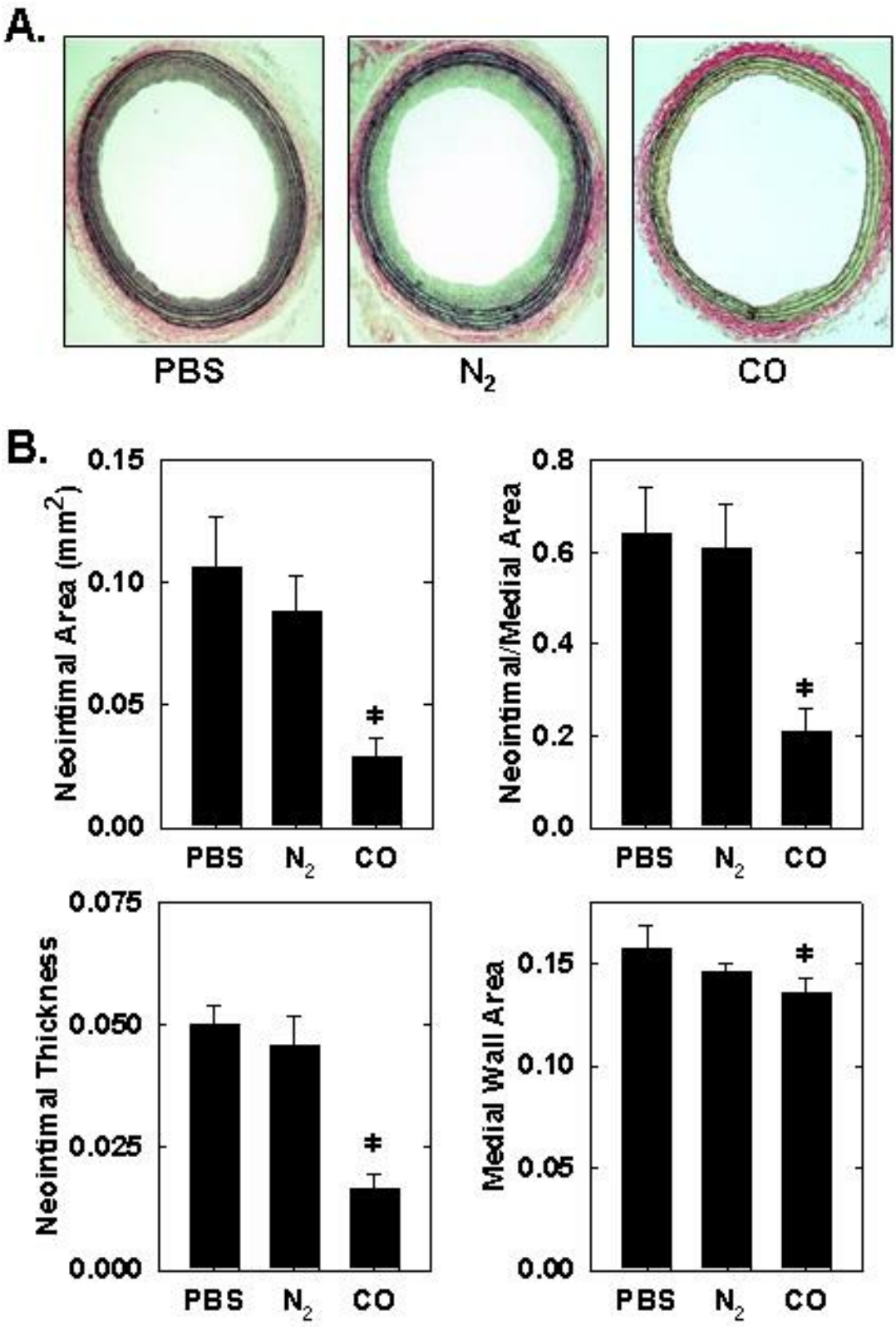

Fig. 1 Effect of CO on neointima formation following rat carotid artery balloon injury. **A**) Representative cross-sections of balloon-injured, perfusion-fixed, Verhoeff-van-Gieson-stained left carotid arteries two weeks after injury. Magn. x 100. Arteries were treated with 50 μl of a saturated solution of CO, N_2, or PBS immediately after injury. **B**) Morphometric analysis of neointima formation two weeks after arterial injury. Results are means ± SEM of between 8 and 13 experiments. *Statistically significant effect of the saturated CO solution.

