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Nitric oxide-generating hydrogels inhibit neointima formation

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Abstract—This study evaluated the effects of localized delivery of nitric oxide (NO) from hydrogels covalently modified with S-nitrosocysteine (Cys-NO) on neointima formation, a key component of restenosis, in a rat balloon-injury model. Soluble Cys-NO was used in preliminary studies to identify dosage ranges that were able to simultaneously inhibit smooth muscle cell proliferation, enhance endothelial cell proliferation, and reduce platelet adhesion. Photo-cross-linked PEG-based hydrogels were formed with covalently immobilized Cys-NO. These materials release NO for approximately 24 h and can be applied to tissues and photo-cross-linked in situ to form local drug-delivery systems. Localized delivery of NO from hydrogels containing Cys-NO inhibited neointima formation in a rat balloon-injury model by approximately 75% at 14 days.

Key words: Nitric oxide; restenosis; thrombosis; S-nitrosocysteine; hydrogel; drug delivery.

INTRODUCTION

Smooth muscle cell (SMC) adhesion, migration and extracellular matrix synthesis have a pronounced role in the progression of neointima formation and restenosis following vascular injury, such as that induced by interventional procedures like angioplasty and stenting [1]. Nitric oxide (NO) is produced by endothelial cells (ECs) of the arterial intima and participates in the maintenance of vascular homeostasis through stimulation of the second messenger guanosine 3′,5′-cyclic...
monophosphate (cGMP) via activation of soluble guanylyl cyclase [2]. cGMP signaling has differing effects in various vascular cell types; for example, inhibition of SMC proliferation, enhancement of endothelial cell proliferation and depression of platelet adhesion.

The monolayer of ECs is often removed or damaged following interventional procedures. Injury to the EC lining interferes with the maintenance of vascular homeostasis, contributing to a damaging cascade of events that may ultimately result in vessel reocclusion, or restenosis. Re-endothelialization of the injured artery, however, can halt the process of restenosis and help restore proper function to the vessel [3]. Administration of various NO donors to essentially replace the NO production lost with damage to the endothelium has been shown to decrease restenosis in animal models [4, 5]. However, local delivery of NO is likely to be required for clinical utility due to the widespread biological effects of NO.

For prevention of restenosis, a key issue to examine is cell proliferation, particularly of SMCs and ECs. SMCs have been identified as one of the principle culprits in the development of the restenotic lesion, while ECs provide a number of factors that help to maintain vascular homeostasis. Ideally, an anti-restenotic therapy would limit SMC proliferation while encouraging EC growth. Studies have shown that NO donors reversibly inhibit SMC proliferation in a cGMP-dependent manner [6], although the effect of NO on ECs remains under debate, they appear to stimulate EC proliferation when given at appropriate dosages [7–10].

The effects of NO on platelet adhesion must also be addressed, as this too plays a prominent role in restenosis. Removal of the naturally non-thrombogenic endothelium during vascular injury leads to immediate platelet activation, adhesion and aggregation [1]. Platelet degranulation results in the release of growth factors such as PDGF, which is chemotactic and mitogenic for smooth muscle cells, and thus encourages a restenotic response [11]. The ability of NO to inhibit platelet activation has important consequences in the restenosis cascade, as it would inhibit the release of platelet-derived mitogens, such as PDGF, and thrombin, both of which contribute to intimal hyperplasia.

The present study examines the effects of one NO donor, S-nitrosocysteine (Cys-NO), on the adhesion, migration, proliferation and ECM production by SMCs and ECs, as well as its effects on platelet adhesion. In the first in vivo application of these materials, the effectiveness of Cys-NO as an anti-restenotic agent is also explored by providing localized delivery of NO from Cys-NO-modified hydrogels to balloon-injured rat arteries and analyzing its effects on neointima formation.

