Benificial Effect of SPM-5185, a Cysteine-Containing Nitric Oxide Donor, in Rat Carotid Artery Intimal Injury

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Abstract We studied the effects of an organic nitric oxide (NO) donor SPM-5185 in a rat carotid artery intimal injury model. Seven days after injury, the two end segments were used for histological measurement of the intimal-to-medial (I/M) ratio and scanning electron microscopy of the luminal surface. The NO donor SPM-5185 or its non-NO-donating control compound SPM-5267 were infused intravenously at 30 μg/d. Full vasorelaxant responses of rat carotid arterial rings were obtained with the endothelium-dependent vasodilators acetylcholine (ACh), A23187, and the endothelium-independent vasodilator acidified NaNO2 in sham-operated control rings. Impaired relaxation occurred with 10 μmol/L ACh and 1 μmol/L A23187 in injured rings but not in rings infused with SPM-5185 for 7 days. Relaxation to 100 μmol/L acidified NaNO2 was not significantly different among any of the groups, indicating a normal vascular smooth muscle response after intimal injury. Morphometric analysis of injured carotid arteries given vehicle and SPM-5267 showed marked intimal thickening with an average I/M ratio of 0.78±0.03 and 0.74±0.05, respectively. SPM-5185 markedly attenuated intimal thickening, resulting in an I/M ratio of 0.13±0.03 (P<.01 from vehicle), representing an =82% inhibition of intimal thickening. SPM-5185 infusion resulted in accelerated regeneration of endothelial cells on the intimal surface at 7 days. SPM-5185 also markedly retarded the proliferation of cultured rat vascular smooth muscle cells at 7 days compared with SPM-5267 (P<.01). We conclude that a constant intravenous infusion of a subvasodilator dose of NO donor SPM-5185 significantly accelerates the functional recovery of the regenerating endothelium and also inhibits vascular smooth muscle cell proliferation, which contributes to myointimal thickening. (Circ Res. 1994;75:77-84.)

Key Words • nitric oxide donor • endothelial regeneration • smooth muscle cell proliferation • endothelium-derived relaxing factor • scanning electron microscopy

A ngioplasty has been widely used in the treatment of occlusive vessel disease in large arteries. However, a high rate of restenosis (ie, ≈45% to 50%) occurs several months later. There is still no fully established therapy to prevent or ameliorate this complication.1,2 This is partially due to poor understanding of the underlying mechanisms of the pathophysiological processes responsible for proliferation of intimal tissue and endothelial dysfunction.

Recently, it has been reported that one of the most important mechanisms for postangioplasty restenosis is myointimal proliferation of the vascular wall after removal of the endothelium. Removal of the endothelium by air desiccation or balloon denudation leads to a sequential series of events, including acute platelet deposition3-5 and vasospasm,6,7 followed by smooth muscle cell activation2 and later smooth muscle migration and proliferation.8-10 Recently, nitric oxide (NO) or nitrovasodilators that release NO were observed to stimulate the proliferation of endothelial cells11 and inhibit the proliferation of smooth muscle cells in vitro.12 These findings suggest that the myointimal proliferative response of the vascular wall in response to endothelial injury or denudation may result partially from reduced production or release of NO by the regenerating endothelial cells.13,14 The purposes of the present study were to (1) study the endothelial morphological and functional changes, including both vasodilator-stimulated and basal NO release, after 1 week of intimal injury, (2) investigate the role of a new organic NO donor, SPM-5185, at a dose below the threshold for vasodilation, in inhibiting myointimal thickening and preserving endothelial function in injured rat carotid arteries,15 and (3) determine the effects of SPM-5185 in cultured rat vascular smooth muscle cells as a potential mechanism for protection against neointimal proliferation.

Materials and Methods
Experimental Procedures
The present study was carried out according to the “Guide for the Care and Use of Laboratory Animals” as adopted and promulgated by the National Institutes of Health.

