SP/W-5186, A Cysteine-Containing Nitric Oxide Donor, Attenuates Postischemic Myocardial Injury

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ABSTRACT

The effects of SP/W-5186, a cysteine-containing nitric oxide (NO) donor, on myocardial reperfusion injury were studied in a rabbit ischemia (45 min) and reperfusion (180 min) model. Five minutes before reperfusion, either low-dose (0.3 μmol/kg) or high-dose (1 μmol/kg) SP/W-5186 was given intravenously as a bolus. Administration of 0.3 μmol/kg SP/W-5186 did not change mean arterial blood pressure, heart rate or pressure-rate index. However, administration of low-dose SP/W-5186 exerted marked cardioprotective effects as evidenced by improved cardiac functional recovery (P < .05 vs. vehicle), decreased plasma creatine kinase concentration (P < .01) and reduced infarct size (P < .01). Moreover, administration of SP/W-5186 significantly decreased platelet aggregation (P < .01 vs. vehicle), attenuated polymorphonuclear leukocyte (PMN) accumulation in myocardial tissue, inhibited PMN adhesion, attenuates platelet aggregation and quenches superoxide anion (Moncada and Higgs, 1995). Replacement of endogenous NO, which has been found to be decreased significantly after reperfusion (Lefer et al., 1991), by an exogenous source of NO at reperfusion could theoretically attenuate reperfusion injury. In this regard, organic nitrates, such as NTG, have been used in myocardial ischemia for >100 years. However, most of these classic nitrates are poor NO donors and quickly induce tolerance, presumably because they must be metabolized intracellularly to release NO. Part of the metabolic degradation of NTG and other nitrates may require reducing equivalents (e.g., thiols), and a lack of reduced thiols has been implicated in tolerance (Needleman et al., 1973; Flaherty, 1989).

SP/W-5186 [N-(3-nitratopivaloyl)-S-(N’-acetylglycyl)-1-cysteine ethylester] is a newly developed NO donor that...
contains both an NO group and a source of thiols (i.e., cysteine) (fig. 1). Preliminary pharmacological experiments have demonstrated that, in contrast to the classic nitrateg TNG, no tolerance developed after a 3-day treatment with high doses of SP/W-5186 administration (Schwarz Pharma AG, unpublished observations). Moreover, as a prodrug, SP/W-5186 has a relatively long half-life, and its vasodilator effect lasts ~3 hr after a single bolus injection at a dose of 1.68 μmol/kg in dog (Schwarz Pharma AG, unpublished observations). The purposes of the present study were (1) to determine the effects of different doses of SP/W-5186 on cardiac functional and myocardial cellular injury associated with ischemia and reperfusion when given at different doses and (2) to investigate the mechanisms by which SP/W-5186 may exert its cardioprotective effects.

**Experimental Procedures**

**Materials.** SP/W-5186 and SP/W-6373 were kindly provided by Schwarz Pharma AG (Monheim, Germany). All other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. A total of 61 adult male New Zealand White rabbits (2.8–3.5 kg) were included in this study. The experiments were performed in adherence to National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care.

**Experimental preparation.** Rabbits were anesthetized with sodium pentobarbital (30 mg/kg body weight) intravenously. An intratracheal tube was inserted through a midline incision, and all rabbits were given intermittent positive-pressure ventilation with O2-enriched room air using a Harvard small animal respirator (Harvard Apparatus, South Natick, MA). Arterial blood gases were measured using a blood gas analyzer (CIBA-CORNINE 288 Blood Gas Analyzer, Ciba-Cornine Corp., Norwood, MA) and arterial PO2 and Pco2 were maintained at 100 to 120 mm Hg and 35 to 45 mm Hg, respectively, by adjusting the oxygen flow and ventilation rates. The pH was adjusted to 7.35 to 7.45 with sodium bicarbonate as necessary. A polyethylene catheter was inserted into the right external jugular vein for supplemental pentobarbital injection to maintain a surgical plane of anesthesia and for administration of drugs. An additional polyethylene catheter was inserted through the left femoral artery and positioned in the abdominal aorta for measurement of ABP via a Statham P23AC pressure transducer (Spectromed, Critical Care Division, Oxnard, CA). After a midline thoracotomy was performed, the pericardium was opened and the heart was exposed. A 4–0 silk ligature was carefully placed around the major marginal branch of the left circumflex coronary artery located on the dorsal surface of the heart, 10 to 12 mm from its origin. A Millar-tip catheter transducer (5F) was inserted into the LV cavity through the apex, and the LVP was obtained. ABP, LVP and standard lead II of the scalar ECG were digitized at 250 Hz using a 12-bit analog-to-digital converter (Data Translation Devices, Marlboro, MA) and a Dell 486–66 computer. MABP, HR, LVSP, LVEDP and the instantaneous first derivative of LVP (dP/dt) were continuously monitored during the entire experimental period. The PRI, calculated as the product of MABP and HR divided by 1000, was used as an approximation of myocardial oxygen demand.