**MATERIALS AND METHODS**

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified.
Cell culture and Cys-NO synthesis

Smooth muscle cells (SMCs) were isolated from rat abdominal aortae via an enzymatic digestion method [12]. Briefly, the intimal layer was gently scraped off and the medial layer was mechanically separated from the adventitia, then digested in an enzymatic digestion solution consisting of 15 mM HEPES, 0.2 mM CaCl₂, 200 U/ml collagenase, 12 U/ml elastase, 0.375 mg/ml trypsin inhibitor and 2 mg/ml bovine serum albumin. After digestion for 2 h at 37°C with shaking, the tissue fragments were strained through a nylon mesh cell strainer (70 μm pore size, Becton-Dickinson, Franklin Lakes, NJ, USA) then cultured in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 500 U penicillin and 100 mg/l streptomycin, at 37°C in a 5% CO₂ environment. Immunohistochemical staining was performed for smooth muscle α-actin in order to verify the presence of SMCs in the culture, while human dermal fibroblasts acted as a negative staining control. Bovine aortic endothelial cells (ECs, provided by T. Scott-Burden, Texas Heart Institute) were cultured in Minimum Essential Medium supplemented with 10% FBS, 2 mM L-glutamine, 500 units penicillin and 100 mg/l streptomycin, at 37°C in a 5% CO₂ environment. S-nitrosocysteine (Cys-NO), a NO donor with a half-life on the order of a few hours [13], was synthesized by reaction of L-cysteine with sodium nitrite in pH 2 buffer. Conversion of thiol groups to S-nitrosothiols was measured using Ellman’s assay [14–16]. Cys-NO solutions were adjusted to pH 7.4 and sterilized by filtration immediately prior to use.

cGMP production

The amount of total cGMP produced by cells exposed to Cys-NO was assessed using a competitive enzyme colorimetric immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ, USA). SMCs were seeded at confluency (3 × 10⁴ cells/cm²) and cultured in 96-well plates for 24 h. Cys-NO was added to the cell-culture media at concentrations of 1.0 and 2.0 mM, and cells were incubated for 5 min. The cells were then lysed with 0.5% dodecyltrimethylammonium bromide in 0.05 M sodium acetate buffer (pH 5.8) containing 0.02% bovine serum albumin, and the cGMP was acetylated with a 1:2 mixture of acetic anhydride in triethylamine. The supernatant, which contained both extra- and intracellular cGMP, was added to a microtitre plate coated with donkey anti-rabbit IgG, along with rabbit anti-cGMP antibody for 2 h at 4°C. Standard solutions consisting of acetylated cGMP were added to additional wells with the anti-cGMP antibody. cGMP conjugated to horse radish peroxidase (HRP) was then added to the wells and incubated at 4°C for 1 h. 3,3’,5’,5’-tetramethylbenzidine (TMB, Amersham Pharmacia Biotech) was added to the mixture and incubated for 30 min to develop a blue color via reaction with the peroxidase enzyme. 1 M sulfuric acid was then added to all wells to stop the reaction, and the optical density at 450 nm was read immediately on a microtitre plate reader (ELx800, Bio-Tek Instruments, Winooski, VT, USA).
Cell viability

SMCs and ECs were seeded separately into 24-well plates at a concentration of $10^4$ cells/cm$^2$. 12 h following seeding, Cys-NO was prepared and added to the cell-culture media at concentrations of 0.1, 0.5, 1.0, 2.0 and 3.0 mM. At 1 and 24 h following the addition of Cys-NO, cell viability was examined using a Live/Dead staining kit (Molecular Probes, Eugene, OR, USA). A 4 $\mu$M solution of ethidium homodimer-1 causes dead cells to fluoresce red, while a 2 $\mu$M solution of calcein AM causes viable cells to fluoresce green. Cells were examined under a fluorescence microscope (Zeiss Axiovert 135, Thornwood, NY, USA) and micrographs were taken using a digital camera (Sony, Tokyo, Japan). The number of viable (green) cells was compared to the total cell number (green plus red cells) in four randomly chosen fields of view per well (3 wells per condition) to assess viability.

Migration

Migration of smooth muscle cells and endothelial cells in the presence of Cys-NO was assessed using a modified Boyden chamber migration assay. SMCs and ECs were seeded into transwell cell-culture inserts (8 $\mu$m pore size, Becton-Dickinson) at $3 \times 10^4$ cells/well and placed in 24-well insert companion plates. Cell-culture media was added to both the top and bottom wells. At 12 h post-seeding, Cys-NO was added to the wells at concentrations of 0.1, 0.5, 1.0 and 2.0 mM. Due to the short half-life of this NO donor, repeated dosages of Cys-NO were added every 12 h for two days, at which time the cells on top of and beneath the transwell were trypsinized and counted using a Coulter Counter. The percentage of cells that migrated through the membrane was calculated.