Adult male Sprague-Dawley rats weighing 275 to 300 g were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). After a longitudinal neck incision, a 1.5-cm-long segment of the left carotid artery was carefully isolated by using the first and the eighth tracheal cartilage as the respective proximal and distal borders of the isolated vascular segment. Two 4-0 silk ligatures were tied in a slipknot around the proximal and distal ends of the artery. After the blood inside the vessel segment was aspirated with a 30-gauge needle placed in the proximal end of the artery, a stream of nitrogen gas was allowed to flow through the lumen for 1 minute at a
flow rate of 2 mL/min. This procedure does not cause a visible distortion of the vessel wall but results in deendothelialization with slight injury to the underlying media. Thereafter, the ligatures were removed, and a direct gentle pressure was applied to the puncture site for several minutes. Once hemostasis was achieved, reestablishment of blood flow in the carotid artery was confirmed. Subsequently, minisomatic infusions pumps (model 2ML1, Alza Corp) were implanted subcutaneously in the neck and connected to the external jugular vein on the contralateral side to the injured carotid artery via a subcutaneously tunneled PE-60 catheter. These pumps are designed to deliver 10 μL/h of fluid for up to 7 days. Rats were randomly assigned to one of the following four experimental groups: (1) six sham-injured rats, (2) six injured rats given vehicle (0.9% NaCl at 240 μL/d), (3) eight injured rats given SPM-5185 (30 μg/d), and (4) seven injured rats given SPM-5267 (30 μg/d). SPM-5185 or SPM-5267, diluted in 0.9% NaCl, or saline alone was intravenously infused continuously for 7 days. This infusion rate (ie, 1.25 μg/h) did not significantly alter mean arterial blood pressure over a 3-hour infusion period in sham rats. Mean arterial blood pressure was 139±7 mm Hg initially and 138±9 mm Hg after 3 hours of SPM-5185 infusion (P=NS). All incisions were aseptically closed, and after recovery from the anesthetic, the rats were returned to the animal facility. After 7 days, the rats were reanesthetized, and the injured carotid arterial segments were removed, with the first and the eighth tracheal cartilage used as markers. The isolated arteries were carefully cleaned of connective tissue and cut into three segments. Segments from the proximal and distal ends were used for physiological assessment of endothelial function in carotid arterial rings, and the middle segment was used for histological and scanning electron microscopy studies. At the end of the experiments, the pumps were removed, and the volume of the remaining pump fluid was measured to determine the delivered drug volume. Rats developing a clot or a blocked catheter after 7 days were excluded from the studies. These included one rat given vehicle, one rat given SPM-5267, and two rats given SPM-5185. As an additional control, a solution of SPM-5185 was maintained in a water bath at 37°C for 7 days, after which it was added to isolated rat carotid rings. At 50 μmol/L, the SPM-5185 incubated for 7 days relaxed isolated rat carotid arterial rings 64±5% (n=4) compared with fresh SPM-5185 76±5% (n=4) carotid arterial rings (P=NS). Five additional rats subjected to air injury were infused with recombinant human superoxide dismutase (hSOD) at a rate of 1 mg/d for 7 days. This corresponded to 5 μg/mL hSOD, which was sufficient to inhibit 80% of the superoxide radicals formed by xanthine-xanthine oxidase.

Isolated Carotid Ring Studies

The two end segments of injured carotid arteries were cut into vascular rings 2 to 3 mm in length and placed in 10-mL chambers filled with Krebs-Henseleit (K-H) solution consisting of (mmol/L) NaCl 118, KCl 4.75, CaCl2·2H2O 2.54, KH2PO4 1.19, MgSO4·7H2O 1.19, NaHCO3 12.5, and glucose 10.0. The rings were then mounted on stainless-steel hooks, suspended in 10-mL tissue baths, and subsequently connected to PT-03 force displacement transducers (Grass Instrument Co) to record changes in force with a Grass model 7 oscillographic recorder. The baths were filled with 10 mL K-H buffer and aerated at 37°C with a gas mixture of 95% O2/5% CO2. Carotid arterial rings were stretched to a preload of 0.5 g of force and equilibrated for 60 to 90 minutes. During this period, the K-H solution in the tissue baths was replaced with fresh K-H solution every 15 to 20 minutes. After equilibration, the rings were then exposed to 10 mmol/L ACh (0.1-epoxyethanoprostaglandin H2, Biomol Laboratories), a histamine H1 mimetic that generates =0.3 to 0.4 g of developed force. After a stable contraction was obtained, 0.1, 1, 10, 100, 1000, and 10 000 mmol/L acetylcholine (ACh), an endothelium-dependent receptor-mediated vasodilator, was added to the bath to assess regenerated endothelial function after injury. Once maximal vasodilation occurred, the rings were washed, and developed force was allowed to return to baseline. The procedure was repeated with A23187, an endotheli-um-dependent non–receptor-mediated vasodilator (1, 10, 100, and 1000 nmol/L). These concentrations of ACh and A23187 failed to relax carotid arterial rings mechanically denuded of endothelium. Then, rings were washed and allowed to equilibrate to baseline again. The procedure was then repeated with acidified NaNO2, an endothelium-independent vasodilator (1, 10, and 100 μmol/L) to assess the ability of smooth muscle to relax in response to endothelium-independent vasodilator. To study endothelial basal NO release, after a stable contraction to U46619 was obtained, 1 mmol/L Nω-nitro-L-arginine methyl ester (L-NAME) (Calbiochem) was added, and basal NO release was assessed by measuring L-NAME–induced vasorelaxation. The L-NAME–induced contraction could be reversed by 1 mmol/L L-arginine but not by 1 mmol/L D-arginine, indicating that L-NAME specifically blocked NO synthesis in these vessels.