After a 20-min period of stabilization after thoracotomy, MI was initiated by complete ligation of the marginal coronary artery. This was designated as time 0. After 45 min of ischemia, the ligature was untied and the ischemic myocardium was reperfused (R) for 3.0 hr. Sham MI/R rabbits were subjected to all the surgical procedures performed on MI/R rabbits, including the placement of silk suture around the coronary artery, except the suture was left untied throughout the observation period. At 5 min before reperfusion, the rabbits were randomly assigned to one of the following 6 groups: (1) sham MI/R + vehicle (0.9% NaCl, 1 ml/kg, n = 6); (2) MI/R + vehicle (n = 11); (3) MI/R + SP/W-5186 (0.3 μmol/kg, n = 12); (4) MI/R + SP/W-5186 (1 μmol/kg, n = 10); (5) MI/R + SP/W-6373 (0.3 μmol/kg, n = 11) or (6) MI/R + SP/W-6373 (1 μmol/kg, n = 11). Each drug or vehicle was given as a single intravenous bolus injection over 1 min. Preliminary experiments with SP/W-5186 at different doses (0.1, 0.3, 0.5 and 1 μmol/kg) indicated that 0.3 μmol/kg was the highest dose that could be administered without alterations in MABP (115 ± 4.6 mm Hg before drug injection and 114 ± 3.4 mm Hg 3 min after drug injection, P > .1, n = 6), whereas a 1 μmol/kg decreased MABP significantly (116 ± 3.2 mm Hg before drug injection and 101 ± 3.1 mm Hg 3 min after drug injection, P < .01, n = 6). These two doses (i.e., 0.3 and 1 μmol/kg) were thus used in this study as a low dose and a high dose, respectively.

**Analysis of myocardial injury.** Ischemia-reperfusion-induced cardiac contractile dysfunction was monitored during the ischemia and reperfusion period. LVP, MABP and ECG were acquired over 10 sec at the following time points: immediately before coronary occlusion (0 min); 20 and 45 min after coronary occlusion; and 5, 30, 60, 120 and 180 min after reperfusion. Data were stored on computer hard disk for later retrieval and analysis. All acquisitions were obtained in duplicate. The LVP, LVSP, dP/dt and HR were obtained using computer algorithms and an interactive videographics program (Bowman Gray School of Medicine, Winston-Salem, NC) (Sato et al., 1995).

The arterial blood samples (1 ml) were drawn immediately before ligation (0 min), 45 min after ischemia and hourly thereafter. The blood was collected in polyethylene tubes containing 200 IU of heparin sodium. Samples were centrifuged at 2000 × g and 4°C for 20 min, and plasma was removed for biochemical analysis. Plasma protein concentration was determined by the BCA method (Pierce). Plasma creatine kinase (CK) activity was measured in a blind manner using the method of Rosalki (1967) and expressed as IU/mg of protein.

At the end of the 3.0-hr reperfusion period, the ligature around the marginal coronary artery was retied, and 20 ml of 5% Evans blue dye was injected into the LV cavity. The dye was circulated uniformly distributed except in that portion of the heart previously perfused by the occluded coronary artery. The heart was quickly excised, and the

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**Fig. 1.** Structures of SP/W-5186 and SP/W-6373.
atria, right ventricle and fatty tissues were subsequently removed from the heart. The coronary artery was isolated for examination of endothelial function as described below. The LV was then sliced into 2- to 3-mm-thick sections perpendicular to the long axis of the heart. The unstained portion of myocardium (i.e., AAR) was separated from the Evans blue-stained portion of the myocardium (i.e., NEC). The AAR was again sliced into about 1-mm-thick sections and incubated at 37°C in 0.1% solution of nitro blue tetrazolium in phosphate buffer for 15 min to detect the presence of coenzyme and dehydrogenase. At the end of this time, the myocardial tissue was examined under a bright field optic illuminator and unstained (i.e., red) NEC was carefully separated from the stained (i.e., the dark blue) NNEC viable tissue. Samples from all three portions of LV cardiac tissue (i.e., ANAR, NNEC and NEC) were weighed. The AAR as a percent of the total LV mass [AAR/LV(100%)] and the NEC as a percentage of total LV mass [NEC/LV(100%)] were calculated (Ma et al., 1996). The three portions of the myocardium were then stored at −70°C for later assay of myeloperoxidase activity.