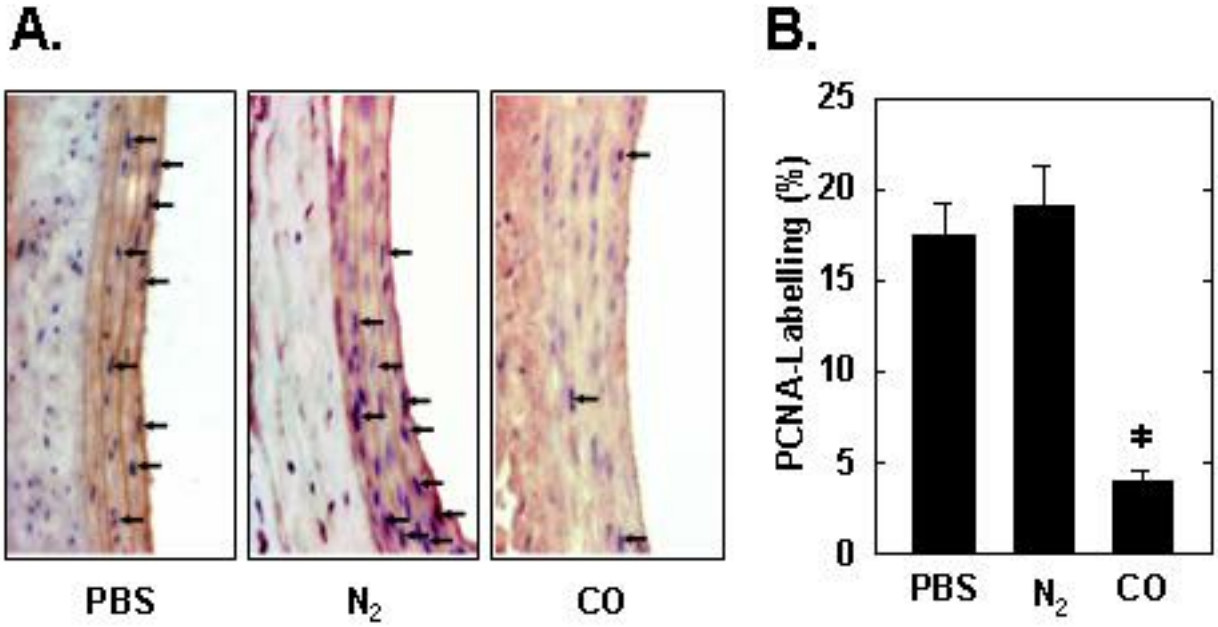

Fig. 2 Effect of CO on PCNA-staining following rat carotid artery balloon injury. **A**) Representative photographs of PCNA-stained medial wall SMC nuclei two days after injury. Magn. x 400. Arteries were treated with 50 μl of a saturated solution of CO, N_2, or PBS immediately after injury. **B**) PCNA LI two days after arterial injury. Results are means ± SEM of 5 experiments. *Statistically significant effect of the saturated CO solution.

circumference of the left carotid external elastic laminae (PBS, 2.96 ± 0.05 mm; N_2, 2.87 ± 0.49 mm; CO, 2.76 ± 0.08 mm) or internal elastic laminae (PBS, 2.59 ± 0.04 mm; N_2, 2.57 ± 0.06 mm; CO, 2.50 ± 0.05 mm).

In subsequent experiments, the effect of CO on SMC proliferation was examined by monitoring nuclear PCNA-staining, a marker of DNA synthesis and SMC mitogenesis (14). Fig. 2A shows representative photomicrographs of PCNA-stained carotid artery medial wall nuclei two days after injury. Preliminary experiments (data not shown) determined that PCNA-LI peaked 2 days after arterial injury. At this time, PBS-treated vessels demonstrated approximately 17% PCNA staining and this was reduced to less than 5% in vessels exposed to CO (Fig. 2B). In contrast, exposure to N_2 had no