Histological Analysis

To confirm the degree of reendothelialization of the luminal surface and to evaluate the morphology of the regenerated endothelial cells 7 days after injury, the carotid arterial rings were collected at the end of the physiological studies for assessment of endothelial surface area of the rings. The technique was modified from that reported by Poole et al6 for staining intercellular junctions. Vessel rings from each group were bathed in 5% glucose for 3 minutes and stained with 0.25% silver nitrate, followed by fixation for 10 minutes in 0.9% NaCl and rinsing with 5% glucose several times. Each vessel was opened longitudinally and mounted flat on glass slides for subsequent light microscopy.

To measure the intimal-to-medial (I/M) ratio, the middle segments of injured carotid arteries isolated from five or six rats in each of the four different groups were also collected and fixed in 2.5% glutaraldehyde. After fixation, the vascular segments were dehydrated in graded ethanol and xylene and embedded in paraffin. Four-micron-thick sections were cut, mounted on glass slides, and stained with hematoxylin and 1% alcoholic eosin. The slides were projected at ×400 magnification with a Nikon 591027 light microscope connected to a high-resolution television monitor (FVM-122, Sony), and the intimal and the medial areas were then calculated. The internal elastic lamina was divided by the area of the media between the internal and external elastic lamina to obtain the I/M ratio. The I/M ratio was used as an index of total intimal thickening or intimal smooth muscle cell proliferation. Specimens that were distorted or torn were excluded.

To evaluate the early effects of injury, 12 additional rats were killed immediately after the injury or 24 hours later. Specimens from each injured carotid arterial segment were stained with silver and hematoxylin-eosin for light microscopy by using the same procedure as described above.

Scanning Electron Microscopy

An additional three rats per group were used for scanning electron microscopy. Specimens were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.13 mol/L cacodylate buffer, pH 7.4. After rinsing twice in buffer, specimens were postfix in 1% osmium tetroxide at 4°C for 1.5 hours, rinsed three times in cacodylate buffer, and dehydrated through graded concentrations of ethanol. The specimens were critical-point–dried from 100% ethanol with liquid CO2. After drying, the specimens were bisected to expose the luminal surface, mounted on stubs, and sputter-coated with gold. The specimens were viewed and photographed at 25 kV in a JEOL 35-C scanning electron microscope. Photomicrographs were taken at ×1000 magnification.
**Vascular Smooth Muscle Cell Culture**

**Cell Culture**

Smooth muscle cells were isolated and cultured from the thoracic aorta of rats. The aorta was isolated, the tunica adventitia was removed, and the lumen was denuded of endothelium. The resulting tissue was cut into 2-mm² cubes, placed on gelatin-coated flasks, and allowed to adhere in the presence of media. The cells were maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B. Cells were subcultured, and the purity of the cultures was determined by α-actin immunofluorescence. The purity of the rat aortic smooth muscle cells was >97%. Unless otherwise noted, all reagents were obtained from GIBCO BRL.

**Assay of Cell Proliferation**

Cultures from the third to fifth passages were established on 24-well plates at an initial density of 5 x 10⁴ cells per square centimeter and maintained on DMEM with 10% FBS for 2 days. Quiescence was achieved by culturing the rat aortic smooth muscle cells for 2 days in DMEM with 0.1% FBS. To determine the effect of the NO donor SPM-5185 on rat aortic smooth muscle cell proliferation, the quiescent cells were cultured in DMEM with 5% FBS, DMEM with 5% FBS plus SPM-5185 (20 µg/mL), or DMEM with 5% FBS plus SPM-5267 (20 µg/mL). The cultures receiving either SPM-5185 or SPM-5267 were dosed on day 0 and day 2 to maintain biological activity of the compound, since the ability of SPM-5185 in the culture medium to relax rat aortic rings was significantly decreased by day 4. On day 7, the cells were visually inspected to ensure that there was no cell detachment or cell death and then removed enzymatically and counted with a Coulter counter (Coulter Electronics Inc).