**Studies on coronary endothelial dysfunction.** After injection of Evans blue, hearts were excised and placed in ice-cold Krebs-Henseleit (K-H) buffer. The marginal coronary artery was carefully isolated and 10- to 12-mm-long segments were removed both above and below the ligature. The segment above the ligature (i.e., the proximal segment) was used as a nonischemic, nonreperfused control, and the portion of the vessel that had undergone ischemia and reperfusion (i.e., the distal segment) was used as an ischemic vessel. These segments were placed in warmed K-H solution consisting of (in mM): NaCl 118; KCl 4.75; CaCl2 2.54; KH2PO4 1.19; MgSO4 1.19; NaHCO3 25, and glucose 10.0. Within 5 min, isolated vessels were cleaned of adhering fat and connective tissue and cut into rings 2 to 3 mm in length. The rings were then quickly mounted on stainless steel hooks, suspended in water-jacketed tissue baths and connected toORT-10 force transducers (WPI, Sarasota, FL) to record changes in force on WindoGraf Recorders (Gould, Valley View, OH). The baths were filled with 7 ml of K-H buffer and aerated with a gas mixture of 95% O2 and 5% CO2. They were then stretched to a tone of 50% of the baseline force. Once a stable contraction was obtained, ACh, an endothelium-dependent vasodilator, was added with the hypoxia-hypoglycemic PBS-BSA solution (300 mO2; 5% CO2) to the EC surface was quantified by measuring MPO activity with a modification of the method of Ohkawa et al. (1979).

**Ex vivo platelet aggregation.** Immediately before and 30 min after each drug or vehicle administration, 5 ml of blood samples were drawn into a polyethylene tube containing 4% sodium citrate (1:9 v/v). PRP was prepared by centrifugation of blood of 200 × g for 15 min, and PPP was obtained by centrifugation of the remaining blood at 1600 × g for 20 min at room temperature. Autologous PPP was used to dilute the PRP to a final concentration of 3 × 108 platelets/ml. All tests were performed within 90 min after blood sampling.

Platelet aggregation was studied by the modified turbidometric method of Born (1962) using a Chrono-Link Aggregometer (Chronolog, Havertown, PA). Then, 500 µl of adjusted PRP or PPP was added into a cuvette, and the initial light transmission of the PPP and PRP was set as 100% and 0%, respectively. After 5 min of incubation at 37°C, platelet aggregation was induced by addition of a submaximal concentration of collagen (10 µg/ml) to PRP. The aggregation reaction was recorded for the following 10 min, and the amplitude and slope of the aggregation were determined automatically by a computer connected to the aggregometer.

**PMN adhesion to cultured ECs.** Rabbit peripheral blood (20 ml) was collected from the central ear artery and mixed with 3.0 ml of anticoagulation agents, which included 1.6% citric acid and 2.5% sodium citrate at pH 5.4, and 17 ml of Hespan solution (6% hetastarch in 0.9% saline; DuPont Pharmaceutical, Wilmington, DE). PMNs were isolated by a procedure originally described by Doerschuk et al. (1989) and modified by Todd et al. (1996). PMN preparations obtained by this method are typically >95% pure (hematoxylin/eosin staining) and >95% viable (trypan blue exclusion).

Microvascular ECs were isolated from rabbit tissue by the methods originally reported by Kern et al. (1983) and Bowman et al. (1982), and modified by Renzi and Flynn (1992). Plated cells reached confluence within 4 to 5 days and were transferred into 24-well plates. All experiments were performed within 48 hr. Glucose-free PBS-BSA (1%) solution was first gassed for 5 min with a hypoxic gas mixture (90% N2-5% CO2-5% O2) in a custom-designed hypoxia-reoxygenization incubator. Normal culture medium was quickly replaced with the hypoxia-hypoglycemic PBS-BSA solution (300 µl per well) within the incubator, and ECs were incubated at 37 ± 0.2°C for 45 min. A continuous flow of a hypoxic gas humidified and warmed to 37°C was maintained during hypoxic incubations. After 45 min of hypoxic-hypoglycemic incubation, the medium was replaced with normal cell culture medium that had been gassed with 20% O2-75% N2-5% CO2. This complete replacement of the medium ensured an immediate return to a normal oxygen environment (Po2 = 145–155 mm Hg). At the onset of reoxygenation, PMNs (5 × 105) and testing compounds were added. The plate was agitated (60 rpm) at 37°C in an incubator that was purged with a 20% O2-75% N2-5% CO2 gas mixture. After another 3 hr of incubation, nonadherent PMNs were removed by three complete washings. The number of PMNs adhering to the EC surface was quantified by measuring MPO activity with a modification of the method described by Bath et al. (1989), as modified by Pietersma et al. (1994) using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

**Statistical analysis.** All values in the text, tables and figures were presented as mean ± S.E.M. of n independent experiments. All data were subjected to ANOVA followed by the Bonferroni correction for post-hoc t tests. Probabilities of P < 0.05 were considered to be statistically significant.
**Results**

**HR, MABP and PRI.** There were no significant differences among the groups in HR and MABP before coronary occlusion. Immediately after occlusion of the coronary artery, the portion of the ventricle that had been perfused by the occluded artery became cyanotic and dyskinetic. Simultaneously with these visual alterations, MABP decreased significantly. When the coronary arterial ligature was loosened after 45 min of ischemia, ventricular reperfusion was confirmed visually by resumption of normal ventricular color and wall motion, as well as a decline in S-T segment elevation on the ECG. MABP slightly declined again during the first 20 min of reperfusion and gradually returned toward the prereperfusion level thereafter. There were no significant differences in MABP after reperfusion among the five groups, with the exception that high-dose SP/W-5186-treated rabbits had a significantly lower MABP after drug injection.