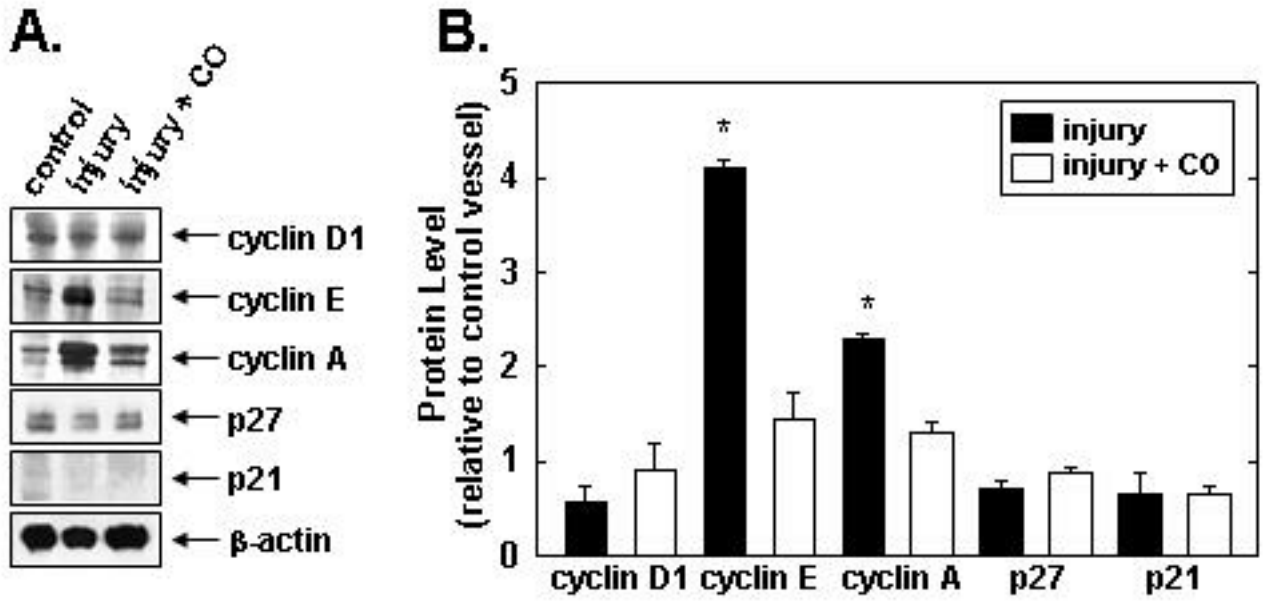

Fig. 3 Effect of CO on the expression of cell cycle regulatory proteins following rat carotid artery balloon injury. **A**) Expression of cyclin D1, cyclin E, cyclin A, p21, p27, and β-actin protein in control and injured arteries treated with or without 50 μl of a saturated solution of CO. Data are representative of four experiments. **B**) Quantification of relative protein levels by laser densitometry. Results are means ± SEM of 4 experiments. *Statistically significant effect of arterial injury.

significant effect on PCNA staining (Fig. 2B).

Finally, the effect of CO on cell cycle regulatory proteins was determined. Two days after arterial injury a marked increase in the expression of the G_1 cyclins, cyclin E and A, was observed in the vessel wall (Fig. 3), which was localized to the SMC-rich medial layer. However, the expression of both cyclin E and A was dramatically reduced following exposure of the vessel to the saturated solution of CO (Fig. 3). In contrast, CO had no effect on the expression of cyclin D1 and failed to induce the expression of the cyclin-dependent kinase inhibitors, p21 or p27 (Fig. 3). Interestingly, arterial injury was also associated with an approximate two-fold increase in the expression of TGF-β1 that was completely suppressed by CO (Fig. 4).

DISCUSSION

The present study demonstrates that acute, local administration of CO immediately after carotid artery balloon injury significantly inhibits neointimal and medial wall remodeling two weeks after arterial injury. The inhibition of neointima formation by CO is associated with a reduction in medial wall DNA replication and with changes in the expression of specific cell cycle regulatory proteins and in the proliferative cytokine, TGF-β1.