**Statistical Analysis**

All data are expressed as mean±SEM. Differences both within and between groups were examined by ANOVA, with multigroup comparisons where appropriate, followed by a t test with Fisher’s exact probability test. Statistical significance was defined at the P<0.05 level and is indicated in the figures and text.

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**Results**

**Effects of an NO Donor on Vascular Morphological Alterations After Injury**

Fig 1 depicts representative photomicrographs of rat carotid arteries at different times after air injury. Compared with the control sham-injured intimal surface, injury produces an immediate endothelial denudation, exposing the media and resulting in slight medial injury. Also, there are numerous adhered platelets to the injured surface. This pattern of injury remains essentially unchanged at 3, 5, and 7 days in untreated vessels. The only change of note is a decline in adhered platelets at 5 and 7 days. However, treatment with SPM-5185 for 7 days resulted in a significant reendothelialization (Fig 2, which did not occur after treatment with the non-NO-donating compound SPM-5267. The reendothelialization is marked but is not complete as compared with the control uninjured segment. Thus, SPM-5185 appears to promote reendothelialization of the artery at 7 days.

The normal control rat carotid arterial intima consists of only a monolayer of endothelial cells (Fig 3). One day after injury, a marked endothelial denudation occurred, as shown in Figs 1 and 3. At 7 days after injury, a marked intimal proliferation developed. SPM-5267, a non-NO-donating compound did not alter these changes (Fig 3). However, rats treated with SPM-5185 (30 µg/d), a substance with an NO moiety, exhibited significantly attenuated intimal thickening at 7 days (Fig 3).

Morphometric analysis of carotid arteries isolated from injured rats given vehicle or SPM-5267 showed an average I/M ratio of 0.78±0.03, and 0.74±0.05, respectively (Fig 4). These values are significantly elevated above control values (P<.001). However, carotid arteries isolated from injured rats given SPM-5185 (30 µg/d) exhibited a markedly reduced I/M ratio of 0.13±0.03 (P<.01) (Fig 4), which represented an 82% attenuation of intimal thickening. These data show that a significant intimal thickening develops after endothelial denudation, and this intimal thickening was reduced by an NO donor (ie, SPM-5185) but not by its analogue lacking...
the NO moiety (ie, SPM-5267). However, hSOD did not attenuate intimal thickening in an additional five rats.

Effects of an NO Donor on Endothelial Functional Recovery After Injury

Basal release of NO was assessed by the addition of 1 mmol/L L-NAME. The addition of 1 mmol/L L-NAME in vitro, a potent inhibitor of endothelial NO synthase, increased a vasoconstriction of 140±10 mg in normal rat carotid arterial rings (P<.01 from pre-L-NAME values). Furthermore, this vasoconstriction was blunted in injured carotid arteries. These data indicate that there is a significant basal NO release in normal rat carotid arteries that modulates vascular tone. The vasoconstriction induced by 10 mmol/L U46619 was not significantly decreased in injured rats 7 days after injury compared with sham-injured rats (ie, 0.3±0.01 versus 0.3±0.2 mg, respectively, in eight preparations). This indicates that vascular smooth muscle tone is comparable between the different groups. As shown in Fig 5, 7 days after injury, 1 mmol/L L-NAME induced a vasoconstriction of only 41±7 mg in the absence of significant numbers of endothelial cells. However, constant intravenous infusion of an active NO donor, SPM-5185 (30 μg/d), significantly restored the L-NAME-induced vasoconstriction to 91±2 mg (P<.05). However, this was not the case for the inactive nitrate, SPM-5267, which lacks the NO moiety. Infusion of SPM-5267 resulted in an L-NAME-induced vasocontraction of only 43±17 mg 7 days after artery injury (P=NS from vehicle). Since the vasocontractions induced by 10 mmol/L U46619 and 40 mmol/L KCl were not significantly different among the groups 7 days after injury, the reduced L-NAME-induced vasocontraction in the vehicle-treated and SPM-5267-treated groups is not the result of vascular smooth muscle injury. Therefore, it is very likely that it is the NO moiety in the SPM-5185 that preserves basal NO release after intimal injury. This is consistent with the effects of SPM-5185 observed via scanning electron microscopy.