To determine whether any of the agents studied significantly altered myocardial oxygen consumption, we used the product of MABP and HR as an index for myocardial oxygen demand. As shown in figure 2A, the PRI for all groups were not significantly different from one another at the start of the experiment and at 20 min after ischemia. In the vehicle-treated MI group, the PRI value slightly recovered at the end of the 45 min of ischemia and decreased again in the first 10 min of reperfusion. Administration of low-dose SP/W5186, and low-dose or high-dose SP/W-6373, did not change the PRI significantly. Therefore, it is unlikely that any protective effect demonstrated in this model by these compounds is the result of a reduction in myocardial oxygen demand. In contrast, administration of high-dose SP/W-5186 specified in the methods resulted in a transient but significant decrease in PRI after drug administration, indicating that this dose of SP/W-5186 may significantly reduce myocardial oxygen demand.

![Graphs showing PRI, LVEDP, and dP/dt max](image)
Cardiac functional injury. The maximal rate of \( dP/dt_{\text{max}} \) is a commonly used index of myocardial contractility. Because \( dP/dt_{\text{max}} \) is critically related to LV preload (i.e., LVEDP) and afterload (i.e., MABP), we measured LVEDP, MABP and \( dP/dt_{\text{max}} \) simultaneously in this experiment. As described above, there were no significant differences among vehicle, low-dose SP/W-5186 and either dose of SP/W-6373-treated groups at any time point observed. Although administration of high-dose SP/W-5186 significantly decreased the MABP, this decrease lasted for a short period only and MABP returned to a level comparable to that in the other groups 30 min after drug administration. Therefore, the difference in \( dP/dt_{\text{max}} \) if any, among the groups, could not be attributed to a difference in afterload (i.e., MABP).

There was no significant difference in the basal readings of LVEDP among groups. After occlusion of the coronary artery, LVEDP increased significantly in all five groups and reached the highest value 20 min after coronary occlusion. On reperfusion, LVEDP decreased slightly in the first 30 min of reperfusion and increased again thereafter. There was no significant difference in drug-treated groups and vehicle-treated group during ischemia and within 30 min of reperfusion. However, administration of either low-dose or high-dose SP/W-5186, but not its inactive control compound SP/W-6373, prevented the LVEDP increase during reperfusion. Thus, LVEDP was significantly lower in these two groups than in either vehicle or SP/W-6373-treated groups at the end of the experimental period (i.e., 180 min after reperfusion) (fig. 2B).

All five groups showed comparable initial values for \( dP/dt_{\text{max}} \). On coronary occlusion, \( dP/dt_{\text{max}} \) decreased dramatically over the first 20 min and recovered slowly thereafter. There was no significant difference among groups during the entire ischemia period and during the first 120 min of reperfusion. However, in SP/W-5186-treated rabbits at either dose level, \( dP/dt_{\text{max}} \) almost recovered back to pre-occlusion values 180 min after reperfusion. Hence, \( dP/dt_{\text{max}} \) was significantly higher compared with either the vehicle-treated or the SP/W-6373-treated groups (fig. 2C).

Myocardial cellular injury. Plasma CK activity was measured before coronary occlusion, at the end of coronary occlusion and hourly thereafter. At the end of the 45-min ischemic period, plasma CK activity only slightly increased and there was no significant difference among groups. However, plasma CK activity increased markedly after reperfusion and reached 173 ± 7.1 U/g protein at the end of reperfusion in the vehicle-treated group (5.2 ± 0.8 U/g protein before ischemia, \( P < .0001 \)). Treatment with inactive control compound, SP/W-6373 at either dose did not change plasma CK activity at any time point observed. In contrast, MI rabbits treated with SP/W-5186 developed significantly lower plasma CK activities compared with MI rabbits treated with either the vehicle or SP/W-6373 at every time point after reperfusion (fig. 3). Moreover, at 180 min after reperfusion, plasma CK activity in the high-dose SP/W-5186-treated group was significantly lower than the low-dose SP/W-5186-treated group, indicating that the higher dose of SP/W-5186 exerted a more pronounced cardioprotective effect. The decrease in plasma CK activity in the SP/W-5186-treated groups was not due to a direct effect of this compound on the CK assay, as addition of SP/W-5186 directly to rabbit plasma did not affect the CK assay results (i.e., duplicate values with and without exogenously added SP/W-5186 were within 5% of each other). CK values in the four plasma samples drawn from untreated MI rabbits at 3 hr after reperfusion were 166 ± 8.7 vs. 172 ± 7.9 IU/g protein in these same samples reassayed in the presence of SP/W-5186 up to a concentration of 100 nM.

