Augmentation of Intrapericardial Nitric Oxide Level by a Prolonged-Release Nitric Oxide Donor Reduces Luminal Narrowing After Porcine Coronary Angioplasty

Sang Hong Baek, MD, PhD; Joseph A. Hrabie, PhD; Larry K. Keefer, PhD; Dongming Hou, MD, PhD; Naomi Fineberg, PhD; Rodney Rhoades, PhD; Keith L. March, MD, PhD

Background—Nitric oxide (NO) is a potent vasodilator and antiplatelet agent that suppresses vascular smooth muscle cell proliferation. Hypothesizing that generating NO in the pericardial space would reduce luminal narrowing after coronary angioplasty without affecting systemic hemodynamics, we have determined the effect of a novel NO donor on vascular healing after balloon overstretch.

Methods and Results—Diazeniumdiolated bovine serum albumin (D-BSA; molecular weight 74 kDa, half-life for NO release 20 days) was radioiodinated and found by intravital \textsuperscript{131}I-imaging to have a longer residence time in pig pericardium than a low-molecular-weight (0.5 kDa) analogue (22 versus 4.6 hours, respectively). Intrapericardial injection of D-BSA immediately before 30% overstretch of normal left anterior descending and left circumflex coronary arteries dose dependently reduced the intimal/medial area ratio by up to 50% relative to controls treated with underivatized albumin when measured 2 weeks after intervention. Positive remodeling was also noted, which increased luminal area relative to control.

Conclusions—Perivascular exposure of coronary arteries to NO via intrapericardial D-BSA administration reduced flow-restricting lesion development after angioplasty in pigs without causing significant systemic effects. The data suggest that intrapericardial delivery of NO donors for which NO release rates and pericardial residence times are matched and optimized might be a beneficial adjunct to coronary angioplasty. (Circulation. 2002;105:2779-2784.)

Key Words: angioplasty ■ restenosis ■ pericardium ■ nitric oxide ■ coronary disease

Restenosis after coronary angioplasty remains a major public health problem. It results from various factors, including proliferation of vascular smooth muscle cells in response to overstretch injury, abnormal migration of these cells out of the media to produce intimal thickening, inward vascular remodeling, and sometimes formation of obstructive thrombus. Therapies based on local nitric oxide (NO) release might significantly improve the prognosis for angioplasty patients.\textsuperscript{1} As the endothelium-derived relaxing factor, NO is both a potent antithrombotic\textsuperscript{2} and an inhibitor of vascular smooth muscle cell proliferation.\textsuperscript{3}

As might be predicted for a bioeffector with these attributes, prophylactic treatment with NO has proved beneficial in several in vivo restenosis models (reviewed in Janero and Ewing\textsuperscript{4}). Transfection of arteries with endothelial\textsuperscript{4,5} or inducible\textsuperscript{6} NO synthase inhibited neointima formation in rats and pigs. Administration of L-arginine, the metabolic precursor of NO, benefited rats and rabbits.\textsuperscript{4,7} Inhaled NO inhibited stenosis in rat carotid arteries,\textsuperscript{4} and NO donor molecules of several structural classes reduced intimal thickening in rabbits, pigs, mice, and rats.\textsuperscript{4,8–10} Treatment of stable coronary patients with infused linsidomine during angioplasty followed by oral molsidomine for 6 months significantly increased minimum luminal diameter relative to controls who were given oral diltiazem, but long-term clinical outcome was not improved.\textsuperscript{11} Interestingly, periadventitial exposure of rat iliofemoral arteries to a gel containing an NO-releasing diazeniumdiolate during and after balloon injury produced a marked reduction of intimal hyperplasia 2 weeks after overstretch.\textsuperscript{12}

We hypothesized that intrapericardial instillation of a high-capacity NO donor drug with sufficiently long pericardial residence time might provide a practical, single-dose approach to restenosis prevention; specifically, concentration of NO exposure perivascularly at the coronary arteries might inhibit the mitogenic platelet responses and vascular smooth muscle proliferation that accompany angioplasty while minimizing unwanted systemic effects. Accordingly, we studied...
the effect of increasing molecular weight (MW) on pericardial residence time and showed that intrapericardial delivery of a novel NO-releasing protein derivative immediately before coronary angioplasty in the pig both reduced intimal and adventitial thickening and produced a positive remodeling effect.

