

Inhibition of Neointimal Proliferation in Rabbits after Vascular Injury by a Single Treatment with a Protein Adduct of Nitric Oxide

David S. Marks,* Joseph A. Vita,[†] John D. Folts,[§] John F. Keaney, Jr.,[†] George N. Welch,[†] and Joseph Loscalzo[†]

*Department of Medicine, Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115; [†]Whitaker Cardiovascular Institute, Evans Memorial Department of Medicine, Boston University Medical Center, Boston, Massachusetts 02118; and [§]University of Wisconsin Department of Medicine, Cardiology Section, Madison, Wisconsin 53706

Abstract

Endothelium-derived relaxing factor is important for vascular homeostasis and possesses qualities that may modulate vascular injury, including vasodilation, platelet inhibition, and inhibition of smooth muscle proliferation. *S*-nitroso-serum albumin is a naturally occurring adduct of nitric oxide (NO) with a prolonged biologic half-life and is a potent vasodilator and platelet inhibitor. Given the avidity of serum albumin for subendothelial matrix and the antiproliferative effects of NO, we investigated the effects of locally delivered *S*-nitroso-bovine serum albumin (*S*-NO-BSA) and a polythiolated form of bovine serum albumin (pS-BSA) modified to carry several *S*-nitrosothiol groups (pS-NO-BSA) on neointimal responses in an animal model of vascular injury.

Locally delivered *S*-NO-BSA bound preferentially to denuded rabbit femoral vessels producing a 26-fold increase in local concentration compared with uninjured vessels ($P = 0.029$). pS-NO-BSA significantly reduced the intimal/medial ratio ($P = 0.038$) and did so in conjunction with elevations in platelet ($P < 0.001$) and vascular cGMP content ($P \leq 0.001$). pS-NO-BSA treatment also inhibited platelet deposition ($P = 0.031$) after denuding injury. Comparison of BSA, *S*-NO-BSA, pS-NO-BSA, and control revealed a dose-response relationship between the amount of displaceable NO delivered and the extent of inhibition of neointimal proliferation at 2 wk ($P \leq 0.001$).

Local administration of a stable protein *S*-nitrosothiol inhibits intimal proliferation and platelet deposition after vascular arterial balloon injury. This strategy for the local delivery of a long-lived NO adduct has potential for preventing restenosis after angioplasty. (*J. Clin. Invest.* 1995; 96:2630–2638.) Key words: endothelium-derived relaxing factor • sulfhydryl groups • vascular smooth muscle cell • cell proliferation

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Address correspondence to Joseph Loscalzo, MD, PhD, Boston University Medical Center, Section of Cardiology, 88 E. Newton Street, Boston, MA 02118. Phone: 617-638-8706; FAX: 617-638-4066.

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Introduction

The vascular endothelium participates in many homeostatic mechanisms important for the regulation of vascular tone and the prevention of thrombosis. A primary mediator of these functions is endothelium-derived relaxing factor (EDRF).¹ First described in 1980 by Furchgott and Zawadzki (1), EDRF is either nitric oxide (2) (NO) or a closely related NO-containing molecule (3–5).

Removal of the endothelium is a potent stimulus for neointimal proliferation and represents an important mechanism for restenosis of atherosclerotic vessels after balloon angioplasty (6, 7). NO dilates blood vessels (8), inhibits platelet activation (9), and limits the proliferation of vascular smooth muscle cells (VSMC) in vitro (10). Similarly, in animal models, suppression of platelet-derived mitogens decreases intimal proliferation (7). The potential importance of endothelium-derived NO in the control of arterial remodeling after injury is further supported by recent preliminary reports in humans suggesting that systemic NO donors reduce angiographic restenosis 6 mo after balloon angioplasty (11).

Biologic thiols react readily with NO (probably as N_2O_3 or NO^+) under physiologic conditions to form stable, biologically active *S*-nitrosothiol species (10, 12). *S*-nitrosothiols exhibit EDRF-like activity in vitro and in vivo, including vasodilation (3) and platelet inhibition via a cyclic 3',5'-guanosine monophosphate (cGMP)-dependent mechanism (13–15).

Balloon arterial injury results in endothelial denudation and subsequent regrowth of dysfunctional endothelium (16) that may contribute to the local smooth muscle cell proliferation and extracellular matrix production underlying restenosis of the arterial lumen. We hypothesized that local delivery of an EDRF-like species to restore or replace the relative deficiency of EDRF observed with dysfunctional endothelium will modulate the effects of vascular injury and potentially reduce intimal proliferation after injury. We, therefore, synthesized a form of serum albumin, a plasma protein avid for subendothelial matrix, specifically designed to carry multiple NO groups, and examined its effect on platelet adhesion and neointimal growth after balloon injury.

Methods

Materials. Sulfanilamide and *N*-(1-naphthyl)ethylenediamine dihydrochloride were purchased from Aldrich Chemical Co. (Milwaukee, WI).

1. **Abbreviations used in this paper:** EDRF, endothelium-derived relaxing factor; NO, nitric oxide; PCNA, proliferating cell nuclear antigen; pS-BSA, polythiolated BSA; pS-NO-BSA, polythiolated *S*-nitroso-BSA; *S*-NO-BSA, *S*-nitroso-BSA; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cell.

Sodium bicarbonate, sodium chloride, sodium phosphate, sodium nitrite, potassium phosphate-monobasic, 40% formaldehyde solution, and sucrose were purchased from Fisher Scientific (Fairlawn, NJ). Sephadex G-25 was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). IODO-BEADS were purchased from Pierce Chemical Co. (Rockford, IL), and Na[¹²⁵I] from New England Nuclear/DuPont (Boston, MA). [¹¹¹In]Oxine was purchased from Amersham (Arlington Heights, IL). Cell culture media (RPMI 1640) and fetal bovine serum were purchased from Gibco Laboratories (Grand Island, NY). Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody was purchased from Dako A/S (Glostrup, Denmark). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Citrate-phosphate-dextrose anticoagulant solution contained 10 mM citric acid, 90 mM trisodium citrate, 15 mM NaH₂PO₄, and 142 mM dextrose, pH 7.35. Tris-buffered saline (TBS) consisted of 10 mM Tris, pH 7.4, and 150 mM NaCl. Acid-citrate-dextrose contained 100 mM trisodium citrate and 142 mM dextrose, pH 6.5. Phosphate-buffered saline contained 10 mM sodium phosphate and 150 mM NaCl, pH 7.4 or 6.8 (in cell migration assays).

