Basic Science Reports

Antineutrophil and Myocardial Protecting Actions of a Novel Nitric Oxide Donor After Acute Myocardial Ischemia and Reperfusion in Dogs

David J. Lefer, PhD; Katsuhiro Nakanishi, MD; William E. Johnston, MD; Jakob Vinten-Johansen, PhD

Background. It has recently been demonstrated that myocardial ischemia and reperfusion results in a marked decrease in the release of nitric oxide (NO) by the coronary endothelium. NO may possess cardioprotective properties, possibly related to inhibition of neutrophil-related activities. We tested the hypothesis that a cysteine-containing nitric oxide donor compound, SPM-5185, would reduce infarct size and inhibit neutrophil-related activities (adherence to coronary vascular endothelium, accumulation).

Methods and Results. The effects of intracoronary infusion of SPM-5185 were investigated in a 5.5-hour model of myocardial ischemia (1 hour) and reperfusion (4.5 hours) (MI-R) in anesthetized, open-chest dogs. SPM-5185 (500 nmol/L) or saline vehicle was infused for 4.5 hours into the left anterior descending coronary artery (LAD) at the time of reperfusion after 1 hour of LAD occlusion. MI-R in dogs receiving saline vehicle resulted in severe myocardial injury characterized by dyskinesia, a profound elevation of plasma creatine kinase, marked myocardial necrosis, and high cardiac myeloperoxidase (MPO) activity in the ischemic and necrotic zones. In contrast, treatment with SPM-5185 resulted in a modest restoration of regional function, a reduction of myocardial necrosis expressed as a percentage of the area at risk (12.5±3.2% versus 41.7±5.4%, P<.001), and significant reductions of MPO activity in the ischemic zone (0.8±0.1 versus 2.5±0.7 U/100 mg tissue, P<.05) and the necrotic zone (1.6±0.2 versus 3.3±0.6 U/100 mg tissue, P<.05). In additional studies, SPM-5185 (500 nmol/L) significantly (P<.001) attenuated the adherence of LTB4-stimulated canine neutrophils to autologous segments of coronary artery and attenuated the neutrophil-induced contraction of isolated coronary arterial rings.

Conclusions. SPM-5185 reduces myocardial necrosis and neutrophil accumulation in an acute model of canine myocardial ischemia and reperfusion. This reduction in myocardial cell injury may be partially related to the inhibitory actions of this novel NO donor on neutrophil adherence to the coronary endothelium. (Circulation. 1993;88[part 1]:2337-2350.)

KEY WORDS • blood flow • neutrophils • myocardial infarction • ischemia • reperfusion

Previous studies of myocardial ischemia and reperfusion have demonstrated marked impairment of endothelium-dependent relaxation of the coronary vasculature. Studies by Ku1 and Van Benthuysen et al2 have demonstrated attenuated responses of canine coronary artery rings to a variety of endothelium-dependent vasodilators acting via release of endothelium-derived relaxing factor (EDRF). Moreover, in vivo studies of intact coronary arteries by Nichols et al3 and by Sobey et al4 have documented impaired EDRF release after ischemia and reperfusion. More recently, studies conducted by Tsao et al5 and Ma et al6 indicate that the impaired release of EDRF by coronary arteries occurs very early after reperfusion and precedes PMN infiltration into the myocardium and subsequent myocardial cell necrosis.

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and their adherence to vascular endothelium may progress unopposed because of the loss of antiaggregatory effects of NO. Therefore, it is likely that diminished NO release by the injured coronary vascular endothelium is a crucial event in the pathogenesis of myocardial ischemia-reperfusion (MI-R) injury.\(^1\)

A number of agents\(^6-19\) including L-arginine, perfluorochemical, human recombinant superoxide dismutase, and taprostene (a stable prostacyclin analog) administered just before or at the time of reperfusion have been shown to preserve agonist-mediated release of NO from the coronary vascular endothelium. This preserved endothelial function was associated with a reduction of postschismic infarct size. Furthermore, Johnson and coworkers\(^9\) demonstrated that authentic NO, administered at a subsasodilator concentration at reperfusion, significantly reduced myocardial necrosis in cats subjected to MI-R. However, because of the difficulties of administering NO in the gaseous form, the administration of authentic NO is impractical for clinical use. Therefore, pharmacological agents that release NO under physiological conditions may provide a more practical therapeutic approach to cardioprotection from MI-R injury.\(^1\)

**Methods**

**Experimental Procedure**

Twenty-eight heartworm-free dogs of either sex (weight, 17.0 to 30.0 kg) were initially anesthetized with 2% sodium thiamylal (20 mg/kg IV) supplemented with fentanyl citrate (25 \(\mu g\)/kg) and diazepam (5 mg bolus) administered intravenously as needed to maintain deep anesthesia. The dogs were endotracheally intubated and ventilated with a Harvard volume-cycled respirator using oxygen-enriched room air. The oxygen flow was adjusted to maintain the arterial oxygen tension above 100 mm Hg, and the ventilatory rate was set to maintain arterial carbon dioxide tension between 30 and 40 mm Hg. Arterial pH was adjusted to 7.40±0.05 using sodium bicarbonate (Abbott Laboratories, North Chicago, Ill) as necessary. A standard lead II electrocardiogram was monitored throughout the experimental protocol as an indication of the severity of myocardial ischemia. Polyethylene catheters were inserted into the right femoral artery for blood sampling and into both femoral veins for fluid and drug administration. Additionally, the left femoral artery was cannulated with a 13-F infant vent catheter (DLP, Inc, Grand Rapids, Mich) to provide an arterial blood source for the extracorporeal coronary artery perfusion circuit at the time of reperfusion.

Through a median sternotomy incision, the pericardium was incised and tented to cradle the heart. Millar MPC-500 temperature-compensating solid-state catheter-tipped transducers (Millar Instruments, Houston, Tex) were placed in the proximal aorta through the right internal mammary artery and in the left ventricular cavity through an apical stab wound to measure arterial and left ventricular pressures, respectively. A polyethylene cannula was inserted into the left atrium for administration of radioactive microspheres. A 1-cm portion of the left anterior descending (LAD) coronary artery distal to the first marginal branch was dissected and loosely encircled with 3-0 silk suture for subsequent LAD coronary artery occlusion. A small right ventricular branch of the LAD artery distal to the site of occlusion was dissected free and cannulated with a 2-F Millar solid-state catheter-tipped transducer (Millar Instruments) to monitor LAD coronary artery pressure. A pair of 5-MHz piezoelectric ultrasonic crystals, 2.5 mm in diameter, were implanted in the subendocardium of the myocardium perfused by the LAD (ischemic-reperfused zone) and the circumflex coronary artery, oriented parallel to the direction of the circumferential fibers to measure instantaneous segment length using a sonomicrometer (model 120, Triton Technology, San Diego, Calif).