NO release can be augmented by stimulation with endothelium-dependent vasodilators, including ACh. Intimal injury impaired the carotid artery rings from relaxing fully to ACh but not to NaNO2, indicating a marked endothelial dysfunction without impairment of vascular smooth muscle responsiveness. Fig 6 summarizes the vasorelaxant responses of isolated carotid arterial rings to ACh. Seven days after injury, carotid arterial rings isolated from sham-injured rats relaxed 90±2% to ACh. In contrast, carotid arterial rings isolated from injured rats given either vehicle or SPM-5267 relaxed only 17±5% and 15±5%, respectively. These data indicate a significant reduction of endothelium-dependent receptor-mediated vasorelaxation in carotid arteries observed from injured rats given either 0.9% NaCl or SPM-5267. However, those carotid arterial rings isolated from injured rats given SPM-5185 showed significantly improved vasorelaxation responses to ACh (67±6%, P<.01 compared with vehicle-treated and SPM-5267-treated groups). Carotid rings isolated from injured rats infused with hSOD over 7 days did not significantly relax to ACh (22±6%, P=NS from vehicle).

Fig 7 summarizes the vasorelaxant responses of isolated carotid arterial rings to the endothelium-dependent non-receptor-mediated vasodilator A23187. Seven days after injury, carotid arterial rings isolated from sham-injured rats relaxed 80±3% to 1 μmol/L A23187. In contrast, carotid arterial rings isolated from injured rats given either vehicle or SPM-5267 relaxed only minimally to 1 μmol/L A23187. These data indicate a significant reduction of endothelium-dependent non-receptor-mediated vasorelaxation in the carotid arteries of injured rats given vehicle or SPM-5267. However, carotid arterial rings isolated from injured rats given SPM-5185 showed significantly improved vasorelaxation responses to 1 μmol/L A23187 (P<.01) compared with vehicle-treated and SPM-5267-treated groups. Therefore, 7 days after injury, there was a significant and selective attenuation of the vasorelaxant response to both endothelium-dependent vasodilators in the carotid arteries of injured rats not given an NO donor. This decreased vasorelaxation to endothelium-dependent vasodilators was not due to a muscarinic receptor defect, since a reduced vasorelaxant response was also obtained to A23187. Carotid rings isolated from injured rats infused with hSOD did not relax to A23187 significantly above vehicle-treated rings (24±7%, P=NS from vehicle).

Fig 8 summarizes the vasorelaxation responses to the endothelium-independent vasodilator acidified NaNO2.
in carotid arterial rings isolated from rats in each experimental group. No impairment in vasorelaxation occurred in any of the groups. These findings indicate that the smooth muscle cells of carotid arterial rings isolated from each group of rats respond normally to direct vasodilators regardless of the status of the endothelium. Therefore, the decreased vasorelaxation to endothelium-dependent vasodilators is not due to refactoriness of smooth muscle to these vasodilators nor to vascular smooth muscle injury. Carotid rings isolated from injured rats infused with hSOD responded comparably to vehicle-treated rings (81±5%, P=NS from vehicle).

Analyses of the effects of SPM-5185 on cultured rat vascular smooth muscle cells indicated a marked and significant antiproliferative effect of this NO donor. Fig 9 summarizes these effects after 7 days of treatment with either SPM-5185 or SPM-5267 at 20 μg/mL. Clearly, SPM-5185 attenuated vascular smooth muscle proliferation by 60% (P<.01 from control untreated cultures), whereas SPM-5267 did not significantly attenuate vascular smooth muscle cell proliferation. Thus, it is the NO that is responsible for the inhibition of vascular smooth muscle cell proliferation. Therefore, a
major consequence of the NO-induced protective effect in carotid arterial injury appears to be due to inhibition of vascular smooth muscle cell proliferation.

**Discussion**

Our present study demonstrates that a brief period of desiccation of a hemodynamically isolated segment of the rat common carotid artery results in virtually a total denudation of the endothelium but with only limited damage to the underlying subendothelial structures and the media (Figs 1 and 3). In accordance with these results, Fishman et al. and Dryski et al. have shown that 12 to 24 hours after injury, the endothelially denuded injured area is covered with a carpet of platelets. Starting at 2 days, the platelet carpet is progressively replaced from both ends by a new sheet of endothelium, which occurs by ingrowth from both ends of the denuded segment. This is consistent with our scanning electron microscopy results. Approximately 4 days after injury, smooth muscle cells began to migrate and proliferate under the layer of platelets and regenerating endothelium. By 7 days, a thickened myointimal region has developed in the central portion of the denuded segment. Obviously, brief injury of an isolated vascular segment elicits a complex sequence of events including platelet deposition and subsequent intimal proliferation of smooth muscle cells that are thought to contribute to the development of postangioplasty restenosis. Therefore, this experimental model may be useful in understanding the pathophysiological processes and the mechanisms of postangioplasty restenosis.