**Methods**

**Animals**

Animal care and handling followed the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, NIH publication No. 86-23, revised 1985). The Animal Care and Use Committee of the Indiana University School of Medicine approved the protocols. Domestic pigs (weight 20 to 25 kg; GI Acres, Mooresville, Ind) were given oral aspirin (325 mg) daily starting the day of the procedure. Procedures were performed under general anesthesia induced with ketamine, xylazine, and atropine (20, 2, and 0.05 mg/kg, respectively) administered intramuscularly, followed by intravenous thiopental sodium (25 mg/kg). After anesthesia was induced, animals were intubated and ventilated with oxygen containing 2% isoflurane to maintain anesthesia.

**Materials**

BSA (Calbiochem) was converted to its diazeniumdiolated derivative (D-BSA) as described previously. The diethylenetriamine/NO adduct (DETA/NO) was synthesized as described previously. Agents were freshly dissolved in PBS to which enough NaHCO₃ was added to maintain physiological pH after dissolution; without such neutralization, the PBS turned acidic when D-BSA was dissolved, which caused its precipitation. D-BSA was labeled with ¹³¹I by Covance Laboratories (Vienna, Va) using the chloramine T method and purified to radiochemical homogeneity by Sephadex chromatography (specific activity 99 mCi/mg). Lysyl-lysine (Sigma) was converted to the γ-(4-hydroxyphenyl)propionamide derivative of lysyl-lysine, a DETA/NO surrogate of similar molecular weight and charge status.

Animals received follow-up angiograms. Heart and vessels were excised rapidly and fixed by infusion of 10% buffered formalin under 100 mm Hg pressure for 60 minutes into the left main coronary artery. Coronary arteries were sectioned into 2- to 3-mm segments in the area of angioplasty for immersion fixation over an additional 24 hours before paraffin embedding and histological sectioning were performed.

**Morphometric Analysis**

Sections were cut at 5 μm, affixed to glass microscope slides, and stained with hematoxylin, eosin, and Verhoeff–van Gieson/elastin combination stain. Cross-sectional parameters of intima and media were measured by an experienced observer blinded to sample origin using computerized morphometric analysis (NIH Image). At low-power (×10) view, borders of the external elastic lamina (EEL), internal elastic lamina (IEL), neointima, and vessel lumen were traced. Areas, circumferences, and maximum intimal thickness were measured. Extent of injury was assessed as ratio of IEL fracture length to IEL circumference. A second observer blinded to treatment reviewed random sections; results were compared with the numbers obtained by the first examiner. Interobserver variability was typically <5%. Only vessel segments with IEL fracture were used in the analysis.

**Selection of NO Donor**

Because diazeniumdiolates generate copious NO without metabolic activation, they make excellent test agents for determining the feasibility of reducing restenosis risk by prophylactically introducing NO into the pericardial fluid. In choosing a specific diazeniumdiolate for the initial experiment, we reasoned that continuous, prolonged action would be important if vascular smooth muscle proliferation were to be optimally suppressed. Two long-lived candidates were considered (Table 1): DETA/NO, a low-MW (163 Da) zwitterion with a 20-hour half-life for NO release in physiological buffer, and D-BSA, a derivatized protein containing 22 diazeniumdiolates per molecule with a 20-day half-life for NO release. These kinetic parameters translate to similar initial rates of NO generation on a mole-for-mole basis (Table 1).

Because clearance rate is also a kinetic parameter of critical importance for drug selection, we studied the pharmacokinetics of distribution from the pericardial space. To probe the effect of MW differences on pericardial residence time, we used γ-imaging to evaluate the delivery efficiency and kinetics of agent elimination after intrapericardial delivery of

**Biological Experiments**

Intrapericardial pharmacokinetics were studied by γ-imaging 20- to 25-μCi boluses of [¹³¹I]-labeled test agent as described previously except that 2 areas of interest were drawn for quantification (1 around the external reference source and 1 restricted to the cardiac region). Total activity was plotted against time to yield a local half-time of agent redistribution with a Sigmaplot fit package (Jandel).