**Experimental Protocol**

The dogs were systemically heparinized with 10 000 U sodium heparin (Lymphomed, Rosemont, Ill), which was supplemented every 60 minutes to achieve an activated clotting time >400 seconds. All animals were allowed to stabilize for 30 minutes after surgery; the experimental protocol is schematically presented in Fig 2. Baseline hemodynamic and segmental function data were collected in triplicate. Myocardial blood flow was determined by injecting 1 to 2 million 15-\(\mu M\) microspheres labeled with either \(^{14}Ce, \ ^{85}Sr, \ ^{99}Nb, \ ^{51}Cr, \) or \(^{113}Sn\) (3M Medical-Surgical Division, St Paul, Minn) through the left atrial cannula. Reference sampling from the femoral artery was started simultaneously with injection at a rate of 11.7 mL/min for a total of 4 minutes. The LAD coronary artery was then ligated for 60 minutes.
Nitric Oxide Donor in Myocardial Ischemia-Reperfusion

Lefer et al

EXPERIMENTAL PROTOCOL

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>ISCHEMIA</th>
<th>REPERFUSION</th>
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<tbody>
<tr>
<td>TIME (minutes)</td>
<td>0</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>SPM-5185 (500 nM)</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Plasma CK Activity</td>
<td>*</td>
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<td>*</td>
</tr>
<tr>
<td>Microspheres</td>
<td>*</td>
<td>*</td>
<td>*</td>
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<tr>
<td>Cardiac MPO</td>
<td>*</td>
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</table>

Fig 2. Schematic diagram of experimental protocol. CK indicates creatine kinase and MPO, myeloperoxidase.

Immediately after LAD ligation, a Deseret 14-gauge Intracath catheter (Parke-Davis & Co, Sandy, Utah) was inserted through the arteriotomy site in the LAD distal to the site of occlusion, and the cannula was opened to the atmosphere to divert collateral blood flow from the ischemic zone. This technique has been shown by Eng et al to result in a uniform depletion of collateral blood to all layers of the dog myocardium to <0.05 mL·min⁻¹·g⁻¹ and produce a consistent degree of necrosis within the area of myocardial placed at risk. The SPM-5185-treated group and the vehicle group undergoing ischemia-reperfusion received equal amounts of lidocaine before ischemia (1.2 mg/kg bolus) as well as an equivalent continuous infusion of lidocaine (0.3 mg·kg⁻¹·min⁻¹) during the 60-minute ischemic period and for the first 30 minutes of reperfusion. After 55 of the 60 minutes of LAD coronary artery occlusion, hemodynamic and segmental length data were again collected, and microspheres were injected to measure collateral blood flow. The LAD coronary artery catheter was attached to an extracorporeal perfusion system drawing arterial blood from the left femoral artery cannula. Reperfusion was abruptly established by adjusting LAD coronary blood flow using a Masterflex model 7013 roller pump (Cole-Farmer Instrument Co, Chicago, Ill). After 60 minutes of ischemia, the dogs were assigned by simple randomization to receive either normal saline or SPM-5185 in the reperfusing blood. In the untreated vehicle group, extracorporeal blood was mixed with 0.9% NaCl (Abbott Laboratories, North Chicago, Ill), while in the SPM group SPM-5185 (Schwarz Pharma AG, Monheim, Germany) was dissolved in 0.9% NaCl in the drug bag and continuously mixed with arterial blood in a ratio of 10 parts blood to 1 part SPM-5185 solution. SPM-5185 infusion was targeted to achieve an LAD plasma concentration of 500 nmol/L. Saline (vehicle) or SPM-5185 was infused throughout the 270-minute reperfusion period. Reperfusion was established by adjusting the Masterflex roller pump so that mean LAD coronary artery pressure immediately equaled mean arterial pressure, thus simulating coronary pressure-flow relations in the intact circulation. Hemodynamic and segmental length data were collected at 30, 120, and 270 minutes of reperfusion.

A total of 28 dogs were initially entered into the ischemia-reperfusion study. Four dogs undergoing MI-R were excluded because collateral blood flow exceeded 0.10 mL·min⁻¹·g⁻¹, 5 dogs died as a result of ventricular fibrillation during the early ischemic period, and 2 dogs died of ventricular fibrillation upon reperfusion. Therefore, 17 MI-R dogs completed the entire protocol. In 9 additional dogs, the entire time course of the surgery and experimental protocol was carried out without applying the LAD ligature and therefore served as sham controls. The purpose of the sham-operated animals was to determine the effects of the surgical procedures and the 270-minute femoral artery-LAD artery extracorporeal perfusion on hemodynamic and functional indices and on plasma CK and MPO activities. These 9 dogs were randomized to either a saline vehicle (n=5) group or an SPM-5185 (500 nmol/L, n=4) group in which extracorporeal circulation was conducted for 270 minutes after 60 minutes of sham ischemia.

Hemodynamic Data Collection and Analysis

Hemodynamic data including instantaneous left ventricular, arterial and LAD pressures, and heart rate were acquired during a 12-second period of respiratory apnea. Hemodynamic and segmental length data were collected during baseline, at 55 minutes of ischemia, and at 30, 120, and 270 minutes of reperfusion. The data from each channel were digitized at 250 Hz using a 12-bit analog-to-digital converter (Data Translation Devices, model DT 2821, Marlborough, Mass) and an IBM-PC AT computer (IBM, Armonk, NY); data were stored on a 80-megabyte hard disk for later analysis. Three acquisitions of steady-state data were obtained at each measurement period. The digitized data were processed by computer algorithms using an interactive videographics program developed in our laboratory. The beginning of systole was marked when instantaneous dP/dt exceeded 300 mm Hg/s, and the end of systole was marked 20 milliseconds before the nadir of negative dP/dt. These points were visually confirmed and manually adjusted if necessary. Percent segmental shortening (SS) was calculated as 100×(EDL minus ESL)/EDL, where EDL is end-diastolic length and ESL is end-systolic length in millimeters. Segmental work (SW) was calculated using point-by-point integration of the pressure-segment length loop over the entire cardiac cycle, taking into account the direction of trajectory and subtracting the diastolic component. The characteristics of diastolic segment length stiffness (the inverse of regional compliance) were determined by dynamic pressure-length analysis of end-diastolic points of variably loaded beats produced by temporary bicaval occlusion (12 seconds) during a period of respiratory apnea. The end-diastolic pressure-segment length loops were fit to the exponential equation \( P = a e^{-bL} \), where \( b \) equals end-diastolic pressure, \( a \) is the \( y \)-axis intercept, \( b \) is the modulus of segmental stiffness, and \( L \) is the end-diastolic length.