To verify plasma CK activity as an index of preservation of ischemic tissue and to ascertain the effects of SP/W-5186 on the degree of myocardial salvage of ischemic/reperfused tissue, we measured the amount of necrotic cardiac tissue and expressed it as a percentage of either the AAR or of the total LV mass (fig. 4). There was no significant difference in the AAR expressed as percentage of total LV among groups, indicating that a comparable degree of ischemic jeopardy existed in all five groups. After 45 min of coronary occlusion and 180 min of reperfusion, ~57% of the ischemic-reperfused myocardial tissue evolved to necrosis in the vehicle group. Administration of SP/W-5186 significantly decreased the necrotic size compared with the vehicle group. Thus, the percent of AAR that actually became necrotic decreased to 30 ± 1.3% in the low-dose SP/W-5186-treated group (\( P < .01 \) vs. vehicle group) and to 23 ± 1.7% in the high-dose SP/W-5186-treated group (\( P < .01 \) vs. vehicle and \( P < .05 \) vs. low-dose SP/W-5186-treated group). In contrast, administration of either low-dose or high-dose SP/W-6373 exerted no protective effects (fig. 4).

Neutrophil accumulation in the myocardial tissue. Accumulation of neutrophils in the ischemic region during reperfusion has been thought to be one of the major mechanisms responsible for reperfusion injury. We therefore measured MPO activity in the three different regions of the myocardium as a marker for neutrophil accumulation in ischemic tissue. In the nonischemic myocardium
AANR, MPO activity was very low in all groups and there was no significant difference among them, indicating that few neutrophils were present in the nonischemic myocardium. However, MPO activity in the ischemic region was markedly increased. Treatment with inactive control compound, SP/W-6373, had no effect on MPO activity either in the ischemic non-necrotic area or necrotic area. In contrast, SP/W-5186-treated ischemic rabbits exhibited a significantly lower MPO activity in ischemic non-necrotic myocardial tissue as well as in necrotic tissue (fig. 5). There was no significant difference in MPO activity between the two SP/W-5186-treated groups. These results indicate that adherence and accumulation of neutrophils in ischemia-reperfused myocardium was markedly inhibited by treatment with SP/W-5186.

**Effect of SP/W-5186 on myocardial lipid peroxidation after ischemia and reperfusion.** In a separate series experiment, the effect of -NO on lipid peroxidation was studied and the results were summarized in table 1. Myocardial ischemia followed by reperfusion caused significant lipid peroxidation, as evidenced by marked increase in MDA content in ischemic-reperfused myocardial tissue (AAR). Administration of SP/W-5186 (0.3 μmol/kg), but not SP/W-6373, before reperfusion significantly attenuated MDA elevation, suggesting that supplementation of exogenous -NO prevented myocardial lipid peroxidation induced by coronary occlusion and reperfusion.

**Isolated neutrophil adhesion to cultured microvessel endothelial cells.** To further determine the effect of SP/W-5186 on PMN-EC interaction, we investigated the effects of SP/W-5186 on isolated PMN adhesion to cultured microvascular ECs. Exposing ECs to hypoxia followed by reoxygenation resulted in a 2.5- to 3-fold increase in PMN adhesion (not shown). Addition of SP/W-5186, but not SP/W-6373, at the time of reoxygenation inhibited PMN adhesion in a concentration-dependent manner with an EC₅₀ of 53 nM. Maximal inhibition (75.4 ± 1.6% inhibition) was observed at a SP/W-5186 concentration of 300 nM (fig. 6). These results further demonstrate that SP/W-5186 is a potent and effective inhibitor of PMN-EC interaction.

**Coronary endothelial function.** Because endothelial dysfunction is an early and critical event in reperfusion and plays an important role in reperfusion injury, we tested endothelial function by comparing the vasoactivity of isolated coronary artery rings in re-
sponse to the endothelium-dependent vasodilator, ACh, with the response of an endothelium-independent vasodilator, acidified NaNO2. Figure 7 summarizes the vasorelaxant responses of isolated coronary artery rings from the five groups of rabbits obtained with ACh and NaNO2. In control coronary artery rings, ACh induced a concentration-dependent vascular relaxation with >90% relaxation occurring at a concentration of 10 μmol/l. In contrast, the concentration-response curve to ACh showed a significant shift to the right in the coronary artery rings from vehicle-treated MI rabbits. At the highest concentration ACh tested, relaxation was markedly impaired (46 ± 4.2%, P < .001 vs. 94 ± 2.9% in sham). Treatment with SP/W-6373 did not improve vasorelaxation responses of coronary artery rings to ACh. However, coronary artery rings from MI rabbits treated with SP/W-5186 demonstrated a significant improvement in endothelium-dependent vasorelaxation. When exposed to 10 μM ACh, the coronary artery rings showed a relaxation of 78.6 ± 2.4 and 80.2 ± 2.9 in low-dose and high-dose SP/W-5186 groups, respectively (P < .001 compared with vehicle-treated MI rabbits).