Synthesis of S-nitroso species. S-nitroso-bovine serum albumin (S-NO-BSA) was synthesized as previously described (12). Fatty acid-free bovine serum albumin (200 mg/ml) was exposed to a 1.4 M-fold excess of NaNO₂ in 0.5 N HCl for 30 min at room temperature and neutralized with an equal volume of TBS and of 0.5 N NaOH. Polythiolated bovine serum albumin (pS-BSA) was prepared after Benesch and Benesch (17). Briefly, essential fatty acid-free bovine serum albumin (50 mg/ml) was dissolved in water with N-acetyl-homocysteine thiolactone (35 mM) and 0.05% polyethylenesorbitan monolaurate. Equimolar silver nitrate was slowly added at room temperature over 90 min at pH 8.5. Excess thiourea (70 mM) was added and the pH was lowered to 2.5. Excess silver nitrate was removed by Dowex 50 chromatography with a mobile phase consisting of 1 M thiourea, pH 2.5, and excess thiourea was removed by Sephadex G-25 chromatography. pS-BSA was prepared within 2 d of nitrosation and stored at 4°C. Nitrosation of pS-BSA was accomplished using 3.6 mM NaNO₂ in 0.5 N HCl for 30 min at room temperature. The solution was adjusted to pH 4.0 with 0.5 N NaOH after nitrosation. In platelet binding studies, 0.1 mM EDTA was added to pS-BSA before nitrosation.

The content of S-nitrosothiol was determined by the method of Saville (18). Protein concentration was determined using the method of Lowry and colleagues (19).

Preparation of [¹²⁵I]-labeled S-NO-BSA, [¹²⁵I]-labeled pS-NO-BSA, and [¹¹¹In]-labeled platelets. BSA or pS-BSA (0.1 mg/ml) was combined with two IODO-BEADS and 0.1 mCi of Na[¹²⁵I] (20). The solution was incubated for 45 min and unincorporated Na[¹²⁵I] was removed by gel filtration with Sephadex G-25 equilibrated with TBS. [¹²⁵I]-Labeled S-BSA had a specific activity of 5.7×10^6 cpm/ μ g and [¹²⁵I]-labeled pS-BSA had a specific activity of 18.2×10^6 cpm/ μ g. Subsequently both molecules were S-nitrosated as described for unlabeled S-BSA and pS-BSA. 0.5 ml of [¹²⁵I]-labeled S-NO-BSA or polythiolated S-nitroso-bovine serum albumin (pS-NO-BSA) with 1×10^6 cpm was administered with saline control on the contralateral vessel. [¹¹¹In]-Labeling was performed after the method of Wistow and colleagues (21).

Animal preparation. All animal preparations were performed within the institutional guidelines of the Brockton/West Roxbury Department of Veteran Affairs Medical Center and Boston University Medical Center and were in accordance with the guiding principles of the American Physiological Society. New Zealand White rabbits (3.5–4.2 kg) of either sex were premedicated with 5 mg/kg intramuscular (i.m.) xylazine hydrochloride (Miles Pharmaceuticals, Shawnee Mission, KS) and 0.1 mg/kg subcutaneous atropine sulfate (Lyphomed, Inc., Deerfield, IL) 15 min before the induction of anesthesia. Anesthesia was induced with 40 mg/kg i.m. ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA) and 5 mg/kg i.m. acepromazine maleate (Aveco Co., Inc., Ford Dodge, IA). Additional doses of ketamine hydrochloride were administered as necessary to maintain anesthesia. For survival

studies, 100,000 U penicillin G (Apothecon of Bristol-Myers Squibb, Princeton, NJ) was administered intramuscularly perioperatively. The skin over the femoral arteries was next infiltrated with 1% lidocaine (Astra Pharmaceuticals, Inc., Westborough, MA), and the common femoral arteries were exposed from the inguinal ligament to the superficial femoral artery. Arteries were cleared of connective tissue, side branches were ligated, and the superficial femoral artery was suspended with silk ties. A 1.5–2.0-cm length of femoral artery was isolated from the circulation proximally and distally with neurosurgical microaneurysm clips. The superficial femoral artery was cannulated with a 2F Fogarty balloon catheter (American Edwards Laboratories, Santa Ana, CA) that was passed into the isolated segment of femoral artery. The balloon was inflated with sufficient air to generate slight resistance and withdrawn three times. A 20-gauge angiocath was then inserted in the arteriotomy, and 1 mg/ml of 25.8 mg/ml pS-NO-BSA or 49.2 mg/ml S-NO-BSA was administered over 15 min. The contralateral femoral artery was prepared identically and an appropriate control (25.8 mg/ml pS-BSA, 49.2 mg/ml BSA, or 0.66 mg/ml sodium nitroprusside [SNP]) was administered. After administration of the agent, the superficial femoral artery was ligated and flow was reestablished. Sham-operated animals underwent surgical exposure and side branch ligation, but no balloon injury was performed or local delivery administered. The area of balloon injury was marked by surgical staples in the adjacent muscle fascia. For chronic studies, the incision was closed with subcuticular absorbable suture and the animals were allowed to recover. For acute studies, blood was allowed to circulate through the treated areas for 15 min before vessel harvest. In some experiments, a distant control vessel, the right carotid artery, was isolated and harvested without any other manipulation.

cGMP analysis. Whole blood was obtained from fasting human volunteers who had not taken any medications in the past 96 h and platelet-rich plasma was prepared by centrifugation at 120 g. Platelet counts were determined using a Coulter counter (model ZM; Coulter Diagnostics, Hialeah, FL). After balloon injury and treatment with pS-NO-BSA or pS-BSA, arterial segments were harvested and 2-mm segments were incubated with 100 μ l platelet-rich plasma containing 10 μ M isobutylmethylxanthine. After 1 min, an equal volume of ice-cold 10% trichloroacetic acid was added to each aliquot and the sample was vortexed. Enzyme-linked immunoassay of cGMP was then performed (Cayman Chemical Co., Ann Arbor, MI). Separate 2-mm vessel segments were also assayed for tissue cGMP after treatment with ice-cold 10% trichloroacetic acid and sonication (Heat Systems Ultrasonics, Inc., Plainville, NY).