Determination of Myocardial Necrosis and Blood Flow

At the end of each experiment, the LAD was again ligated and 5 mL of Unisperse Blue dye (CIBA-GEIGY, Newport, Del) was injected into the left atrium to stain the nonischemic zone blue, thereby demarcating the in vivo area at risk. After ventricular fibrillation was electrically induced with direct application of 9 V DC, the heart was
TABLE 1. Hemodynamics and Segmental Systolic and Diastolic Function Data

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vehicle</th>
<th>SPM-5185</th>
<th>Ischemia, 5 min</th>
<th>Vehicle</th>
<th>SPM-5185</th>
<th>Reperfusion, 30 min</th>
<th>Vehicle</th>
<th>SPM-5185</th>
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<tr>
<td>HR, bpm</td>
<td>120±5</td>
<td>95±5</td>
<td></td>
<td>138±11</td>
<td>109±6*</td>
<td></td>
<td>154±11</td>
<td>106±5*</td>
<td></td>
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<tr>
<td>MABP, mm Hg</td>
<td>94±3</td>
<td>95±4</td>
<td></td>
<td>106±4</td>
<td>93±4</td>
<td></td>
<td>96±2</td>
<td>94±1</td>
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<tr>
<td>PRIx10^2, mm Hg/min</td>
<td>13.0±0.5</td>
<td>10.0±0.8*</td>
<td></td>
<td>15.5±0.9</td>
<td>11.4±0.7</td>
<td></td>
<td>15.8±0.9</td>
<td>11.3±0.6*</td>
<td></td>
</tr>
<tr>
<td>LVSP, mm Hg</td>
<td>105±3</td>
<td>108±3</td>
<td></td>
<td>114±3</td>
<td>104±2*</td>
<td></td>
<td>103±2</td>
<td>106±2</td>
<td></td>
</tr>
<tr>
<td>LVESP, mm Hg</td>
<td>6±1</td>
<td>9±1</td>
<td></td>
<td>10±2</td>
<td>10±1</td>
<td></td>
<td>8±1</td>
<td>12±1*</td>
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<tr>
<td>dP/dt, mm Hg/s</td>
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<td>1209±50</td>
<td></td>
<td>1233±128</td>
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<td>1137±69</td>
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<td>LADP, mm Hg</td>
<td>87±5</td>
<td>90±4</td>
<td></td>
<td>23±6</td>
<td>9±5</td>
<td></td>
<td>95±4</td>
<td>93±1</td>
<td></td>
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<tr>
<td>EDL, mm</td>
<td>14.4±0.4</td>
<td>18.0±0.8*</td>
<td></td>
<td>18.7±0.8</td>
<td>19.9±0.8</td>
<td></td>
<td>16.4±0.9</td>
<td>19.4±1.0*</td>
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<tr>
<td>ESL, mm</td>
<td>12.6±0.4</td>
<td>15.7±0.5*</td>
<td></td>
<td>20.2±0.8</td>
<td>21.2±0.8</td>
<td></td>
<td>16.9±0.9</td>
<td>20.1±1.0*</td>
<td></td>
</tr>
<tr>
<td>SS, %</td>
<td>12.5±1.9</td>
<td>12.2±1.8</td>
<td></td>
<td>-8.3±0.6</td>
<td>-6.7±0.4</td>
<td></td>
<td>-3.7±0.4</td>
<td>-3.6±1.0</td>
<td></td>
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<tr>
<td>SW, mm Hg: mm</td>
<td>160.0±12.4</td>
<td>268.5±28.4*</td>
<td></td>
<td>-1.0±7.2</td>
<td>9.7±4.8</td>
<td></td>
<td>7.4±4.0</td>
<td>44.3±5.9*</td>
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<tr>
<td>Stiffness</td>
<td>0.31±0.05</td>
<td>0.20±0.04</td>
<td></td>
<td>0.45±0.06</td>
<td>0.22±0.04*</td>
<td></td>
<td>0.90±0.16</td>
<td>1.16±0.20*</td>
<td></td>
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</tbody>
</table>

HR indicates heart rate; bpm, beats per minute; MABP, mean arterial blood pressure; PRI, pressure-rate index; LVSP, left ventricular peak systolic pressure; LVESP, left ventricular end-diastolic pressure; dP/dt, maximum of first derivative of LV pressure; LADP, left anterior descending coronary artery mean pressure; EDL, end-diastolic segment length; ESL, end-systolic segment length; SS, segment shortening; and SW, segmental work.

*P < .05 versus vehicle group.

RAPIDLY EXCISED. One liter of a 1% solution of triphenyltetrazolium chloride (TTC; Aldrich Chemical Co, Milwaukee, Wis) at 37°C was infused through the LAD perfusion cannula at 100 mm Hg. The atria, right ventricle, great vessels, and valves were then removed to isolate the left ventricle and septum, which were sectioned into five transverse slices. The area at risk was separated from the nonischemic zone, and the necrotic area at risk (pale, TTC-negative) was distinguished from the nonnecrotic area at risk (red, TTC-positive). Each of the three zones was then subdivided into subepicardial, midmyocardial, and subendocardial regions. Each tissue sample was then placed in a tared vial marked according to its anatomic location and staining pattern. Myocardial samples and reference samples were counted in an LKB 1282 CompuGamma gamma counter (Turku, Finland) correcting for background radiation, spillover from overlapping channels, and radiation loss due to sample height. Myocardial blood flow data were analyzed using an interactive computer program developed in our laboratory.26 Transmural collateral blood flow in the area at risk was calculated by integrating blood flow in milliliters per minute from all area at risk samples and correcting for the collective weights to obtain mL·min⁻¹·g⁻¹ of the entire area at risk.

The area at risk (AR) expressed as a percent of the left ventricular free wall was determined gravimetrically by [(weight of area at risk/weight of total left ventricular free wall)×100]. Area of necrosis (AN) relative to the left ventricle free wall was determined by [(weight of necrotic area/weight of total left ventricle free wall)×100] and relative to the area at risk (AN:AR) expressed as a percent was calculated as [(weight of necrotic/weight of total area at risk)×100]. The gravimetric method has been found to correlate closely with the planimetric method.22,27

To evaluate if SPM-5185 had any effect on the TTC staining of the ischemic-reperfused myocardium, two additional dogs were subjected to equivalent periods of MI-R. After ischemia and reperfusion, the left ventricle was cut transversely into 2-mm sections from the base to the apex. Alternate transverse sections were then incubated with TTC alone or incubated in TTC along with SPM-5185 (500 nmol/L). The extent of TTC-apparent necrosis was virtually identical between the slices incubated in TTC alone and those incubated in TTC+SPM-5185 (ie, 47% versus 45% AN:AR, respectively). This observation indicates that SPM-5185 does not interfere with the TTC staining technique.

Plasma Creatine Kinase Activity

Blood samples (5 mL) were withdrawn from the femoral artery at baseline, 30 and 60 minutes of ischemia, and at 30, 60, 120, 180, 240, and 270 minutes of reperfusion. The blood samples were transferred into Vacutainer collection tubes (Becton-Dickinson, Rutherford, NJ) containing 15% EDTA and stored on ice. Samples were centrifuged at 2000g and 4°C for 20 minutes, and the plasma was analyzed spectrophotometrically for CK activity28 and protein concentration.29 Plasma CK was expressed as IU per microgram of protein. In two additional experiments, SPM-5185 (500 nmol/L) was added to aliquots of the final two plasma samples to determine interference with the CK assay. All samples were determined to be within 5% of each other whether SPM-5185 was present or not.