Extracellular matrix production

The effects of a range of NO concentrations on extracellular matrix (ECM) production by SMCs were investigated through incorporation of $[^3]$H]glycine into glycoprotein, elastin and collagen portions of the ECM [17]. Cys-NO was added at concentrations ranging from 100 $\mu$M to 3 mM. Cells were cultured in 24-well tissue-culture dishes as described above, with 5 $\mu$g/ml ascorbic acid added to the tissue-culture medium to stimulate matrix production. Cys-NO was added 24 h following cell seeding, with the addition of 1 $\mu$Ci/ml $[^3]$H]glycine accompanying the addition of Cys-NO. The same procedure was followed for cells intended for counting, except that the media was not supplemented with $[^3]$H]glycine. Two days following exposure to Cys-NO, the cells in non-radioactive plates were trypsinized, and cells were counted on a Coulter Counter. The cells on the remaining plates were lysed in 25 mM ammonium hydroxide for 30 min, and the plates were then dehydrated by rinsing with 70% ethanol and air-dried. A sequential digestion of extracellular matrix was performed by incubating the plates with 20 $\mu$g/ml trypsin
Nitric oxide-generating hydrogels inhibit neointima formation for 4 h, 58 U/ml elastase for 4 h and 76 U/ml collagenase for 8 h, all at 37°C, in order to digest glycoproteins, elastin and collagen, respectively. All enzyme solutions were made in 25 mM Tris-HCl with 5 mM CaCl₂ (pH 8). The plates were then incubated in 1 M NaOH for 1 h at room temperature to verify that the ECM had been completely digested. Radioactivity in samples from each digestion step was determined using a scintillation counter (Minaxiβ Tri-Carb 4000, Packard Instrument, Meridien, CT, USA).

Proliferation of SMCs and ECs

Proliferation of SMCs and ECs following exposure to Cys-NO was examined through immunohistochemical staining for proliferating cell nuclear antigen (PCNA), which is present during the S-phase of mitosis [18]. SMCs and ECs were seeded into 24-well plates at 10⁴ cells/cm² and allowed to adhere for 12 h, at which point Cys-NO was added to the cell-culture media at concentrations of 0.1, 0.5, 1.0 and 2.0 mM. One day after the addition of Cys-NO, the cells were fixed in 10% formalin and permeabilized in methanol. Anti-PCNA (Dako, Carpinteria, CA, USA) was diluted 1:100 in PBS containing 3% FBS and added to the wells for 1 h. The wells were rinsed in PBS, and HRP-labeled goat anti-rabbit secondary antibody (diluted 1:100) was added to the wells for 45 min. After rinsing, 3-amino-9-ethylcarbazole (AEC, Dako) was added as the enzyme substrate that causes PCNA-positive cells to stain red. Cells were counterstained with Mayer’s hematoxylin and mounted in aqueous mounting medium (Gel Mount, Biomeda, Foster City, CA, USA) for examination under the light microscope (Zeiss Axiovert 135). Two wells per condition were kept as staining controls; one well with no primary (anti-PCNA) antibody and one well with neither primary nor secondary antibody.

Platelet adhesion

Blood was obtained from a healthy volunteer by venipuncture and anticoagulated with 10 U/ml heparin. Platelets and white blood cells were fluorescently labeled by incubation with 10 μM mepacrine for 20 min at 37°C. A solution of 2.5 mg/ml collagen I in 3% glacial acetic acid in diH₂O was prepared and applied to glass slides for 45 min in a humidified environment at room temperature. Cys-NO was prepared as described above and incubated with the labeled whole blood at 37°C for 30 min at concentrations of 0.5 and 2 mM. The blood was transferred to the collagen-coated glass slides (two per group), incubated at 37°C for 20 min, and then rinsed with HEPES-buffered saline (pH 7.4, HBS). Platelet counts per field of view at 400× were determined under a fluorescent microscope (Zeiss Axiovert 135) in five randomly chosen areas per slide.