Tissue processing and analysis. On the 14th postoperative day, animals were killed with 120 mg/kg intravenous sodium pentobarbital (Anpro Pharmaceuticals, Arcadia, CA), and the abdominal aorta and inferior vena cava were interrupted by silk ties. A 7F plastic cannula was inserted into the abdominal aorta and the vessels were perfused clear with saline followed by fixation at 100 mmHg pressure with 10% buffered formalin. The vessels were stored in 10% buffered formalin and the samples were paraffin-embedded and microtome-sectioned. Six sections were made along the length of each injured segment of vessel and the specimens were stained with Verhoeff's stain for elastic tissue. The areas within the lumen, internal elastic membrane, and external elastic membrane were measured by a blinded observer using computerized digital planimetry (Zeiss, Oberkochen, Germany).

In a separate set of animals, vessels were perfusion-fixed with 10% buffered formalin 7 d after injury and processed for analysis of proliferating cells within 12 h as described above. Sections were stained for PCNA and adjacent sections were stained with hematoxylin and eosin. Five representative sections from each segment were examined. Total nuclei were counted from the hematoxylin and eosin slides and percent PCNA-positive cells were defined as the number of PCNA-positive nuclei divided by the total nuclei multiplied by 100.

[¹¹¹In]-Labeled platelet studies. Animals were prepared and treated with pS-NO-BSA or pS-BSA as described above. 5 min before the release of the vascular clamps, autologous [¹¹¹In]-platelets were infused

via the femoral vein, and blood was allowed to recirculate for 15 min before harvest. Platelet adhesion was quantified with a gamma counter (Capintec Instruments, Inc., Pittsburgh, PA) and normalized to tissue wet weight.

VSMC migration assay. Neonatal rat aortic VSMC were isolated and incubated as previously described (22, 23). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin G sodium (100 U/ml), and streptomycin (100 µg/ml) and incubated at 37°C under 5% CO₂ in air. VSMC from the ninth passage were used for cell migration assays. Migration of VSMC was assayed by a modification of Boyden's chamber method using microhemotaxis chambers (Costar Corp., Cambridge, MA) with polycarbonate filters having pores of 5.0 µm in diameter (24, 25). Cultured VSMC were treated with 0.05% trypsin and 0.53 mM EDTA, and were suspended at a concentration of 1 × 10⁶ cells/ml. A 100-µl volume suspension of cells was placed in the upper chamber and a 600-µl volume of varying concentrations of pS-NO-BSA and pS-BSA diluted in PBS (pH 6.8) with 10 ng/ml PDGF-BB in the lower chamber (25). The chambers were incubated for 4 h at 37°C under 5% CO₂ in air. The filter was removed, cells from the upper side of the chamber were gently scraped off, and the filter was fixed in 10% buffered formaldehyde, stained with 0.18% crystal violet, and examined microscopically (×400). Cells were counted and migration was expressed as the mean number of cells that migrated per high power field.

Statistics. Data are presented as mean ± SEM. Treatments were administered in a paired fashion with one femoral artery receiving S-nitrosated protein and the contralateral artery receiving the appropriate nonnitrosated control. pS-NO-BSA bearing the two intermediate concentrations of displaceable NO was administered in paired fashion with pS-BSA, but not the highest or lowest concentration; this difference accounts for the difference in vessels used between treated and control groups described in the figure legends. SNP was given to a separate set of animals. Data were tested for normality using the Kolmogorov-Smirnov algorithm and for equal variance with the Levene median test. Normally distributed variables were compared using the paired t test and nonnormally distributed variables were compared using the Wilcoxon sign-rank test or the Mann-Whitney rank-sum test. Nonpaired data were compared using an independent t test. Statistical analysis of dose-response was performed by one-way ANOVA. Statistical significance was accepted if the null hypothesis was rejected with *P* < 0.05.

Results

NO content of S-nitrosothiol species. Using total protein concentration of 755 µM (49.2 mg/ml), the synthesis of S-NO-BSA resulted in a displaceable NO concentration of 230 ± 60 µM, yielding a stoichiometry of 0.3 ± 0.08 mol NO/mol albumin (*n* = 11). Polythiolation and S-nitrosation of BSA at a final protein concentration of 391 µM (25.8 mg/ml, *n* = 8) yielded a 5.9-fold increase in displaceable NO content with a maximum of 2,300 µM displaceable NO or 5.9 mol NO/mol albumin. This stock solution was used with dilution in the experiments described below.

S-NO-BSA and pS-NO-BSA binding. The binding of locally and systemically delivered [¹²⁵I]-labeled S-NO-BSA and pS-NO-BSA to balloon-injured rabbit femoral artery is shown in Fig. 1. Compared with systemic administration to an injured artery, local delivery of [¹²⁵I]-labeled S-NO-BSA to the site of injury was associated with a 26-fold increase in binding (140.4 ± 3.9 × 10³ cpm/gram vs. 5.4 ± 0.9 × 10³ cpm/gram, *n* = 4; *P* = 0.029). Endothelial denudation facilitated S-NO-BSA binding as systemic administration of [¹²⁵I]-S-NO-BSA resulted in significantly greater deposition at the site of balloon injury compared with an uninjured control vessel both exposed