Cardiac Myeloperoxidase Activity

Myeloperoxidase (MPO) activity, an enzyme virtually exclusive for PMNs, was determined in myocardial tissue using the method of Bradley et al30 as modified by Mullane et al.31 MPO activity was used as an index of PMN accumulation in the heart. Tissue was stored at
−70°C until processed. Myocardial tissue samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemical Co, St Louis, Mo), solubilized in 50 mM/L potassium phosphate buffer at pH 6.0 using a Polytron (PCU-2) homogenizer (Kinetumica GmbH, Lucerne, Switzerland) for 15 seconds×2 at 7000 rpm. Homogenates were centrifuged at 2°C for 20 minutes at 12,000 g. The supernatant was added to 0.167 mg/mL O-dianisidine dihydrochloride (Sigma Chemical Co) and 0.005% H2O2 in 50 mM/L phosphate buffer at pH 6. The change in absorbance was measured spectrophotometrically at 460 nm/L. One unit of MPO activity is defined as the quantity of enzyme degrading 1 μmol/L peroxide/min at 25°C.

In Vitro PMN Adherence Assay

Peripheral blood (500 mL) was collected from the femoral artery of seven additional pentobarbital-anesthetized (30 mg/kg IV) dogs (weight, 15.0 to 17.0 kg) and anticoagulated with citrate-phosphate-dextrose solution (Sigma Chemical Co) (1.4·10⁻³ vol:vol anticoagulant to whole blood) into round-bottom polycarbonate centrifuge tubes (Nalge, Rochester, NY). PMNs were isolated using the technique of Laffrado and colleagues. Platelet-rich plasma (PRP) was obtained by centrifuging blood at 400g for 10 minutes. The PRP was then centrifuged at 2500g for 10 minutes to obtain platelet-poor plasma (PPP). The PPP was then mixed with isotonic Percoll (9 vol Percoll:1 vol 1.5 M NaCl, Sigma Chemical Co) to produce Percoll-PPP density gradients of 80%, 62%, and 50%. Five milliliters of 4% dextran (average molecular weight, 6000 to 9000, Sigma Chemical Co) was added to the erythrocyte-leukocyte pellet from the initial 400g centrifugation. After mixture by inversion, the erythrocytes were allowed to settle for 50 minutes. The leukocyte-rich upper layer was collected and centrifuged at 1500g for 10 minutes. The pellets were resuspended in 1 mL of 1.2% NaCl and layered onto the Percoll:PPP gradient. Centrifugation was then performed at 400g for 40 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, Inc, Newton, Conn). PMNs were collected from the 62% to 82% interface and washed twice with 0.9% NaCl before being assayed for viability using trypan blue exclusion. PMN preparations obtained by this method were typically >85% pure and >95% viable. Isolated autologous PMNs were then labeled with Zynaxis PKH-2 GL fluorescent dye according to the method of Yuan and Fleming. This labeling procedure does not change the function or viability of PMNs. The LAD coronary artery was carefully removed from 4 control dogs so as not to disturb the endothelium and was placed in warmed Krebs-Henseleit solution. Isolated coronary vessels were cleaned of adipose and connective tissue and cut into rings 2 to 3 mm in length. These rings were then opened carefully and placed in 5-mL round dishes containing 3 mL of Krebs-Henseleit solution. The Krebs-Henseleit solution consisted of (in mmol/L): 118 NaCl; 4.7 KCl; 1.2 KH2PO4; 1.2 MgSO4·7H2O; 2.5 CaCl2; 2H2O; 12.5 NaHCO3; and 11 glucose at pH 7.4.

After preincubation of the coronary artery segments and labeled PMNs at 37°C separately, labeled PMNs were added to Krebs-Henseleit solution at a final concentration of 4×10⁴ cells/mL. The labeled PMNs were randomly divided into the following groups: (1) PMNs alone; (2) PMNs and LTB4 (100 nmol/L; Sigma Chemical Co); (3) PMNs, LTB4 (100 nmol/L), and SPM-5185 (500 nmol/L); (4) PMNs, LTB4 (100 nmol/L), and the nonnitroso compound, SPM-5267 (500 nmol/L, N-[3-hydroxypivaloyll-S-[N'-acetylalanoyl]-L-cysteine ethyl ester, Schwarz Pharma AG, Monheim, Germany) (Fig 1); (5) PMNs, LTB4, (100 nmol/L), and MAb R15.7 (20 mg/mL) (Boehringer Ingelheim Pharmaceuticals, Ridgefield, Conn), a monoclonal antibody to the common β-chain of the CD11/CD18 neutrophil adhesion complex; and (6) PMNs, LTB4, (100 nmol/L), and nitroglycerin (NTG, 500 nmol/L). PMNs were added to the dishes containing unstimulated coronary artery rings. The dishes were placed in a Dubnoff metabolic shaking incubator and shaken for 20 minutes at 0.5 Hz at 37°C. After 20 minutes of incubation, coronary artery segments were removed and dipped three to four times in fresh Krebs-Henseleit solution and were then placed on a glass slide with the endothelial side up. The number of PMNs adhering to the endothelial surface in five separate microscopic fields were counted under epifluorescent microscopy at a magnification ×100.

To study the adherence of unstimulated PMNs to ischemic-reperfused coronary arterial segments, 4 additional dogs were subjected to 60 minutes of LAD coronary artery occlusion and 120 minutes of reperfusion. The ischemic-reperfused LAD coronary artery was carefully removed from the hearts and placed in warmed Krebs-Henseleit solution. Autologous PMNs were isolated as described above from these dogs before ischemia, and the PMNs and coronary arterial segments were randomly divided into the following groups: (1) PMNs alone; (2) PMNs and SPM-5185 (500 nmol/L); (3) PMNs and SPM-5267 (500 nmol/L); (4) PMNs and NTG (500 nmol/L); (5) PMNs and MAb R15.7 (20 μg/mL). After 20 minutes of incubation in a shaker bath, the coronary artery segments were removed, gently washed, and counted under epifluorescent microscopy as described earlier.