PEG-Cys-NO hydrogels

Hydrogels containing a covalently bound S-nitrosocysteine (Cys-NO) were synthesized as previously described [16]. Briefly, polyethylene glycol N-hydroxysucci-
nimide monoacrylate (ACRL-PEG-NHS, MW 3400, Shearwater Polymers, Huntington, AL, USA) was reacted with L-cysteine at a 1:2 molar ratio in 50 mM sodium bicarbonate (pH 8.5). The solution was dialyzed in a cellulose ester membrane (Molecular weight cutoff 500, Spectrum Labs, Laguna Hills, CA, USA) against diH$_2$O overnight, lyophilized, and molecular weight was analyzed using gel permeation chromatography with a light scattering detector and a UV detector at 260 nm (Polymer Laboratories, Amherst, MA, USA). The copolymer was then reacted with an equimolar amount of NaNO$_2$ at pH 2 and incubated at 37°C for 20 min to form S-nitrosocysteine. Conversion of thiol groups to S-nitrosothiols was measured as described for Cys-NO in solution. PEG (MW 6000, Fluka, Milwaukee, WI, USA) was acrylated to form PEG-diacylate via a method described previously [19]; this forms a water-soluble, biocompatible precursor polymer that can be photo-cross-linked in situ to form a hydrogel. The pH was adjusted to 7.4, and the PEG-Cys-NO was combined with PEG hydrogel precursor solution consisting of 30% (w/v) PEG-diacylate, 0.15% N-vinylpyrrolidone as a solvent, and 1500 ppm 2,2-dimethoxy-2-phenyl acetophenone as a long-wavelength ultraviolet initiator. The polymer solution was sterilized by filtration prior to gelation. An additional set of PEG-Cys-NO hydrogels was made for the purpose of validating that the theoretical amount of NO released from the gels matched the actual amount released. These hydrogels were incubated in pH 7.4 HEPES buffered saline solution for analysis of NO release via the Griess reaction [16].

**Rat carotid balloon injury**

Balloon denudation injury of the carotid artery of male Sprague–Dawley rats (425–450 g, Harlan, Indianapolis, IN, USA) was used as an experimental model to assess the ability of PEG-Cys-NO hydrogels to inhibit intimal thickening. After induction of anesthesia with isoflurane, the left carotid artery was surgically exposed. An inflated 2F Fogarty embolectomy catheter (Edwards Lifesciences, Irvine, CA, USA) was passed three times through the common carotid to denude and injure the vessel. Either a NO-generating PEG-Cys-NO hydrogel precursor solution (200 µl, 1.25 µmol NO delivered over 12 h) or control PEG-diacylate hydrogel precursor solution was applied perivascularly immediately following the injury and was photo-cross-linked for 1 min under a UV lamp (Blak-Ray B100AP, UVP, Upland, CA, USA, 8 cm distance, 365 nm, 10 mW/cm$^2$) to form the hydrogel material in situ. The injured segment of the left carotid artery was explanted 14 days after injury for histological analysis. Sections (20 µm thick, cryosectioned) were stained with Gieson’s elastin stain, and digital image processing was used for morphometric analysis of neointimal area.

**Statistical analysis**

Data sets were compared using two-tailed, unpaired $t$-tests or one-way analysis of variance (ANOVA) where indicated. $P$-values less than 0.05 were considered significant. Data are presented as mean±standard deviation.
RESULTS

cGMP production

Treatment of SMCs with 1 mM Cys-NO for 5 min resulted in a more than 50-fold increase in cGMP production and 2 mM Cys-NO provided a more than 100-fold increase (P < 0.004), as measured by a competitive binding enzyme immunoassay for cGMP. This result confirms the bioactivity of the Cys-NO used as the model NO donor for these studies.

Viability

There was no decrease in either EC or SMC viability upon addition of Cys-NO to cell-culture media, as evidenced by staining with ethidium homodimer-1 and calcein AM. More than 95% viability was seen in all cells exposed to Cys-NO concentrations in the range of 0.1 mM to 2.0 mM at all time points, which was similar to viability observed in control wells (P > 0.71).