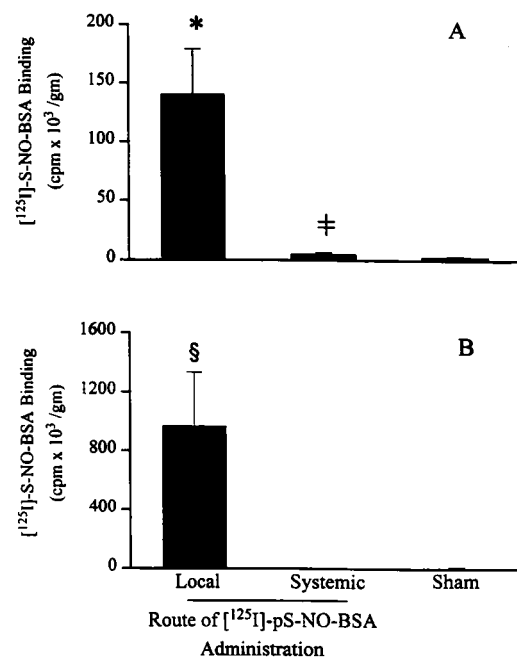


Figure 1. [¹²⁵I]-S-NO-BSA (A) and [¹²⁵I]-pS-NO-BSA (B) binding as a function of the method of delivery. Rabbit femoral arteries were isolated and balloon-injured as described in Methods and the [¹²⁵I]-labeled species applied either directly into the injured artery (*Local*) or injected intraarterially (*Systemic*). [¹²⁵I]-S-NO-BSA had a specific activity of 5.7 × 10⁶ cpm/µg and pS-NO-BSA had a specific activity of 18.2 × 10⁶ cpm/µg. [¹²⁵I]-S-NO-BSA or [¹²⁵I]-pS-NO-BSA binding (*Sham*) was determined from an uninjured carotid artery harvested simultaneously with the femoral arteries. Data are presented as mean ± SEM per gram of wet tissue weight and are derived from four animals for [¹²⁵I]-S-NO-BSA and six animals for [¹²⁵I]-pS-NO-BSA. * *P* < 0.05, local vs. systemic delivery [¹²⁵I]-S-NO-BSA, † *P* < 0.05, systemic injured vs. sham, [¹²⁵I]-S-NO-BSA, and § *P* < 0.05 local vs. systemic delivery, [¹²⁵I]-pS-NO-BSA.

to systemically delivered [¹²⁵I]-S-NO-BSA (5.4 ± 0.9 × 10³ cpm/gram vs. 3.0 ± 0.3 × 10³ cpm/gram, *n* = 4; *P* = 0.038). Local delivery of [¹²⁵I]-labeled pS-NO-BSA was also associated with increased binding compared with systemic delivery (976.1 ± 365.6 vs. 1.2 ± 0.3 cpm/gram, *n* = 6; *P* = 0.045). There was no significant difference in the binding of locally delivered [¹²⁵I]-S-NO-BSA and [¹²⁵I]-pS-NO-BSA (0.054 vs. 0.055 µg/gram, respectively).

pS-NO-BSA effect on platelet binding to injured vessel. Since platelet adhesion to the injured arterial surface is important in the proliferative response to injury, we investigated the effects of pS-NO-BSA on platelet deposition after balloon injury, the results of which are shown in Fig. 2. The local administration of pS-NO-BSA reduced the adhesion of [¹¹¹In]-labeled platelets to the injured vessels more than fourfold compared with control (71.3 ± 40.4 × 10³ cpm/gram vs. 16.3 ± 6.2 × 10³ cpm/gram, *n* = 6, *P* < 0.031).

S-NO-BSA and pS-NO-BSA effects on neointimal proliferation. Neointimal proliferation after local delivery of S-nitrosated proteins and appropriate controls was evaluated by comparing the lumen area, absolute neointimal area, and neointima/media

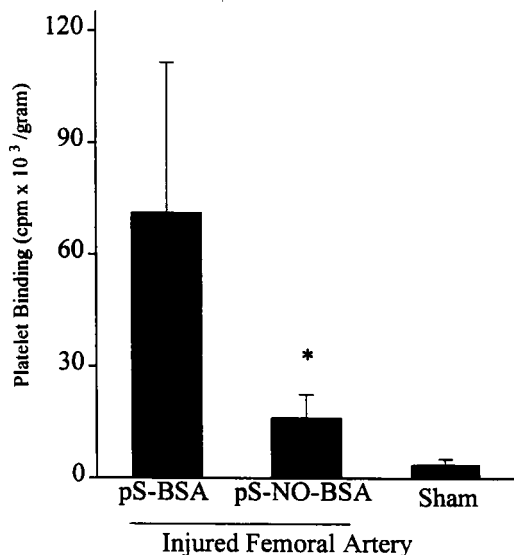


Figure 2. The effect of pS-NO-BSA and pS-BSA on [¹¹¹In]-labeled platelet binding to injured rabbit femoral artery. Femoral arteries were isolated and balloon-injured as described in Methods. During paired local administration of pS-NO-BSA and pS-BSA, [¹¹¹In]-labeled platelets were administered intravenously and allowed to circulate after flow was reestablished in the treated arteries. [¹¹¹In]-Labeled platelet binding was determined by quantification of radioactivity after flow was reestablished for a period of 15 min. Nonspecific [¹¹¹In]-labeled platelet binding (Sham) was determined from uninjured carotid artery harvested with femoral arteries. Data presented are mean±SEM per gram of wet tissue weight and are derived from six animals. * $P < 0.05$, pS-BSA vs. pS-NO-BSA.

ratios, as shown in Fig. 3. Compared with BSA, the administration of S-NO-BSA did not significantly increase lumen area ($0.85 \pm 0.059 \times 10^5 \mu\text{m}^2$ vs. $1.47 \pm 0.57 \times 10^5 \mu\text{m}^2$, $n = 15$), reduce neointimal area ($2.54 \pm 0.33 \times 10^5 \mu\text{m}^2$ vs. $2.54 \pm 0.33 \times 10^5 \mu\text{m}^2$, $n = 15$), or reduce neointima/media ratio (1.07 ± 0.17 vs. 0.72 ± 0.084 , $n = 15$), although a trend was noted. By contrast, compared with pS-BSA, the administration of pS-NO-BSA with greater displaceable NO content maintained the lumen area ($0.31 \pm 0.061 \times 10^5 \mu\text{m}^2$ vs. $0.86 \pm 0.12 \times 10^5 \mu\text{m}^2$, $n = 7$, $P = 0.010$), reduced neointimal area by 81% ($2.24 \pm 0.33 \times 10^5 \mu\text{m}^2$ vs. $0.41 \pm 0.11 \times 10^5 \mu\text{m}^2$, $n = 7$, $P = 0.022$), and reduced the neointima/media ratio by 77% (0.85 ± 0.12 vs. 0.20 ± 0.066 , $n = 7$, $P = 0.025$). The lumen area ($1.22 \pm 0.80 \times 10^5 \mu\text{m}^2$, $n = 7$), neointimal area ($0.23 \pm 0.07 \times 10^5 \mu\text{m}^2$, $n = 7$), and the neointima/media ratio (0.12 ± 0.041 , $n = 7$) in the sham-operated animals were comparable with those of the vessels treated with pS-NO-BSA. Using a relatively high concentration of a conventional NO donor, SNP (2,300 μM), we noted a trend toward inhibition of neointimal proliferation in luminal area ($1.13 \pm 0.091 \times 10^5 \mu\text{m}^2$, $n = 5$), neointimal area ($1.47 \pm 4.15 \times 10^5 \mu\text{m}^2$, $n = 5$, $P = 0.056$), and the neointima/media ratio (0.60 ± 0.19 , $n = 5$, $P = 0.11$) compared with control. Illustrative photomicrographs of vessels treated with S-nitrosated albumins are shown in Fig. 4.