### Table 1. Continued

<table>
<thead>
<tr>
<th>Reperfusion, 120 min</th>
<th>Reperfusion, 270 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vehicle</strong></td>
<td><strong>SPM-5185</strong></td>
</tr>
<tr>
<td>127±6</td>
<td>102±5*</td>
</tr>
<tr>
<td>93±3</td>
<td>98±2</td>
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<td>13.2±0.9</td>
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<td>16.8±0.7</td>
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<td>17.7±0.7</td>
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<td>−5.5±0.4</td>
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<td>1.25±0.23</td>
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Neutrophil-Induced Contraction and Endothelial Dysfunction Assay

Canine PMNs were isolated from the arterial blood of 6 sodium pentobarbital-anesthetized (35 mg/kg IV) dogs as previously described. During the PMN isolation, the LAD coronary artery was carefully removed and placed into 37°C Krebs-Henseleit solution. The LAD coronary artery was cleaned of fat and connective tissue and cut into rings 2 to 3 mm in width and then mounted on stainless steel hooks and placed into 10-mL organ chambers (Radnotti Glass, Monrovia, Calif). The coronary artery rings were initially stretched to give a preload of 1.0 g of force and allowed to equilibrate for 90 to 120 minutes until a stable baseline was achieved. After this equilibration period, the coronary artery rings were exposed to 100 nmol/L U-46619 (9,11-methanoepoxy-PGH₂, The Upjohn Co, Kalamazoo, Mich) to precontract the vascular ring. Once a stable contraction was observed, acetylcholine (ACH) was added to the bath to achieve the following final concentrations: 0.1, 1, 10, and 100 nmol/L. After the response stabilized, the rings were washed several times with fresh Krebs-Henseleit solution and allowed to equilibrate to baseline again. This procedure was repeated with the calcium ionophore A23187 (Sigma Chemical Co) at final organ bath concentrations of 1, 10, 100, and 1000 nmol/L and with acidified sodium nitrite (NaNO₂ at pH 2, Sigma Chemical Co) at 0.1, 1, 10, and 100 μmol/L final bath concentrations. These data were referred to as pre-PMN responses. Once the original baseline was restored after washing the LAD coronary artery rings several times with warm Krebs-Henseleit solution, autologous PMN suspensions (4×10⁶ cells/mL final concentration) were added into the organ baths alone or in combination with SPM-5185 (500 nmol/L), nonnitrosylated SPM-5267 (1 μmol/L), or MAb R 15.7 (20 μg/mL). After 3 to 5 minutes, 100 nmol/L of LTB₄ (Biomol Inc, Plymouth Meeting, Pa) was added to the organ chambers, and vascular responses were recorded for 20 minutes. The vascular responses obtained during this period (ie, in the presence of PMNs) were defined as the PMN-induced contraction responses.

After exposure to PMNs for 20 minutes, the coronary artery rings were washed three times with Krebs-Henseleit solution and again contracted with U-46619 (100 nmol/L). Once a stable contraction was obtained, vasorelaxation responses to ACh, A23187, and NaNO₂ were recorded and defined as the post-PMN responses. All responses were recorded using Grass FT-03 force-displacement transducers (Grass Instrument Co, Quincy, Mass) coupled with a Grass model 7 oscillographic recorder.

In additional control coronary artery rings without PMNs, the dose-dependent vasorelaxing effects of SPM-5185 and SPM-5267 were studied after precontraction of the coronary rings with U-46619.

Statistical Analysis

All data were analyzed with the STATISTICAL ANALYSIS SYSTEM program (PC-SAS; SAS Institute, Cary, NC). Time-related differences and group-time interactions were analyzed by two-way ANOVA for repeated measures followed by Holm’s sequential rejection method to reduce the chance of type I errors in multiple comparisons to <.05.²⁴ Hemodynamics, infarct size, PMN adherence, and MPO data were analyzed for group differences by ANOVA. The sources of differences were located with Duncan’s multiple range post hoc test. All data are presented as mean±SEM.

Results

Cardiac Hemodynamic Data

Hemodynamic data including heart rate (HR), left ventricular pressures, mean arterial blood pressure (MABP), mean LAD pressure, and pressure rate index (PRI), are shown in Table 1. No significant group differences were observed in MABP, dP/dt, or LAD pressure at any time point. Heart rates were comparable between the SPM-5185 and vehicle groups at control. Heart rate was lower in the SPM-5185 group after ischemia and reperfusion. The PRI was higher in the SPM-5185 group at control but was comparable to the vehicle group during ischemia. PRI was significantly lower than vehicle at 30 minutes and 270 minutes of reperfusion. Therefore, O₂ demand indexed by PRI was comparable during ischemia and lower during reperfusion. LVEDP was similar between the two groups at control and was transiently increased in the SPM-5185 group at 30 minutes of reperfusion.

Regional Myocardial Blood Flow

Transmural myocardial blood flow is shown in Fig 3A for the ischemic-reperfused zone and in Fig 3B for the nonischemic zone for baseline, 55 minutes of ischemia, and 270 minutes of reperfusion. There were no differences in baseline blood flows between groups or zones. In the ischemic-reperfused zone, coronary occlusion significantly reduced blood flow to comparable values in both groups (P=.98 versus groups), averaging 5.4±1.4 mL·min⁻¹·100 g⁻¹ in the vehicle group and 5.4±1.3 mL·min⁻¹·100 g⁻¹ in the SPM-5185 group. After 270 minutes of reperfusion, blood flow in the vehicle group returned to 63% of its baseline value (P=.10 versus baseline). Blood flow in the SPM-5185 group was restored to values threefold greater than the vehicle group (P=.04). The modestly elevated blood flow in the nonischemic region (Fig 3B) of the SPM-5185 group relative to the vehicle group (which may have received some SPM-5185 from LAD coronary venous effluent spillover into the periphery) suggests that some vasodilation may have occurred secondary to SPM-5185 or that microvascular injury was reduced. Fig 3C shows the in vitro vasodilator dose-response curve to SPM-5185. These data confirm that SPM-5185 at 500 nmol/L concentration has a modest dilator capability compared with its nonnitrosylated counterpart.

Regional Function

The segmental function data for the vehicle and SPM-5185 groups are summarized in Table 1. Although end-diastolic length, end-systolic length, and segmental work were significantly greater in the SPM-5185 group, systolic shortening was comparable between groups. Coronary occlusion converted systolic shortening to a comparable level of dyskinesis in both groups and reduced segmental work by 101% in the vehicle group and by 96% in the SPM-5185 group. During the first 30 minutes of reperfusion, there was significantly greater
systolic shortening is due to a hysteresis in the pressure-segment loop with systolic shortening insufficient to overcome initial systolic bulging as described previously. Therefore, SPM-5185 treatment caused a modest but significant recovery of posts ischemic segmental work.

Segmental stiffness determined by end-diastolic pressure-segment length relations is summarized in Table 1. All four groups were comparable at baseline. After 55 minutes of ischemia, there was an increase in stiffness in the vehicle group relative to its baseline value. After 30 minutes of reperfusion, segmental stiffness increased significantly in the vehicle and SPM-5185 groups, with a significantly greater stiffness attained in the SPM-5185 group. However, segmental stiffness decreased to near baseline levels by 270 minutes of reperfusion in the SPM-5185-treated group, while it remained significantly elevated in the vehicle group. Therefore, SPM-5185 during reperfusion prevented the development of posts ischemic segmental stiffness despite relative hyperemic blood flow in the ischemic-reperfused area.