Migration

Cell migration was measured using a modified Boyden chamber assay. The migration index is expressed as the number of cells that migrated to the bottom well divided by the total number of cells in both top and bottom wells. The migration of ECs and SMCs in response to varying Cys-NO concentrations is shown in Fig. 1. Migration of ECs peaked at 0.1 mM Cys-NO, although this was not statistically significant from the control. A concentration of 0.5 mM Cys-NO caused significant inhibition of migration (P < 0.02) in SMCs.

Extracellular matrix production

When Cys-NO was added to SMCs 24 h after cell seeding, the amount of matrix proteins synthesized on a per cell basis increased with increasing Cys-NO dosage for concentrations greater than 0.5 mM (Fig. 2). However, the matrix composition (relative amounts of glycoprotein (G), elastin (E) and collagen (C)) was not significantly affected by the NO dosage when NO was added after cell seeding (67 ± 2% G, 18 ± 2% E, 9 ± 1% C for control vs. 65 ± 3% G, 17 ± 4% E, 11 ± 2% C for 0.5 mM Cys-NO). Thus, one must limit the NO dosage from a local delivery device, since overproduction of ECM proteins can lead to restenosis.

Proliferation

Immunohistochemical staining for PCNA, a marker for cells in the S-phase of the cell cycle, revealed that the proliferation of endothelial cells peaked at a Cys-NO concentration of 1 mM, while the proliferation of smooth muscle cells decreased with increasing Cys-NO concentration (Fig. 3). Again, this result of opposing functions of NO on SMCs versus ECs demonstrates the potential of NO for the
Figure 1. The effect of Cys-NO on the migration of (a) ECs and (b) SMCs was examined using a modified Boyden chamber apparatus. ECs displayed a slight increase in migration at low Cys-NO concentrations, while a minimum in SMC migration was achieved at a Cys-NO concentration of 0.5 mM. *P < 0.02 compared to 0 mM control; n = 3 per treatment group.

prevention of restenosis, since SMC proliferation contributes to the genesis of neointimal hyperplasia but the regeneration of ECs would help restore vascular homeostasis.

Platelet adhesion

Exposure of platelets to Cys-NO led to a significant decrease (P < 0.001) in platelet adhesion to collagen-coated surfaces when compared to controls (Fig. 4). No difference was observed between Cys-NO concentrations of 0.5 and 2 mM.

Rat carotid artery balloon injury

Representative histological sections of balloon-injured left carotid arteries at 2 weeks post-injury are shown in Fig. 5. The perivascular application of NO-generating hydrogels significantly reduced neointimal formation (P < 0.05) in this
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Figure 2. Effect of Cys-NO added 24 h following SMC seeding on the total matrix produced per cell, assessed by sequential enzymatic digestion of the radiolabeled ECM. The matrix produced per cell significantly increased ($P < 0.001$) with increasing Cys-NO concentration, while the ECM composition remained unchanged. One-way ANOVA; $n = 3$ per treatment group.

experimental balloon-injury model, as compared to treatment with control hydrogels that did not release NO. Intimal thickness was reduced by approx. 75% 14 days after injury (Fig. 6) by providing NO therapy to the injured artery. Previous studies have shown that neither the PEG-diacrylate hydrogel nor the photo-polymerization process alters the vascular response when applied perivascularly [20].

DISCUSSION

A complete analysis of the effects of NO on endothelial and smooth muscle cells has not yet been described in the literature, making it difficult to determine appropriate dosages for local delivery systems for anti-restenotic therapy. Thus, the purpose of this study was to further understand the actions of an NO donor, S-nitrosocysteine, on SMCs, ECs and platelets with the goal of identifying an optimal therapeutic strategy for prevention of restenosis. We then investigated localized perivascular delivery of NO from PEG hydrogels covalently modified with Cys-NO in a rat model similar to balloon angioplasty.