Displaceable NO effect on neointimal proliferation. Since S-NO-BSA exhibited a trend toward inhibition and pS-NO-BSA clearly inhibited neointimal proliferation, we examined

the relationship between the amount of displaceable NO and the extent of neointimal response after vascular injury; these results are presented in Fig. 5. A trend toward preservation of lumen area was noted with increasing concentrations of displaceable NO in the S-nitrosated albumin ($P = 0.192$). However, there was a significant inverse relationship between displaceable NO and neointimal proliferation as quantified by absolute neointimal area ($P = 0.001$) and the neointima/media area ratio ($P = 0.001$).

p-NO-BSA effects on cellular proliferation. Mouse monoclonal antibody staining against PCNA was used to assay the degree of S1-phase activity at 7 d after injury, the time point at which PCNA-positive cells are maximally present. At this time point, no difference in the percentage of proliferating cells was noted between vessels treated with pS-BSA ($30.1 \pm 5.9\%$, $n = 5$) and vessels treated with pS-NO-BSA ($37.8 \pm 5.9\%$, $n = 6$). Similarly, no significant difference was noted in the S1-phase activity of the pS-NO-BSA-treated vessels compared with the pS-BSA-treated controls (neointimal area: $0.124 \pm 0.06 \times 10^5 \mu\text{m}^2$ vs. $0.258 \pm 0.19 \times 10^5 \mu\text{m}^2$, $n = 5$, $P = 0.54$, and neointima/media ratio: 0.32 ± 0.005 vs. 0.068 ± 0.027 , $n = 6$, $P = 0.15$).

pS-NO-BSA-treated vessel effect on platelet cGMP and vessel cGMP. NO inhibits platelets and relaxes smooth muscle cells through a cGMP-mediated mechanism. Thus, we tested the ability of pS-NO-BSA-treated vessels to increase platelet and vascular cGMP. Platelet cGMP was significantly increased after a 1-min exposure to pS-NO-BSA-treated vessels compared with pS-BSA controls (19.9 ± 3.3 vs. 4.11 ± 0.9 pmol/ 10^8 platelets, $n = 14$, $P = 0.001$); each vessel was washed three times with normal saline before incubation with platelets. In addition, vascular cGMP levels were also elevated after treatment with pS-NO-BSA compared with pS-BSA control (0.253 ± 0.092 vs. 0.041 ± 0.008 pmol/mg protein, $n = 13$, $P = 0.002$), demonstrating a direct effect on vascular smooth muscle as well. The results are shown in Fig. 6.

VSMC migration. As VSMC migration to the site of injury may be important in the reparative and proliferative response of the vessel after injury, we examined the effect of pS-NO-BSA on VSMC migration stimulated by a physiologically relevant agonist, PDGF-BB. pS-NO-BSA inhibited VSMC migration compared with pS-BSA (2.5 ± 0.42 vs. 14.2 ± 3.65 cells/high power field, $n = 6$, $P = 0.002$) and PBS controls (27.5 ± 3.58 cells/high power field, $n = 6$, $P = 0.002$), as demonstrated in Fig. 7.

Discussion

We have demonstrated previously that NO combines with protein sulfhydryl groups in the presence of molecular oxygen to form stable, biologically active molecules with cGMP-dependent vasodilatory and antiplatelet properties, both in vitro and in vivo (12, 13). The data presented here demonstrate that serum albumin, after S-nitrosation, can bind avidly to balloon-injured femoral arteries and inhibit neointimal proliferation. This phenomenon is associated with diminished platelet deposition at the site of injury through a cGMP-dependent mechanism and elevation of the vessel cGMP. Moreover, the extent of inhibition of neointima formation is directly related to the quantity of displaceable NO carried by albumin.