The response to arterial injury is a complex process that depends on numerous factors, including the proliferation of vascular SMC and restructuring of the vessel wall. Indeed, inward remodeling of the vessel wall following arterial injury is a major contributor to restenosis (5,22). However, CO does not alter the negative remodeling following arterial injury since the circumferential length of the both the inner and outer elastic laminae is not affected by CO. Instead, CO appears to target SMC proliferation. CO inhibits early medial wall DNA synthesis which leads to a diminished cell population in the medial wall and reduced neointimal development. Consistent with a role for CO in blocking SMC growth, we found that CO blocks injury-induced expression of the G_1 cyclins, cyclin E and A, which are critical for entry and progression of cells through S phase of the cell cycle and DNA synthesis (24). The failure of CO to induce vessel wall p21 expression in our study may be explained by the early transitory nature of the induction of p21 by CO (20). In addition to directly inhibiting the proliferation of vascular SMCs, CO may indirectly influence SMC growth by regulating the synthesis of growth factors. Interestingly, we found that CO completely inhibits the induction of TGF-β1 expression following injury. This cytokine is released at sites of vascular damage and contributes to intimal thickening by stimulating SMC proliferation and collagen synthesis (13,19,29). Furthermore, CO has been reported to inhibit the expression of platelet-derived growth factor (15), another key peptide that has been implicated in neointima formation (11). Thus, CO may prevent intimal thickening following arterial injury via multiple mechanisms.

Our finding that acute administration of CO immediately after injury inhibits neointima formation complements a recent finding demonstrating that inhalation of CO for one hour prior to balloon injury is sufficient to block intimal hyperplasia (20), further reinforcing CO as a potent negative regulator of vascular lesion formation. The local targeting of CO to the vessel wall used in our study may be advantageous over an inhalational approach since it circumvents potential problems with tissue hypoxia associated with the systemic delivery of CO. In this respect, the recent development of CO-releasing molecules that exhibit diverse physicochemical properties and release kinetics offers a promising approach in treating endovascular injury since these compounds could be readily incorporated into coronary stents (4,17,18). Interestingly, the drug-eluting stents that are used to prevent coronary artery restenosis release rapamycin and paclitaxel, which have been shown to induce HO-1 gene expression in vascular

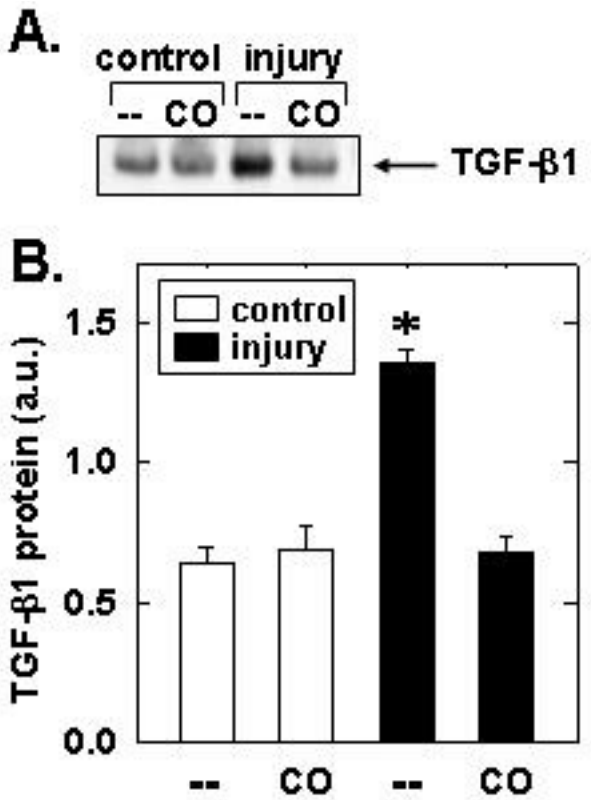

Fig. 4 Effect of CO on the expression of transforming growth factor-β1 (TGF-β1) following rat carotid artery balloon injury. **A**) Expression of TGF-β1 protein in control and injured arteries treated with or without 50 μl of a saturated solution of CO. Data are representative of three experiments. **B**) Quantification of TGF-β1 protein levels [in arbitrary units (a.u.)] by laser densitometry in control and injured arteries treated with 50 μl of a saturated solution of CO. Results are means ± SEM of 3 experiments. *Statistically significant effect of arterial injury.

SMC (3,28). Moreover, the induction of HO-1 contributes to the antiproliferation action of these compounds (3,28). Thus, the HO-1-mediated release of CO may, in part, underlie the clinical efficacy of the currently available drug eluting stents.

In conclusion, the present study demonstrates that the acute delivery of CO immediately after arterial balloon injury inhibits neointima formation and medial wall hypertrophy by blocking SMC proliferation. CO represents an attractive therapeutic agent capable of attenuating post-injury arterial remodeling.

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