To test for antirestenotic effectiveness, D-BSA was delivered percutaneously as described previously. Briefly, a catheter inserted half-time of agent redistribution with a Sigmaplot fit package around the external reference source and 1 restricted to the cardiac except that 2 areas of interest were drawn for quantification (1

<table>
<thead>
<tr>
<th>TABLE 1. Pharmacodynamic Parameters for the 2 NO Donor Drugs Considered for Use in This Investigation</th>
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<tr>
<td><strong>DETA/NO</strong></td>
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<tr>
<td>Molecules of NO available/molecule of drug</td>
</tr>
<tr>
<td>Half-life of NO release in pH 7.4 phosphate buffer at 37°C</td>
</tr>
<tr>
<td>Initial NO flux (mol of NO per mol of agent per h)</td>
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<tr>
<td>In vivo intrapericardial residence half-life, h</td>
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<tr>
<td>Calculated intrapericardial NO flux 24 hours after delivery (mol of NO per h per mol of agent dosed)</td>
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*Residence half-life in the pericardial space for the iodinated γ-(4-hydroxyphenyl)propionamide derivative of lysyl-lysine, a DETA/NO surrogate of similar molecular weight and charge status.
Figure 1. Clearance of radiolabeled test agents from pericardium as function of MW. 131I-labeled lysyl-lysine (A; MW 479 Da) and D-BSA (B; MW 74 200 Da) were injected as boluses into the pericardial sac, and their disappearance was monitored by \( \gamma \)-imaging. Semilog plots of radioactivity remaining vs time were used to estimate pericardial residence lifetimes. Half-lives of clearance for 3 animals receiving radiiodinated lysyl-lysine were 3.8, 4.4, and 5.6 hours (mean 4.6 hours). Half-lives for labeled D-BSA were 14.3, 25.0, and 27.3 hours (mean 22.2 hours).

2 agents with widely divergent MWs: 131I-labeled D-BSA and 131I-labeled lysyl-lysine. The latter was chosen as a surrogate for DETA/NO, a substitution made because radiiodination of DETA/NO by the Bolton-Hunter procedure would have changed its physicochemical characteristics; performance of the procedure on lysyl-lysine produced a radiolabeled species of similar charge status to DETA/NO that we considered a suitable model for studying the effect of MW on pericardial residence time.

Experiments were performed in 6 animals to compare in vivo local residence times for these 2 agents (3 animals each) as a basis for determining which NO donor (DETA/NO or D-BSA) to use in studying postangioplasty healing. Imaging immediately after intrapericardial delivery confirmed that essentially all counts in each injection reached the target region circumscribed by the pericardium. Intrapericardial residence half-times were 3.8 to 5.6 hours (average 4.6 hours) for lysyl-lysine and 14.3 to 25.0 hours (average 22.2 hours) for D-BSA. These data (Figure 1) suggested that the latter would provide more prolonged exposure of balloon-injured arteries to NO, so D-BSA was chosen for study.

Feasibility and Tolerability of Pericardial Access and D-BSA Delivery

Percutaneous delivery of D-BSA and its control (BSA) to the pericardial space was well tolerated in all animals over the time period evaluated in the present study. There were no hemodynamic differences between control and treatment groups. All animals were well and without complications at necropsy 14 days after drug administration. Twelve-lead ECGs obtained before agent instillation (baseline), immediately after instillation, and at necropsy revealed no changes in ECG rhythm or morphology or findings consistent with pericardial inflammation or myocardial damage. Intrapericardial delivery via the percutaneous approach was confirmed in each case by fluorographic imaging of instilled contrast media and typically required <5 minutes from the time of initial catheter introduction into the left ventricle.

Effect of Intrapericardial D-BSA on Vascular Response to Balloon Injury

Morphometric analysis was performed 14 days after PTCA on all injured segments of coronary arteries from pigs that received BSA (control), 40 mg (low dose, LD) of D-BSA, or 400 mg (high dose, HD) of D-BSA (n=12 arteries in each group; Table 2). Average degree of injury estimated by IEL fracture length was similar in all groups (control, 1.00±0.075 mm; LD, 1.10±0.057 mm; HD, 1.01±0.085 mm; P=NS). Fracture length normalized to vessel size was also similar between groups.