The endothelium is essential for vascular integrity, control

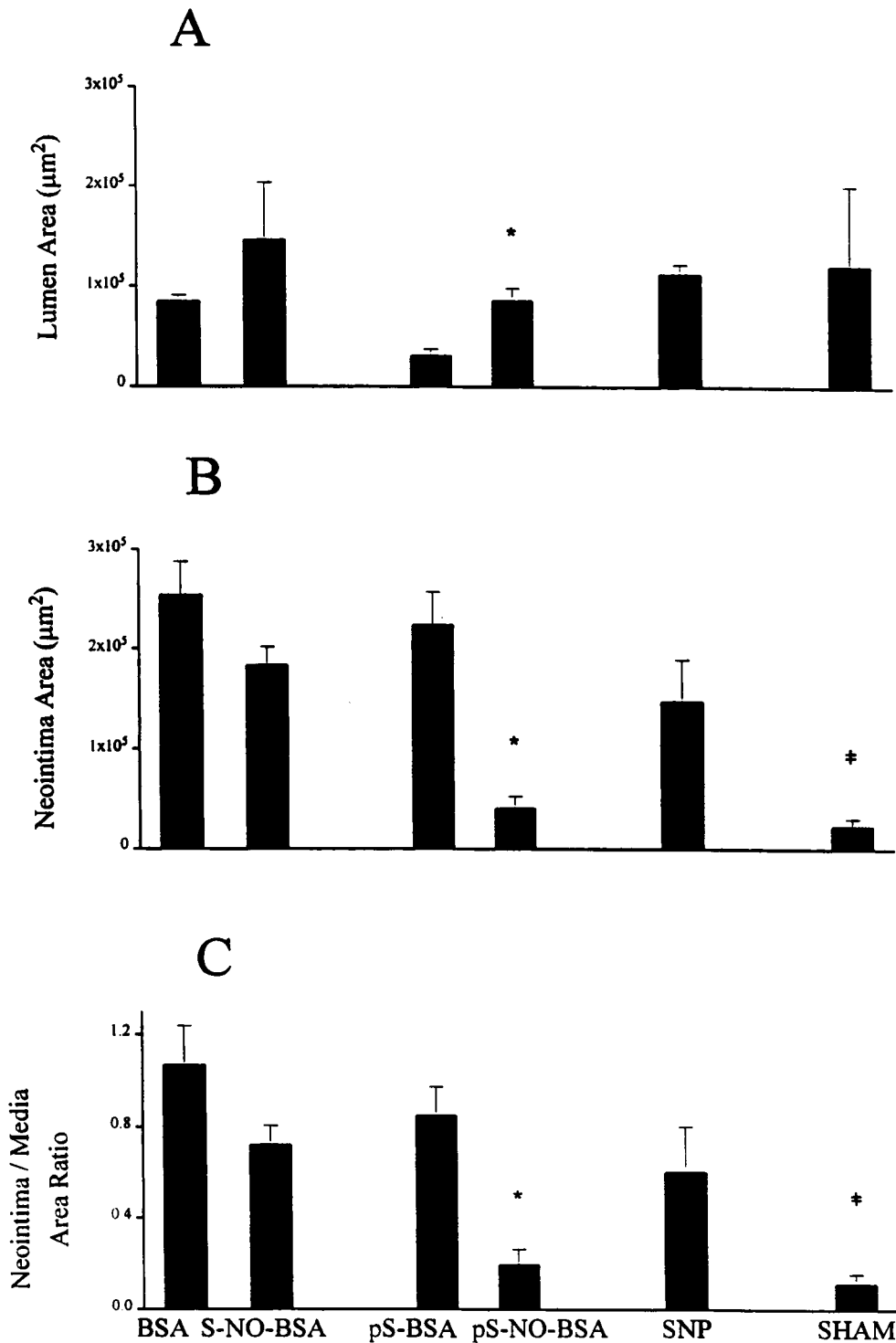


Figure 3. The effect of S-NO-BSA or pS-NO-BSA and BSA or pS-BSA on neointimal proliferation 14 d after balloon injury of rabbit femoral artery. Femoral arteries were isolated and balloon-injured as described in Methods. pS-BSA and pS-NO-BSA were applied in a paired fashion directly into the arterial lumen for 15 min and then blood flow was reestablished. After 14 d, arteries were harvested, perfusion-fixed, stained, and subjected to morphometric analysis of the lumen, intimal, and medial areas. Neointimal proliferation is reported as the lumen area (A), the absolute neointimal area (B), and as a ratio of neointima/media (C) in three to six segments from each artery. Data are expressed as mean \pm SEM and are derived from 15 vessels in the BSA and S-NO-BSA groups, 11 vessels in the pS-BSA group, 7 vessels in the pS-NO-BSA and SHAM groups, and 5 in the SNP group. * $P < 0.05$, pS-NO-BSA vs. pS-BSA for all panels, † $P < 0.05$ SHAM vs. pS-BSA for B and C.

of thrombosis (26, 27), and the regulation of intimal growth (28). The endothelium serves these functions by the production of locally active effector molecules including EDRF, a compound that has been identified as NO or a closely related NO-containing molecule. EDRF is responsible, in part, for many biologic actions via the activation of guanylyl cyclase, including relaxation of vascular smooth muscle (3, 29), inhibition of platelets (7), control of leukocyte adhesion to the subendothelium (30), modulation of vascular permeability (31), and, perhaps, local control of vascular smooth muscle growth. Since balloon angioplasty removes the endothelium from arterial smooth muscle, these endothelial functions are lost after the procedure. In particular, removal of the endothelium and damage to the smooth muscle cells are associated with intimal proliferation (32). The mechanism for this response is complex and involves platelet deposition and activation, cytokine elaboration

of thrombosis (26, 27), and the regulation of intimal growth (28). The endothelium serves these functions by the production of locally active effector molecules including EDRF, a compound that has been identified as NO or a closely related NO-containing molecule. EDRF is responsible, in part, for many biologic actions via the activation of guanylyl cyclase, including relaxation of vascular smooth muscle (3, 29), inhibition of platelets (7), control of leukocyte adhesion to the subendothelium (30), modulation of vascular permeability (31), and, perhaps, local control of vascular smooth muscle growth. Since balloon angioplasty removes the endothelium from arterial smooth muscle, these endothelial functions are lost after the procedure. In particular, removal of the endothelium and damage to the smooth muscle cells are associated with intimal proliferation (32). The mechanism for this response is complex and involves platelet deposition and activation, cytokine elaboration