**Effects on Plasma Creatine Kinase Accumulation**

Fig 4 summarizes the changes in plasma CK activity in all groups of dogs studied. In the sham MI-R and the sham MI-R+SPM-5185 groups, only modest increases in plasma CK activity were observed throughout the 5.5-hour experimental protocol. These data suggest that the surgical procedures contribute modestly to a predictable background level of plasma CK activity. There was a trend for the sham SPM group to have lower plasma CK levels compared with the sham group. The vehicle and SPM groups showed only modest elevations of plasma CK activity after 60 minutes of ischemia. However, MI-R dogs receiving only saline vehicle in the reperfusion blood demonstrated a significant elevation in plasma CK activity relative to the SPM group beginning at 30 minutes of reperfusion. CK levels at the end of reperfusion averaged 71.84±2.71 IU/µg protein. In contrast, dogs treated with SPM-5185 at the time of reperfusion exhibited significantly (P<.01) lower plasma CK values during the course of reperfusion to a maximum value 60% below that in the vehicle group.

**Fig 3.** Bar graphs of regional myocardial blood flow (mL·min⁻¹·g⁻¹ of tissue) at control, ischemia, and 270 minutes of reperfusion (Rep) in the area at risk (A) and nonischemic zone (B) as determined with radioactive microspheres. C, Line graph of the vasorelaxation of isolated dog coronary artery rings induced by SPM-5185 and its analog, SPM-5267. Vasorelaxation responses were recorded in U-46619-contracted (100 nmol/L) arterial rings (n=10) harvested from control hearts. SPM-5185 induced 100% relaxation at 1 µmol/L, whereas SPM-5267 had no effect of vascular tone at concentrations up to 100 µmol/L.

**Fig 4.** Plasma creatine kinase (CK) activity (IU/µg protein). All values are mean±SEM. Occlusion took place at 0 minutes; reperfusion (Rep) was instituted after 60 minutes of occlusion and was observed for 270 minutes.
(29.27±4.22 IU/mg protein) at the end of reperfusion. Plasma CK values in the SPM-5185-treated dogs differed significantly from the sham MI-R+SPM-5185 group only at 120 and 270 minutes of reperfusion. Thus, treatment with SPM-5185 significantly reduced accumulation of CK activity, an indication that SPM-5185 exerted a salutary effect on MI-R injury.

Effects on Myocardial Necrosis

The mass of left ventricle was similar between vehicle and SPM-5185 groups, averaging 84.4±5.0 g and 81.6±4.5 g, respectively (P=.69) (Fig 5A). The mass of left ventricle placed at risk was similar (P=.11) between the vehicle group (28.4±2.1 g) and the SPM-5185 group (23.7±1.3 g). When the area at risk was expressed as a percentage of the left ventricle, there was likewise no significant difference between groups (Fig 5B). The mass of necrotic tissue was significantly less whether expressed as mass (SPM-5185=3.1±0.8 g versus vehicle=13.6±3.1 g, P=.009) or as a percentage of the left ventricular mass. Infarct size expressed as AN:AR was significantly less in the SPM-5185-treated group compared with the vehicle group (Fig 5B); AN:AR was 70% less using the NO donor agent during reperfusion. Myocardial necrosis was present in both midmyocardial and subendocardial regions in the vehicle group, whereas it was confined primarily to the subendocardium in the SPM-5185 group. Thus, SPM-5185 treatment during reperfusion significantly reduced the extent of myocardial necrosis. Fig 5C shows the AN:AR plotted against the corresponding collateral blood flow in the area at risk during ischemia. The regression lines had similar slope; however, the y-axis intercept was significantly smaller in the SPM-5185-treated group, suggesting that infarct size was reduced at any given level of collateral blood flow relative to the vehicle group. These data are consistent with the plasma CK data shown in Fig 4.

Effects on Cardiac MPO Activity

Fig 6 summarizes MPO activity in the nonischemic zone and the area at risk in the four groups. In the nonischemic zone, MPO activity was similar for all groups. This indicates that neutrophils are present either intravascularly or interstitially to a small extent in nonischemic tissue after completion of the experiment. MPO activity in the nonnecrotic (ischemic zone) area at risk was significantly elevated in the vehicle group over that in the other three groups. MPO activity in the ischemic zone of the SPM-5185-treated dogs was not different than in the sham or sham SPM-5185 groups. SPM-5185 treatment significantly (P<.05) decreased MPO activity within the nonnecrotic and necrotic area at risk by 50% compared with the vehicle group. These data suggest that SPM-5185 treatment during reperfusion significantly inhibited neutrophil accumulation in both the ischemic and necrotic zones after ischemia and reperfusion.

SPM-5185 and PMN Adherence to Coronary Endothelium

To further investigate the mechanism of neutrophil inhibition by SPM-5185, we examined the effect of SPM-5185 on canine neutrophil adherence to normal (Fig 7A) and ischemic-reperfused (Fig 7B) isolated dog coronary artery endothelium. When unstimulated PMNs were added to the baths containing normal unstimulated coronary endothelium (PMN alone), very little adherence of PMNs (30±4 PMNs/mm²) was observed (Fig 7A). However, stimulation of the PMNs

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Fig 5. Bar graphs of myocardial necrosis in ischemic-reperfused dogs expressed gravimetrically (A) and as a percentage (B). Areas at risk were comparable between dogs receiving vehicle or SPM-5185 (500 nmol/L). Area of necrosis as a percent of left ventricle (AN/LV) was significantly reduced by SPM-5185 treatment. Area of necrosis indexed to area at risk (AN/AR) was also significantly reduced with SPM-5185. C, Line graph of infarct size is correlated with collateral blood flow for each experiment. Equation for the SPM-5185 group (solid line) was AN/AR=−1.72 (blood flow)+21.8, with correlation coefficient of .72, SE of Y=4.7% of AN/AR. Equation for vehicle group (dashed line) is AN/AR=−3.1 (blood flow)+57.1 with correlation coefficient of .77 and SE of Y=5.8% AN/AR.
with LTB₄ resulted in the adherence of 293±16 PMNs/mm² (P<.001 versus unstimulated). SPM-5185 at the same concentration used in vivo (500 nmol/L) inhibited PMN adherence to the endothelium by 72% (P<.001 versus control LTB₄ stimulated). These results suggest that NO liberated from this compound specifically attenuates the adherence of stimulated PMNs to coronary endothelium. The analog of SPM-5185, SPM-5267, which lacks the NO moiety, failed to inhibit LTB₄-induced PMN adherence at a concentration of 1 μmol/L. In additional studies with SPM-5267, we did not observe any effect on PMN adherence at concentrations as high as 5 μmol/L. The effect of the monoclonal antibody MAb R15.7 inhibited LTB₄-stimulated PMN adherence to LAD endothelium by nearly 100%.

The adherence of unstimulated PMNs to ischemic-reperfused coronary arteries was investigated (Fig 7). The adherence of unstimulated PMNs to coronary arteries subjected to 90 minutes of occlusion and 120 minutes of reperfusion was 115±15 PMNs/mm² greater than that observed in the unstimulated coronary arteries. PMN adherence to ischemic-reperfused LAD coronary artery segments was very significantly (P<.001) reduced by SPM-5185 (27±5 PMNs/mm²), whereas SPM-5267 had no effect. Interestingly, nitroglycerin (500 nmol/L) did not influence PMN adherence to ischemic-reperfused LAD arteries (103±4 PMNs/mm²).