To determine if MI/R may have altered the responsiveness of the vascular smooth muscle to exogenous NO, we investigated the vasorelaxant effect of acidified NaNO2 in the coronary artery rings isolated from all five groups. As summarized in figure 7, acidified NaNO2 induced a concentration-dependent vascular relaxation, with full relaxation occurring at an NaNO2 concentration of 100 μmol/l. There were no significant differences among any of the five groups at any concentration of NaNO2 tested. These findings indicate that the smooth muscle cells of coronary artery rings isolated from each rabbit group were functionally intact and respond normally to exogenously applied NO. Thus, MI/R resulted in a selective vascular endothelial dysfunction with no alteration in the sensitivity or effectiveness of NO on the smooth muscle, and this endothelial dysfunction was significantly attenuated by SP/W-5186 but not SP/W-6373.

**Effects of SP/W-5186 on platelet aggregation.** To determine whether administration of SP/W-5186 may inhibit the platelet aggregation that may contribute to the attenuation of ischemia/reperfusion injury, two blood samples were taken from each animal (i.e., immediately before and 30 min after each drug or vehicle administration). The effects of SP/W-5186 on platelet aggregation were then tested in PRP ex vivo. As illustrated in figure 8 and summarized in figure 9, collagen-stimulated platelet aggregation was comparable in PRP obtained before or after vehicle administration, indicating that platelet function did not significantly change during the observation period. Moreover, because the first blood was sampled ~6 min before reperfusion and the second 25 min after reperfusion, this result also suggested that reperfusion per se did not significantly alter platelet aggregability. However, when 0.3 μmol/kg SP/W-5186 was administered 5 min before reperfusion, platelet aggregation was significantly inhibited compared with blood withdrawn before drug administration. The inhibitory effect of SP/W-5186 on platelet aggregation was slightly increased when 1 μmol/kg SP/W-5186 was given. This difference, however, was not statistically significant compared with low-dose SP/W-5186. Administration of SP/W-6373, the inactive control compound of SP/W-5186, had no effect on platelet aggregation.

**Discussion**

SP/W-5186 was designed as a produg and does not release NO directly. The compound is stable in solution at pH 5 but decomposes at pH 7.4 and 37°C with a half-life of ~10 hr. The major hydrolysis product of SP/W-5186, SP/W-3672, a molecule with a free SH group, releases NO spontaneously (Kojda et al., 1995). In contrast to the classic organic nitrates, addition of cysteine does not further increase NO release. After a bolus i.v. injection of SP/W-5186, SP/W-3672 is detectable in plasma within 1 min and peaks at 5 min, indicating that SP/W-5186 is quickly converted to SP/W-3672 in plasma (Schwarz Pharma, unpublished data).

The data presented in this study demonstrate that SP/W-5186 exerts significant protective effects against reperfusion injury as evidenced by improved cardiac functional recovery, decreased plasma creatine kinase concentration and reduced infarct size. Although improvement of cardiac function was not significantly different between the low- and high-dose treatment groups, myocardial cellular injury as measured by plasma CK activity and necrotic size was more potently attenuated in the group treated with the high dose of SP/W-5186. Administration of SP/W-6373, a nitrate-free analog of SP/W-5186, at either dose failed to exert significant protective effects in this ischemia-reperfusion model. Since the only chemical difference between SP/W-5186 and SP/W-6373 is that the latter lacks the nitrate moiety, the present results clearly demonstrate that NO released from this type of NO donor is responsible for the cardioprotective effects observed in this study.

There are several possible explanations for the marked improvement of cardiac function and the attenuation of myocardial injury seen with SP/W-5186 treatment. It is well documented that reperfusion involves a typical inflammatory response and PMNs are known to play a pivotal role in tissue injury. Many studies have documented a correlation between infarct size secondary to reperfusion and extent of PMN infiltration (Lucchesi, 1994). PMNs can induce endothelial and myocardial reperfusion injury by various mechanisms. Activated PMNs release a variety of cytotoxic substances, including proteases, collagenases, cytokines, leukotrienes and cationic proteins, capable of causing tissue damage (Lucchesi, 1994). Adhering and aggregating PMNs can physically obstruct capillary flow and induce a “no-reflow phenomenon.” This mechanical obstruction causes a regional permanent ischemia and ultimately leads to an increase in necrosis (Duran et al., 1994). A large body of evidence exists that indicates that PMNs are the major source of reactive oxygen species (ROS) and thus are primarily responsible for free radical-induced endothelial and myocardial injury after myocardial ischemia and reperfusion (Lucchesi, 1994; Duran et al., 1994). ROS not only cause direct myocardial injury through the formation of hydroxyl radical (OH)· (Duncan, 1994), but more importantly, activate ECs and PMNs, thus promoting further PMN adhesion and initiating a vicious cycle.