As described previously,16 injured arteries showed marked neointimal lesions that consisted predominantly of \( \alpha \)-actin–positive spindle-shaped cells in a loose extracellular matrix. The LD and HD groups showed progressively less neointimal thickness than control vessels for given fracture lengths (Figure 2). Furthermore, vessels appeared somewhat larger in overall circumference in the LD group and especially in the HD group.

Neointimal response as a proportion of degree of injury was maintained in each of the 3 groups, with \( R \) values of 0.45 to 0.67 and \( P \) values of <0.001 (LD and HD) and 0.003 (control). Scatterplots and fit lines depicting the relationship between fracture length and neointimal area are shown in Figure 3, A through C. As described previously,16 injured arteries showed marked neointimal lesions that consisted predominantly of \( \alpha \)-actin–positive spindle-shaped cells in a loose extracellular matrix. The LD and HD groups showed progressively less neointimal thickness than control vessels for given fracture lengths (Figure 2). Furthermore, vessels appeared somewhat larger in overall circumference in the LD group and especially in the HD group.

TABLE 2. Morphometric Analysis of Effect of Intrapericardial D-BSA on Vascular Healing 2 Weeks After Coronary Balloon Overstretch in Pigs

<table>
<thead>
<tr>
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<th>Control</th>
<th>LD</th>
<th>HD</th>
<th>( P^* )</th>
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<tbody>
<tr>
<td>FL, mm</td>
<td>1.00±0.07</td>
<td>1.10±0.06</td>
<td>1.01±0.08</td>
<td>NS</td>
</tr>
<tr>
<td>FL/(FL+IEL length)</td>
<td>0.21±0.01</td>
<td>0.22±0.01</td>
<td>0.18±0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Neointimal area, mm²</td>
<td>0.39±0.02</td>
<td>0.34±0.03</td>
<td>0.21±0.02*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adventitial area, mm²</td>
<td>1.71±0.11</td>
<td>1.49±0.07</td>
<td>1.03±0.06*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neointimal area/FL, mm²</td>
<td>0.42±0.03</td>
<td>0.30±0.04*</td>
<td>0.21±0.02*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adventitial area/FL, mm²</td>
<td>1.91±0.12</td>
<td>1.49±0.11*</td>
<td>1.15±0.09*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EEL length, mm</td>
<td>5.64±0.14</td>
<td>5.98±0.28*</td>
<td>6.53±0.37*</td>
<td>&lt;0.017</td>
</tr>
</tbody>
</table>

FL indicates fracture length.

*\( P \) relative to control; NS indicates not significant at the \( P=0.05 \) level.
The data for both the neointimal and the adventitial areas clearly demonstrate that intrapericardial delivery of D-BSA dose dependently inhibited injury-induced growth. This was manifested by dose-related decreases in slope of both area plots as a function of fracture length. Neointimal area (Table 2) normalized to fracture length averaged over all segments and vessels 14 days after balloon injury (Figure 4A) was smaller in both treated groups (HD, 0.21±0.02 mm and LD, 0.30±0.04 mm) than in controls (0.42±0.03 mm, ANOVA HD<LD<control, P<0.001). Corresponding adventitial values were also significantly smaller in treated groups (LD, 1.49±0.11 mm and HD, 1.15±0.09 mm) versus control (1.91±0.12 mm; ANOVA HD<LD<control, P<0.001; Figure 4B).

To assess possible vessel remodeling, total area circumscribed by the EEL and EEL circumferential length were measured. In contrast to findings for the neointimal and adventitial areas, the area circumscribed by the EEL was larger in the D-BSA groups (3.53 mm²) relative to control (3.00 mm²). This positive remodeling effect (area increase of 0.53 mm² in HD versus control) was thus a more important determinant of the treatment group’s increased luminal area than was reduction of neointima (reduced area by 0.18 mm² in HD versus control).