In contrast, MAB R15.7 inhibited PMN adherence by nearly 70% (P<.001), a level that was comparable to PMN inhibition afforded by SPM-5185.

Effects of SPM-5185 on PMN-Induced Contraction

To determine the role of SPM-5185 on neutrophil-mediated vasoconstriction, we studied the effects of the NO donor in an in vitro system of PMN-induced endothelial injury. Representative tracings from canine LAD coronary artery rings exposed to LTB₄-activated PMNs are shown in Fig 8. In control rings (Fig 8A), the addition of unstimulated canine PMNs (4×10⁶ cells/mL) to the organ bath did not elicit any contraction. The addition of 100 nmol/L LTB₄ in the absence of PMNs produced no significant contraction of the coronary artery rings (data not shown). However, stimulation of PMN with the addition of LTB₄ (100 nmol/L) resulted in a significant contraction of 1.20±0.150 g. SPM-5185 at a concentration of 500 nmol/L significantly inhibited the PMN-induced contraction by 58% (P<.01 compared with control, Fig 8B). SPM-5267 (1 μmol/L) failed to attenuate the PMN-mediated coronary artery contraction (Fig 8C), suggesting that the donated NO was the active moiety. In addition, MAB R15.7 (20 μg/mL) significantly inhibited the PMN-induced coronary artery contraction by 83% (P<.001 compared with control, Fig 8D), substantiating that inhibition of PMN adherence to vascular endothelium...
rather than interference with elaborated neutrophil products could attenuate PMN-induced endothelial damage. The maximum constrictor responses for all coronary arteries studied in this manner are summarized in Fig 9.

The dilator responses to ACh, A23187, and NaNO2 for all coronary artery rings tested are summarized in Table 2. LTB4-stimulated PMNs significantly attenuated endothelium-dependent vasorelaxation responses to ACh and A23187. In contrast, SPM-5185 (500 nmol/L) significantly reduced PMN-induced endothelial dysfunction. However, the nitrate-deficient analog SPM-5267 failed to demonstrate any preservation of the coronary endothelium relaxation. In addition, MA bR15.7 (20 μg/mL) completely prevented endothelial dysfunction by activated PMNs and restored post-PMN vasodilator capacity to pre-PMN levels. No significant changes were observed in the responses to the endothelium-independent vasodilator sodium nitrate at any time during the experimental protocol. Therefore, SPM-5185 prevented PMN-induced endothelial damage in a manner parallel to inhibition of PMN adherence.

Discussion

The results of the present study demonstrate that the organic NO-donating compound SPM-5185 exerts a cardioprotective effect in a canine model of ischemia and reperfusion. Treatment with 500 nmol/L intracoronary SPM-5185 at reperfusion only resulted in a significant (ie, 70%) reduction in myocardial necrosis relative to the untreated vehicle group. This reduction of infarct size was independent of collateral blood flow since infarct size was lower in the SPM-5185-treated group than in the vehicle group at comparable collateral blood flow. Group differences were not due to any interference of SPM-5185 with the TTC staining process per se. The infarct size reduction was confirmed by significant attenuation of CK release throughout the reperfusion period. In addition, intracoronary SPM-5185 treatment was associated with improved posts ischemic segmental work generation and avoidance of posts ischemic stiffness. Reduction of acute myocardial infarct size and avoidance of segmental systolic and diastolic dysfunction may be attributed in part to inhibition of neutrophil accumulation, possibly by directly inhibiting neutrophil adherence to coronary endothelium and attenuating neutrophil-dependent endothelial cell damage. The PMN-related effects most likely are due to released NO, since SPM-5267 had no effect on vasodilation or neutrophil activity.

SPM-5185 is a recently developed cysteine-containing mononitrate (Fig 1) that has been shown to release NO.35 In addition, it has also been shown that SPM-5185 does not exert tolerance as readily as nitroglycerin or other traditional nitrates.36 At a concentration of 500 nmol/L, SPM-5185 induced a 20% vasorelaxation of isolated dog coronary artery rings and a modest increase in blood flow (in the contralateral myocardium). At this modestly vasodilatory concentration, SPM-5185 exerted potent antineutrophil and myocardial protective effects. These findings are in agreement with those of Johnson et al,30 who administered authentic NO to ischemic-reperfused cat hearts at a subvasodilator concentration just before reperfusion and observed a significant reduction of myocardial necrosis. Furthermore, the cardioprotective effects of NO are also in agreement with studies in which other NO donors30 or precursors to NO (ie, L-arginine) were administered at the onset of reperfusion.16,37

Posts ischemic blood flow defects have been observed in the area at risk, primarily within the necrotic tissue.16,22 This “no-reflow” phenomenon,38,39 or more appropriately, low-reflow phenomenon, was observed in the vehicle group. Proposed mechanisms of posts ischemic blood flow defects include neutrophil embolization (adherence or plugging),40,41 microvascular endothelial
debris embolization, and extravascular compression from tissue edema. Treatment with intracoronary SPM-5185 restored postischemic blood flow to values threefold greater than in the vehicle group. Because the NO donor has been shown in the present study to inhibit neutrophil activity, attenuation of neutrophil adherence or superoxide anion generation are likely mechanisms. However, we cannot rule out that a part of this greater postischemic blood flow in the area at risk is due partially to NO-induced vasodilation. However, a sustained postischemic hyperemia is often observed after short (ie, up to 1 hour) of ischemia followed by salvage pharmacological or mechanical interventions that do not have primary vasodilator activity. Therefore, the reversal of postischemic blood flow defects with SPM-5185 may be the combined action of neutrophil inhibition and vasodilation.

A major mechanism of cardioprotection exerted by SPM-5185 may be related to attenuated neutrophil accumulation in the ischemic-reperfused myocardium. SPM-5185 treatment reduced MPO activity by 70% in the ischemic zone and by 50% in the necrotic zone compared with the untreated vehicle group. Previous studies by Engler and Covell and Dreyer et al have demonstrated that reperfusion markedly enhances the infiltration of neutrophils into the ischemic region. Moreover, this emigration of neutrophils into the ischemic-reperfused myocardium is associated with microvascular injury and myocyte damage. A number of studies have shown a correlation between the extent of neutrophil accumulation in ischemic-reperfused myocardium and infarct size. Studies by Mullane et al used MPO activities specifically as an index of neutrophil accumulation and observed a very strong relation between MPO activity and infarct size. Furthermore, various treatment strategies aimed at the removal or inhibition of neutrophil-mediated injury have been quite effective in limiting myocardial cell necrosis after myocardial ischemia and reperfusion. Our MPO data show that SPM-5185 inhibited neutrophil accumulation in the ischemic-reperfused myocardium. This attenuated neutrophil accumulation may be secondary to reduced neutrophil adherence to the coronary endothelium induced by SPM-5185 as demonstrated by the adherence data (Fig 7). The reduced infarct size may therefore be due to inhibition of neutrophil-mediated endothelial and myocyte damage, possibly related to oxygen free radical generation.