Numerous studies have demonstrated that NO possesses potent antineutrophil activity (Provost et al., 1994), and thus may exert its cardioprotective effects through attenuation of PMN-mediated reperfusion injury (Edgell et al., 1994). In a previous study, we have shown that diminished basal NO release from coronary endothelium after myocardial isch-
Embolization and reperfusion promotes adherence of PMNs in vitro through a CD11/CD18-dependent mechanism (Ma et al., 1993). Increasing endogenous \( \cdot \)NO production by supplying exogenous \( \text{L-arginine} \) (Weyrich et al., 1992) has been shown to decrease PMN accumulation and attenuate myocardial injury. The cellular mechanism for the antiadhesive effects of \( \cdot \)NO is not clearly understood at present. However, recent studies indicate that \( \cdot \)NO may attenuate PMN-EC adhesion.

Fig. 6. Concentration-inhibition effect of SP/W-5186 or SP/W-6373 on isolated rabbit PMN adhesion to cultured microvascular endothelial cells. Confluent endothelial cells were exposed to hypoxia (45 min) and reoxygenation (180 min). At the time of reoxygenation, PMNs (5 \( \times \) 10\(^5\)/well) and drugs were added. At the end of the 3-hr reoxygenation period, nonadherent PMNs were removed by three consecutive washings. The number of adhered PMNs to ECs was quantified by measuring MPO activity using a Thermomax microplate reader. **P < .01 vs. the vehicle-treated group.

Fig. 7. Concentration-relaxation response of coronary artery rings to the endothelium-dependent vasodilator ACh (left) and to the endothelium-independent vasodilator acidified NaNO\(_2\) (right). The rings were constricted by U-46619 (50 nM). Once a stable vasoconstriction was obtained, cumulative concentrations of ACh (1 nM to 10 \( \mu \)M) or acidified N\( \text{A} \)O\(_2\) (10 nM to 100 \( \mu \)M) were added, and the vasorelaxation to each concentration of vasodilator was calculated as a percent of the maximal vasoconstriction induced by U-46619. Each point represents the mean value of 13 to 15 rings isolated from 4 or 5 rabbits from each experimental group. **P < .01 vs. the vehicle group.
by decreasing endothelial expression of adhesion molecules. Gauthier et al. (1994) reported that \( \text{NO} \) decreases P-selectin expression after splanchnic ischemia and reperfusion. De Caterina et al. (1995) demonstrated that \( \text{NO} \) significantly inhibited cytokine-induced up-regulation of the vascular cell adhesion molecule-1 (VCAM-1) as well as ICAM-1 in cultured human saphenous vein ECs. In the present study, we have clearly shown that SP/W-5186 markedly decreases PMN accumulation in ischemia/reperfused myocardial tissue when administered \textit{in vivo} and significantly inhibits PMN adhesion when exposed to hypoxia/reoxygenated ECs \textit{in vitro}. The novel \( \text{NO} \) donor SP/W-5186 may thus protect myocardial tissue from reperfusion injury \textit{via} its antineutrophil effects. Interestingly, it appears that the anti-PMN effects of SP/W-5186 are more potent when administered \textit{in vivo} than \textit{in vitro}. Although the administration of SP/W-5186 at a dose of as low as 0.3 \( \mu \text{mol/kg} \) markedly decreased MPO activity in ischemic-reperfused myocardial tissue, the minimal concentration that exerted significant inhibition of PMN adhesion to endothelial cells \textit{in vitro} was 30 nM, a concentration \( \sim 6 \) to 8 times higher than the estimated minimal plasma concentration after 0.3 \( \mu \text{mol/kg} \) administration. Because SP/W-5186 needs to be converted to the active metabolite SP/W-3672 to release \( \text{NO} \), it is possible that the differences in potency of SP/W-5186 needed to inhibit the PMN-EC interaction \textit{in vitro} and \textit{in vivo} reflect the faster metabolism of SP/W-5186 \textit{in vivo}.