With regard to the critical issue of potentially adverse long-term effects, we found no evidence for myocardial necrosis or inflammatory cell infiltration between treatment and control groups, which suggests that treatment was microscopically benign in the myocardium. Visceral pericardium was not significantly thicker in the treated groups, although the HD group showed a trend in this direction (0.39±0.08 versus 0.25±0.03 mm for LD and 0.26±0.03 mm for control). No significant change in heart rate accompanied
anesthesia either before drug administration or at 14 days. Mean arterial pressure decreased 9 mm Hg on average after delivery and 3 mm Hg more over the next 2 weeks, but there was no difference among the 3 groups, which suggests that it was the procedure, not the agent, that affected mean arterial pressure.

### Discussion

In planning this test of intrapericardial administration of NO donor drugs to reduce restenosis risk after coronary angioplasty, we considered 2 long-lived diazeniumdiolates as possible antirestenosis agents: DETA/NO, a well-characterized NO donor of low MW (163 Da) with a half-life for NO release of 20 hours that has previously been shown to suppress vascular smooth muscle cell proliferation in culture completely but without evidence of toxicity, and D-BSA, a recently introduced prodrug with a 20-day half-life for NO release.

We next calculated the total fluxes of NO expected on intrapericardial injection of DETA/NO and D-BSA as equimolar boluses. As shown in Table 1, initial NO generation rates should be almost equal, assuming that the rates in pericardial fluid equal those in pH 7.4 phosphate. When the different offsetting clearance rates from the pericardium are factored in, the expected flux of molecular NO in pericardial fluid 24 hours after administration should be roughly 30-fold greater for D-BSA (Table 1).

We thus selected D-BSA for the initial test of our hypothesis that administration of an NO-releasing diazeniumdiolate intrapericardially might promote proper healing of the fractured coronary artery after angioplasty. A bolus of 40 mg per pig, injected just before balloon overstretch, produced a suggestive but statistically insignificant reduction of both intimal and adventitial hyperplasia relative to that in albumin-treated controls when the animals were killed 2 weeks after balloon treatment. However, when 400 mg was injected into pigs in 10 mL of buffered saline (making the final concentration roughly 40 mg/mL, the approximate concentration of albumin in normal serum), a statistically significant improvement in vascular repair was seen by every index we measured. Particularly impressive was the much greater area of the lumen in the D-BSA–treated pigs than in the albumin controls, a factor attributable to both inhibition of neointima formation and substantial positive remodeling.

We conclude that the intrapericardial injection of drugs that spontaneously (ie, without enzymatic or redox activation) generate NO in the pericardial fluid that periadventitially bathes the coronary artery undergoing angioplasty merits further study as a possible method for reducing clinical restenosis risk. Although decreases of 50% in extent of injury-induced hyperplasia were seen in these first-generation experiments, the results can hardly be viewed as optimized. Given what we now know about clearance from the pericardium, including its apparent dependence on MW (Figure 1), it is obvious that a better match between pericardial residence time and half-life at physiological pH could be found. In particular, a protein with a much larger MW whose prolonged pericardial residence time is roughly equivalent to the half-life of NO release should be even more beneficial than D-BSA. Such drugs should be relatively easy to prepare, given the chemical versatility of the diazeniumdiolates. It would also be judicious to address issues of immunogenicity in the next tests of this hypothesis, although no adverse effects of injecting a bovine protein derivative into a pig were seen in the present experiments. Future work will focus on potential improvements in the present results, possibly utilizing macroaggregated porcine serum albumin derivatized with diazeniumdiolate functions that have half-lives of a few days rather than weeks and monitoring the course of recovery for longer time periods.

In summary, augmentation of intrapericardial NO levels with the novel prodrug D-BSA significantly reduced neointima formation and luminal narrowing 14 days after PTCA. This study confirms our initial description of the effectiveness of percutaneous instillation of material into a normal
pericardial space with a helical needle-tipped catheter.16,17 as well as by a transthoracic approach.20 This method appears to be reasonably safe, with no evidence of adverse sequelae over several days after the procedure. We suggest that percutaneous transluminal intrapericardial delivery constitutes a useful tool to study key molecular events in the injured vessel wall and may lead to potential new therapeutic approaches for restenosis after PTCA.

Acknowledgments

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References

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