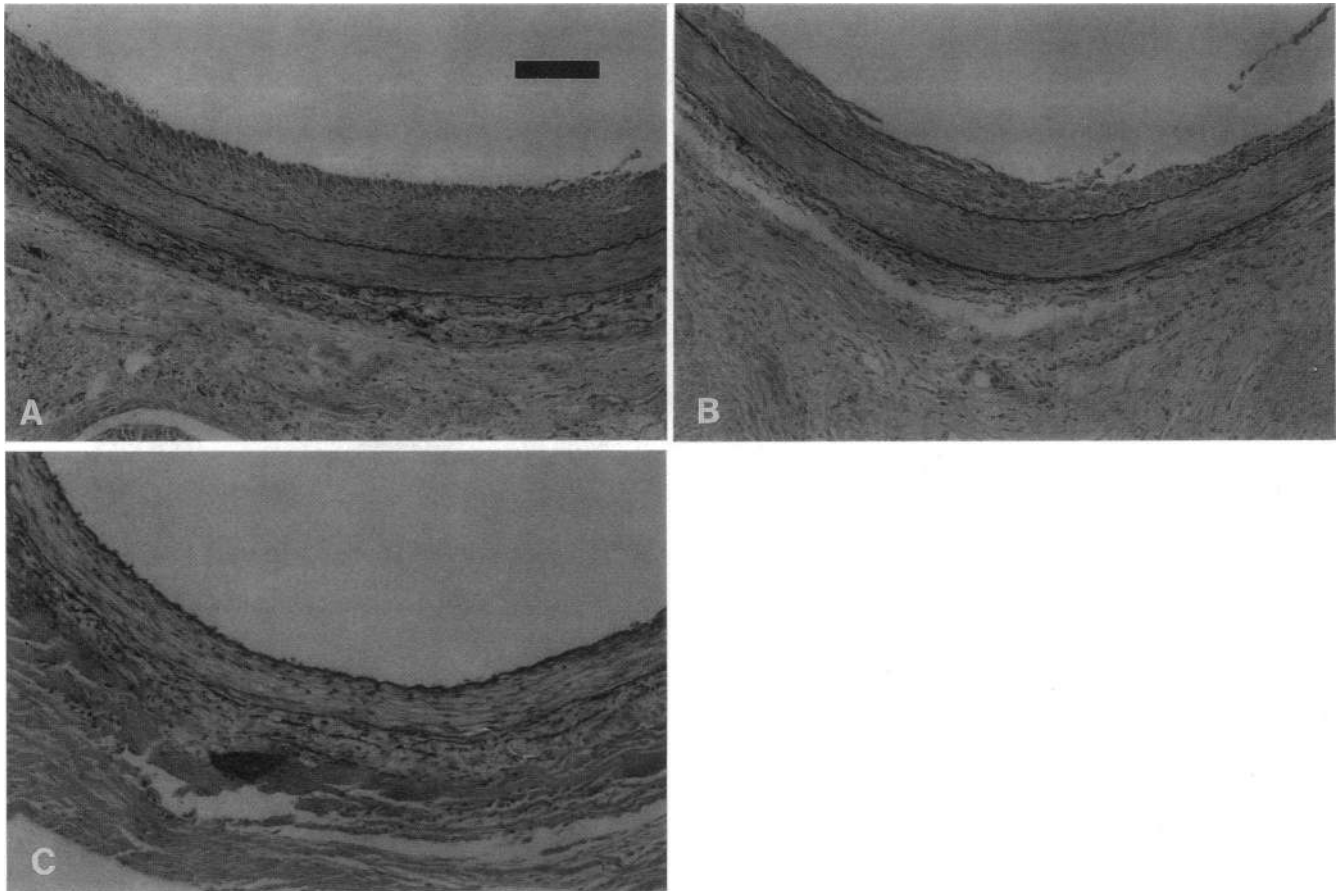


Figure 4. Illustrative photomicrographs of the effects of control and *S*-nitrosated serum albumin on neointimal proliferation. Femoral arteries were isolated and balloon-injured as described in Fig. 2. Vessels were exposed to three different agents differing in amount of displacable NO: (A) BSA alone, (B) *S*-NO-BSA containing 0.3 ± 0.1 mol displacable NO per mole albumin, or (C) *pS*-NO-BSA containing 3.2 ± 1.3 mol displacable NO per albumin molecule and flow was reestablished. After 14 d, arteries were harvested, perfusion-fixed, paraffin-embedded, sectioned, and stained with Verhoeff's stain for elastic tissue. Bar, 100 μ m for all panels. $\times 10$.

tion, smooth muscle cell migration and proliferation, and extracellular matrix production. After balloon injury, the endothelium regenerates rapidly but is often dysfunctional and presumably is unable to maintain an adequate antithrombotic, vasodilating, and antiproliferative phenotype (16).

NO donors have been used with some success in the setting of balloon injury to produce decreases in intimal proliferation and in platelet deposition. In the porcine carotid model, Groves and colleagues (33) demonstrated reduced platelet adhesion and thrombus formation locally after systemic administration of SIN-1, a spontaneous NO donor and metabolite of molsidomine. These authors showed a 2.3-fold reduction in platelet deposition without any significant hemodynamic changes. Because administration of this agent was associated with an increase in template bleeding time and in platelet cGMP, it is possible that SIN-1 exerted its effects through systemic platelet inhibition. A preliminary report from the ACCORD trial also suggests that NO donors might be effective adjuncts for balloon angioplasty in humans (9). This multicenter study evaluated SIN-1 acutely and molsidomine chronically over 6 mo with diltiazem treatment as a control arm in patients undergoing balloon angioplasty. The loss index and binary restenosis rate were significantly improved in the NO treatment group, although the late

loss was not significantly different between groups. Chronic supplementation with L-arginine, a precursor of endothelium-derived NO, has been shown to reduce intimal hyperplasia in rabbit thoracic aorta (34) and rat carotid artery (35). By contrast, administration of an inhibitor of NO synthase, *N*^G-nitro-L-arginine methyl ester, accelerated neointimal formation in the setting of balloon injury (36).

von der Leyen and colleagues (37) recently reported successful transfection of the constitutive endothelial-type NO synthase (eNOS) gene in a rat carotid injury model. In that preliminary study, eNOS incorporation and NO production were demonstrable 4 d after transfection, and neointimal proliferation was partially inhibited 2 wk after injury and transfection. In our study, *S*-nitrosated albumin was administered acutely, and given its half-life of 12 h (14), it is unlikely that significant amounts of displacable NO were still present 4 d after injury. The effectiveness of both early and late administration of NO suggests that NO may influence the complex response to injury by multiple mechanisms. In addition to modifying the development of platelet thrombus and the release of growth factors from platelets, local delivery of *S*-nitrosothiols could modulate gene transcription in VSMC (38) as well as smooth muscle metabolism after injury. In addition, by inducing VSMC cyto-

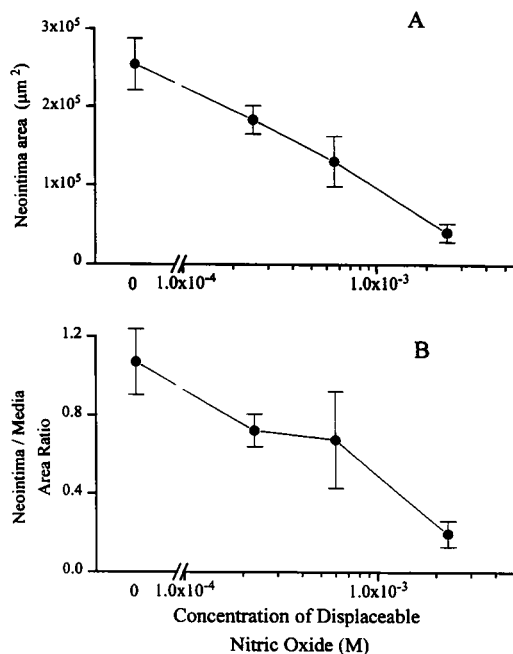


Figure 5. The relationship between neointimal proliferation and the quantity of displaceable NO in preparations of *S*-nitrosated albumin. Femoral arteries were isolated and balloon-injured as described in Fig. 3. Vessels were exposed to different preparations of *S*-nitrosated albumin with different stoichiometric amounts of displaceable NO. After 14 d vessels were harvested and analyzed as described in Fig. 3. Data are expressed as mean ± SEM and are derived from 4–15 animals in each group. $P = 0.001$ for trend.

toxicity directly, NO may facilitate release of basic fibroblast growth factor from VSMC which, in turn, stimulates endothelial cell growth (39).