Substantial evidence exists indicating that endothelial dysfunction manifested as decreased vasorelaxant response to endothelium-dependent vasodilators and increased microvascular leakiness is one of the earliest pathological responses after reperfusion (Lefer et al., 1991). It contributes significantly to the subsequent myocardial functional and cellular injury in a variety of pathological pathways. Endothelial dysfunction distorts the balance between vasorelaxation and vasoconstriction and thus may promote vasoconstriction and contribute to the "no-reflow phenomena" seen after myocardial ischemia and reperfusion. Endothelial dysfunction may also exacerbate myocardial injury indirectly by increasing platelet aggregation and promoting PMN accumulation in ischemia/reperfused myocardial tissue. Although we cannot determine precisely the mechanisms by which SP/W-5186 preserves endothelial function, it is very likely that this effect of SP/W-5186 plays an important part in ultimately protecting against the myocardial dysfunction and cellular injury associated with ischemia and reperfusion.

It is well known that platelet and platelet-derived mediators play a significant role in acute myocardial ischemic injury in the absence of reperfusion (Stamler and Loscalzo, 1991). Recent experiments, however, have revealed that platelets may also contribute significantly to reperfusion injury \textit{via} a platelet-PMN interaction (Nash, 1994). It has been reported that platelet activation significantly facilitates PMN adhesion to endothelial cells and thus increases PMN accumulation in inflammatory tissue (Diacovo et al., 1996). Moreover, the platelet-PMN interaction markedly increases superoxide anion generation by PMNs (Colli et al., 1996). Our present results clearly demonstrated that the administration of SP/W-5186 significantly inhibits platelet aggregation. Therefore, SP/W-5186 might protect myocardial tissue from...
Inhibiting NO synthase thus may reduce peroxynitrite (ONOO\textsuperscript{-}) formation and attenuates reperfusion injury in vivo (Gutierrez et al., 1996; Laskey and Mathews, 1996). Moreover, Engelman et al. (1995) has recently reported that \textalpha-arginine infusion significantly reduces lipid peroxidation and attenuates reperfusion injury in vivo. Our present study has demonstrated that administration of SP/W-5186 significantly reduced myocardial lipid peroxidation associated with ischemia and reperfusion. This result provides direct evidence that in addition to its well-established vasodilatory and antiinflammatory effects, NO may inhibit free radical-induced lipid peroxidation and thus reduces myocardial reperfusion injury.

In summary, our present study demonstrates that the novel NO donor SP/W-5186 exerts marked cardioprotective effects in rabbit ischemia-reperfusion injury. Several lines of experimental evidence indicate that this protective effect may be mediated by a combination of decreased PMN adherence to the coronary endothelium, decreased platelet aggregation, preserved endothelial function and direct antioxidant effects. At higher doses, a vasodilator component may also contribute to the observed cardioprotective effects of this NO donor.

References
Crow JP and Beckman JS (1996) The importance of superoxide in cardioprotective effects through systemic or coronary vasodilatation. However, it is unlikely that this vasodilator effect contributes significantly to the protective effects observed with the low dose of SP/W-5186 because no significant hemodynamic effects were observed at this dose regimen. In contrast, when high-dose SP/W-5186 was administered, a significant vasodilation effect occurred, and the PRI, an index of cardiac oxygen demand, was significantly decreased. This may explain the more significant decrease in plasma CK activity and necrotic size observed in the high-dose SP/W-5186-treated group.

Free radical-induced lipid peroxidation is a major component of reperfusion injury. Recent studies have suggested that NO may simultaneously have both pro- and antioxidant effects depending on the relative concentration of individual reactive species present (Lipton et al., 1993; Crow and Beckman, 1996). At lower relative concentrations, NO may act as a pro-oxidant by reacting with O\textsubscript{2}\textsuperscript{-}, resulting in the formation of a more reactive oxidant, peroxynitrite (ONOO\textsuperscript{-}), and enhancing lipid peroxidation (Yang and Mehta, 1997). Inhibiting NO synthase thus may prevent ONOO\textsuperscript{-} formation and reduce lipid peroxidation. In contrast, at high relative concentrations, NO can act as an antioxidant by reacting with lipid-derived radicals and thus resulting in termination of the lipid peroxidation chain reaction and stop further initiation. By this mechanism, NO has been shown to inhibit O\textsubscript{2}\textsuperscript{-}, ONOO\textsuperscript{-}, H\textsubscript{2}O\textsubscript{2}, \textalpha-0H and lipoxygenase-dependent lipid peroxidation in vitro (Gutierrez et al., 1996; Laskey and Mathews, 1996). Moreover, Engelman et al. (1995) has recently reported that \textalpha-arginine infusion significantly reduces lipid peroxidation and attenuates reperfusion injury in vivo. Our present study has demonstrated that administration of SP/W-5186 significantly reduced myocardial lipid peroxidation associated with ischemia and reperfusion. This result provides direct evidence that in addition to its well-established vasodilatory and antiinflammatory effects, NO may inhibit free radical-induced lipid peroxidation and thus reduces myocardial reperfusion injury.

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