When treated with Cys-NO concentrations ranging from 0.1 to 2 mM, ECs experienced a maximum in proliferation at 1 mM Cys-NO, while SMC proliferation steadily decreased with increasing Cys-NO concentration over the same concentration range, suggesting that it may be possible to simultaneously block SMC proliferation while stimulating EC proliferation in a therapeutic application.

Previous studies that have examined the rate of synthesis of ECM proteins by smooth muscle cells in the presence of NO have led to an incomplete characterization of these events. Decreased collagen synthesis by SMCs following exposure to NO has been reported [26, 27]. However, the only matrix component measured
Figure 3. The effect of various Cys-NO concentrations on the proliferation of (a) ECs and (b) SMCs, as measured by staining for proliferating cell nuclear antigen (PCNA). Opposite trends are displayed by the two cell types, as Cys-NO stimulates the proliferation of ECs while decreasing the proliferation of SMCs. * \( P < 0.01 \) compared to 0 mM control; \( n = 3 \) per treatment group.

was collagen and, more specifically, only soluble collagen in the cell-culture media. These results do not reflect incorporation of collagen into the crosslinked ECM structure. In fact, decreased levels of soluble collagen may indicate an increase in the processing of collagen into the ECM structure. The results of this study show an increase in total ECM protein produced on a per cell basis. This difference from prior work may be due to taking the anti-proliferative effects of NO into account and expressing the data as matrix protein per cell. The significance of the vastly increased matrix per cell for high NO dosages is that it indicates that the benefits and drawbacks of NO therapy for restenosis prevention must be carefully examined. For instance, while a concentration of 2 mM Cys-NO may successfully inhibit smooth muscle cell proliferation, it may also greatly enhance ECM protein production by these cells, which could potentially increase the size of the restenotic lesion.
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Figure 4. Inhibition of platelet adhesion to thrombogenic surfaces was accomplished by pre-incubation of blood with Cys-NO. Thrombogenic surfaces were created by coating glass with collagen I, while platelets were fluorescently labeled with mepacrine and counted following adhesion to the surfaces. *$P < 0.001$ compared to 0 mM control; $n = 5$ per treatment group. Data are reported as platelets/field of view at 400×.

The efficacy of Cys-NO as an anti-thrombotic agent was evaluated by measuring its effect on \textit{in vitro} platelet adhesion to thrombogenic surfaces. The observation that maximal inhibition of platelet adhesion can be achieved at a Cys-NO concentration of 0.5 mM is particularly useful, as this dosage decreased SMC proliferation and migration while enhancing EC functions.

The studies performed here intend to provide a more complete picture of how one NO donor affects many aspects of cell function, with the ultimate goal of designing more effective anti-restenosis therapies based upon NO delivery. Development of NO-releasing materials may have the potential for use as a localized NO-delivery system in order to overcome complications associated with systemic delivery of anti-platelet and anti-proliferative drugs. Although careful animal trials have demonstrated high efficacy of many systemically delivered anti-restenotic drugs, human trials have not displayed any major beneficial effect of drug therapy on the instance of restenosis or overall mortality rate [28]. This lack of success is believed to have little to do with species variation [29]. Instead, it has been concluded that effective dosage regimens in animals may cause systemic side effects in humans [28, 30]. In the current work, hydrogels containing covalently bound Cys-NO were formed in the perivascular space and locally delivered NO to the injury site for several hours post-angioplasty. After two weeks, histologic analysis of explanted arteries displayed significant attenuation of neointima formation in arteries treated with NO-releasing hydrogels. These results suggest the potential for use of Cys-NO-modified biomaterials in the prevention of restenosis.
Figure 5. Histological sections of balloon-injured rat carotid arteries explanted 14 days following injury. Injured arteries were treated with perivascular application of (a) NO-releasing hydrogels or (b) control hydrogels that did not release NO. Neointima formation was significantly reduced by treatment with NO-releasing hydrogels. Arrows indicate the position of the internal elastic lamina. Tissue above this structure is the neointimal layer that reduces the area available for blood flow. Length of bar = 50 µm.
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Figure 6. At 14 days following injury, intimal thickness of arteries treated with NO-releasing hydrogels was reduced by approximately 75% compared to treatment with control hydrogels. *$P < 0.05$; $n \geq 5$ per treatment group.

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