Our data demonstrate a profound limitation of neointimal proliferation after a single, local administration of a durable, potent *S*-nitrosothiol. Antiplatelet activity may explain these findings, in part, since we observed a fourfold reduction in platelet deposition to injured arterial segments after treatment with p*S*-NO-BSA. Similarly, we also demonstrated direct platelet inhibition by the p*S*-NO-BSA-treated vessel rings. Inhibition of platelet binding would result in many effects that are likely to reduce the proliferative response after injury. For example, platelet adhesion and aggregation are associated with the release of PDGF, basic fibroblast growth receptor, epidermal growth factor, and transforming growth factor- β , potent stimuli for smooth muscle cell proliferation and matrix production. p*S*-NO-BSA could also exert its effect by modulating leukocytes through downregulated expression of either monocyte chemoattractant protein-1 (40) or adhesion molecules (41).

Moreover, we demonstrate that *S*-nitrosothiols can modulate VSMC migration. The role of cell migration in the formation of neointima in both animal studies and clinical restenosis is important, but the extent of its contribution to neointima formation is unclear (42). As NO is a multifunctional regulator of the vascular milieu, it is likely that multiple mechanisms exist whereby inhibition of neointima formation is achieved. We cannot exclude a direct inhibitory effect of NO on vascular smooth muscle gene expression, proliferation, or synthesis of extracellular matrix.

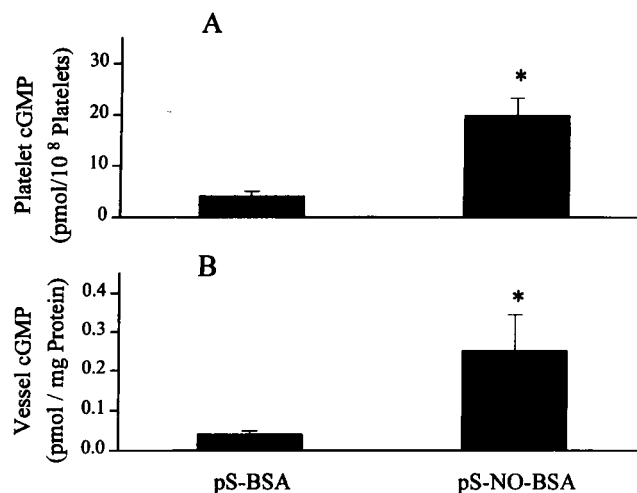


Figure 6. The effect of p*S*-NO-BSA- and p*S*-BSA-treated vessels on platelet (A) and vessel (B) cGMP. Rabbit femoral arteries were isolated and balloon-injured as described in Fig. 2. For platelet studies, paired local administration of p*S*-NO-BSA and p*S*-BSA to vessels for 15 min was followed by harvesting, rinsing, and dividing the segments into 2-mm rings. The rings were then immersed in 100 μ l platelet-rich plasma containing 10 μ M 3-isobutyl-1-methylxanthine and were incubated for 1 min *ex vivo*. An equal volume of ice-cold 10% trichloroacetic acid was added to each aliquot and the sample was vortexed. Platelet cGMP assay was then performed as described in Methods. For vessel studies, rabbit femoral arteries were isolated, treated, and harvested as described for A. The rings were then immersed in 500 μ l of ice-cold 10% trichloroacetic acid. The samples were sonicated and cGMP assays were performed as described in Methods. * $P < 0.05$ for both panels.

The demonstration of PCNA-positive cells in vessels treated with p*S*-NO-BSA compared with control vessels is intriguing. Hanke (43) demonstrated significant DNA synthesis in the neo-

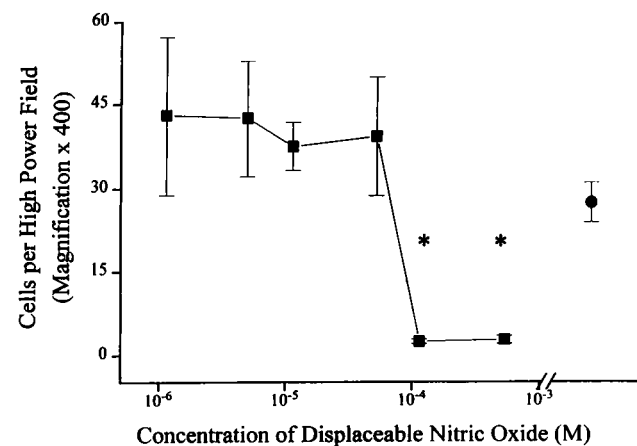


Figure 7. The effect of polythiolated *S*-nitroso-albumin on PDGF-BB-stimulated VSMC migration. Cell migration studies were performed in a modified Boyden chamber seeded with 100,000 cells/ml. Cell migration was directed against PDGF-BB (10 ng/ml) in PBS control (filled circles) or in the presence of varying concentrations of p*S*-NO-BSA (filled squares). Data are expressed as mean ± SEM for three high power fields per chamber. All experiments were performed in duplicate. * $P < 0.05$ PBS vs. p*S*-NO-BSA.

intima and media of a rabbit carotid model using electrical stimulation. Maximal DNA synthesis occurred at ~ 7 d (43) and lasted for at least 14 d. Our observations suggest a mechanism other than the inhibition of local cell replication by which to explain the inhibition of neointimal proliferation in the rabbit injury model. We demonstrate a significant decrease in VSMC migration after exposure to pS-NO-BSA which may, in part, explain these findings. Yet, given the complexity of the reparative process, other mechanisms could include transient inhibition of DNA synthesis, which is not evident on day 7 after injury; inhibition of extracellular matrix production; or inhibition of another factor(s) required for neointima formation.

These findings have several implications for the treatment of human disease. Mechanical removal of the endothelium abolishes the vasodilator responses to endothelium-dependent vasoactive stimuli, while leaving the vasoconstrictor effects of agonists to smooth muscle unopposed (1). This process occurs with balloon angioplasty especially at sites where platelet thrombus is noted (44, 45). Thus the strategy of local replacement of an important endothelial product as therapy for acute thrombotic phenomena and restenosis after angioplasty is suggested by our results.

In summary, our results demonstrate that a stable NO adduct of serum albumin binds avidly to balloon-injured subendothelium when delivered locally. Modified to carry multiple NO groups, pS-NO-BSA markedly decreases neointimal proliferation after balloon injury. Local delivery of this molecule decreases platelet adhesion to the injured subendothelium and directly inhibits the platelet, interrupting a common pathway through which growth responses are initiated. This molecule markedly decreases VSMC migration, an important element in the reparative response to vessel injury. The implications of these findings suggest that local delivery of S-nitrosothiols may be an effective treatment for disease states marked by abnormal or absent endothelium, including restenosis after angioplasty.

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