Recently, McCall and coworkers have demonstrated that NO directly inhibits in vitro aggregation of rabbit PMNs. Consistent with these findings, Kubes et al reported that monomethyl arginine, an inhibitor of NO synthesis, markedly increased PMN adherence to the vascular endothelium. These findings support the concept that NO modulates PMN adherence to the endothelium. After myocardial ischemia and reperfusion, acetycholine- or calcium ionophore-stimulated release of NO by cat coronary artery rings is markedly attenuated as early as 2.5 minutes after reperfusion and remains impaired for several hours. Additional studies conducted by Nichols et al and Van Benthuyzen et al provide strong evidence that canine endothelial cells are also subject to injury after myocardial ischemia-reperfusion and suffer from an impairment of EDRF release. Therefore, it is likely that, after impaired NO release by the endothelium and accumulation of chemotactic factors at the time of reperfusion, local conditions favor the adherence of PMNs to the coronary endothelium. Our adherence data confirm the enhanced adherence of neutrophils to ischemic-reperfused coronary artery vascular endothelium (Fig 9B).

To further define the antineutrophil properties of SPM-5185, the adherence of LTB_4-stimulated PMNs to naive coronary artery endothelium was also investigated. The initial step in PMN infiltration and cellular injury in ischemic-reperfused myocardium involves interaction with and adherence to vascular endothelial cells. Once the PMN has adhered to the vascular endothelium, a series of events involving activation, diapedesis, and extravascular migration into neighboring cardiac myocytes can occur. Any treatment that interferes with the adherence of activated PMNs to coronary endothelial cells would most likely be of tremendous benefit during reperfusion of the ischemic myocardium. In the present study, SPM-5185 reduced the adherence of stimulated dog PMNs to autologous, isolated coronary artery segments by 72% compared with the NO-deficient analog of SPM-5185 (SPM-5267) or the vehicle group. SPM-5185 but not SPM-5267 also inhibited the adherence of PMNs to ischemic-reperfused coronary arterial segments. Although nitroglycerin inhibited the adherence of LTB_4-stimulated PMNs to coronary arteries by 30% under control conditions, we did not observe any attenuation by nitroglycerin in PMN adherence to coronary arteries after MI-R. Nitroglycerin is prone to tolerance that is mediated by the depletion of tissue sulfhydryl groups, and it is likely that sulfhydryl groups might be diminished in ischemic-reperfused coronary arteries. These data suggest that SPM-5185 is a potent inhibitor of PMN adherence to the endothelium, a property that may have been involved in infarct size reduction. Although PMNs predominantly adhere to capillaries and postcapillary venules and larger epicardial veins after ischemia and reperfusion, Kloner et al have recently demonstrated that neutrophils do adhere to the walls of large epicard-
dial coronary arteries in response to myocardial ischemia and reperfusion. Our findings of enhanced PMN adherence to the coronary vascular endothelium of ischemic-reperfused LAD coronary arteries in the present study are consistent with this concept and provide evidence that reperfusion injury occurs throughout the coronary arterial circulation. More important, reduction in PMN adherence and activation at epicardial endothelial sites may reduce the triggering of PMN-related events upon reperfusion.

In additional in vitro studies, we examined the effects of SPM-5185 on neutrophil-induced coronary endothelial dysfunction, manifested as vascular ring contraction produced by neutrophil-generated superoxide anions. This oxygen free radical production overwhelms the basal release of NO by the coronary vascular endothelium. Previous studies using isolated coronary arterial preparations and autologous PMNs from cats, dogs, and humans have demonstrated profound vasoconstriction upon activation of the PMNs by a number of stimuli, including low-flow perfusion followed by a return to normal perfusion. Furthermore, these PMN-induced coronary artery contractions have been shown to be associated with endothelial dysfunction characterized by attenuated responses to endothelium-dependent vasodilators and are comparable to the PMN-mediated coronary endothelial injury that occurs after MI-R. In the present study, SPM-5185 significantly inhibited the PMN-induced contraction of coronary artery rings and provided a significant degree of endothelial protection as assessed by the vasorelaxation responses to ACh and A23187. In contrast, the NO-deficient analog of SPM-5185 (SPM-5267) failed to provide any protection against the PMN-mediated vasoconstriction or subsequent endothelial injury to the coronary endothelium. These results provide additional evidence for a neutrophil-inhibitory effect of SPM-5185 that may have contributed significantly to the reduction of infarct size after ischemia-reperfusion.

Although authentic NO and this cysteine-containing NO donor clearly protect the ischemic myocardium from reperfusion injury, there are reports that NO can combine with superoxide radicals to form peroxynitrite, which is a toxic free radical species. It is highly unlikely that peroxynitrite could be involved in these experiments since very high concentrations of peroxynitrite (ie, 500 μmol/L) are required to produce cytotoxic effects in isolated liposomes. In the present study a 1000-fold lower intracoronary concentration (500 nmol/L) of this novel NO donor was found to be extremely cardioprotective in the early phase of reperfusion, which would suggest that peroxynitrite is not involved in the pathogenesis of myocardial reperfusion injury. Furthermore, peroxynitrite possesses a half-life on the order of 1 second, and it is unlikely that this short-lived free radical plays a significant role in reperfusion injury, which occurs over a number of hours. Most important, peroxynitrite has never been shown to be produced in an in vivo system during myocardial ischemia or reperfusion. Clearly, in our experiments, NO liberated from an NO-releasing agent, SPM-5185, was beneficial.

Conclusions
The present study demonstrates that the administration of the novel NO donor SPM-5185 at the time of reperfusion reduces the extent of cardiac cellular injury induced by severe regional myocardial ischemia. The mechanism of this cardioprotection appears to be largely related to inhibition of PMN adherence to the coronary endothelium and a reduction in the subsequent accumulation of activated PMNs in the ischemic myocardium. However, we cannot rule out that the modest vasodilation induced by SPM-5185 during reperfusion had a therapeutic effect, possibly by improving oxygen delivery or recruiting additional flow-dependent NO release. However, a recent study by Vinten-Johansen et al. would rule out augmented flow as a primary mechanism of infarct size reduction during reperfusion. Since SPM-5185 may act at the microvascular level where bioconversion of nitroglycerin is limited, this cysteine-containing NO donor may be useful in targeting microvascular components of reperfusion injury. However, additional studies are necessary to further elucidate the precise mechanisms of cardioprotection exerted by NO donors in reperfusion injury. Additional studies using longer periods of reperfusion (ie, 24 or 48 hours) and histological assessment of infarct size are also clearly necessary to determine if SPM-5185 actually reduces the extent of myocardial cell injury or merely delays the onset of myocardial injury and infarct development.

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