The data obtained in the present study demonstrate a significant protective effect of infusion of an exogenous NO donor in a 7-day rat model of intimal injury to the carotid artery. The dose of SPM-5185 used in the present study (ie, 1.25 μg/h) did not alter arterial blood pressure when infused over a 3-hour period in rats. Thus, SPM-5185 protected carotid arteries at subvasodilator doses. These results are consistent with a cytoprotective effect of this same NO donor in myocardial ischemia/reperfusion. Infusion of either normal saline or of an organic nitrate having the same molec-
ular backbone without the NO group failed to protect against intimal injury and failed to stimulate NO release, as indicated by (1) a comparable degree of intimal thickening, (2) comparable decreases in L-NAME-induced vasoconstriction, and (3) comparable decreases in endothelium-dependent vasorelaxation. In contrast, SPM-5185, an active NO donor, attenuated intimal thickening by ≈82% of that observed in the SPM-5267- or 0.9% NaCl-treated groups. Moreover, SPM-5185 significantly increased basal NO release compared with vehicle or inactive NO donor treatment and restored responsiveness to endothelium-dependent vasodilators (ie, ACh and A23187).

There are several potential mechanisms for these protective effects of the NO donor in our model of vascular injury and neointimal formation. First, the NO may act as an antithrombotic agent, limiting platelet adhesion to the endothelium-denuded vessel wall (ie, subendothelium) and preventing platelet aggregation (ie, microthrombi) and the subsequent release of vasoconstrictor agents produced by the aggregating platelets. These platelet-derived vasoconstrictors (eg, serotonin and thromboxane A2) could result in vasospasm of the vessel,21 potentiating its injury. In addition, platelets can release growth factors, including platelet-derived growth factor, transforming growth factor-β, and epidermal growth factor,22,23 which may promote the proliferation of vascular smooth muscle cells, leading to neointimal proliferation. Since this model of vascular injury has been shown to result in a monolayer of platelets on the intimal surface5,9 and since NO is known to inhibit platelet aggregation and adhesion,24-26 SPM-5185 may partially protect in this model of arterial injury by an antiplatelet action. However, this is probably not the major protective mechanism of this NO donor.

Second, NO may act to stimulate the regeneration of endothelial cells from the endothelium-denuded intima of the injured carotid arteries. The capacity of endothelial cells to regenerate is quite limited soon after denudation, but these endothelial cells can slowly cover the denuded area.27 Our data from scanning electron microscopy of injured rat carotid arteries are consistent with this possibility as one important mechanism of protection of SPM-5185. Recently, data have been obtained indicating that nitrovasodilators releasing NO promote DNA synthesis and stimulate proliferation of endothelial cells in culture.13 Thus, SPM-5185 stimulates endothelial regeneration in our carotid injury model, accelerating the reendothelialization of the carotid artery. Along these lines, SPM-5185 may also improve the ability of existing endothelial cells to produce and release NO.

Third, NO may directly suppress the proliferation of vascular smooth muscle cells and thus attenuate neointimal formation (ie, reduce the I/M ratio). In this regard, nitrovasodilators have been shown to inhibit proliferation of smooth muscle cells in vitro.12 Moreover, NO has been implicated as the agent inhibiting neointimal formation after the administration of angiotensin-converting enzyme inhibitors.28 Additionally, L-arginine, the precursor of NO, also inhibits intimal hyperplasia after balloon injury.29,30 These effects of L-arginine can be blocked by the NO synthase inhibitor L-NAME, further implicating NO in this process.29,30 In the present data, we have provided direct evidence that SPM-5185 markedly attenuates the rate of proliferation of cultured rat vascular smooth muscle cells. Thus, an important component of the protection afforded by SPM-5185 in carotid arterial injury appears to be by inhibition of vascular smooth muscle proliferation.

Fourth, NO may inhibit leukocytes, which may be involved in intimal hyperplasia and the resulting neointimal formation. Chemotaxis and recruitment of leukocytes have been postulated to be critical events in postangioplasty restenosis.31 In this scenario, leukocytes migrate to the site of injury, adhere to the subendothelium, and stimulate the migration of vascular smooth cells from the media to the intima of the arterial wall. Activation of leukocytes results in the release of key mediators of the inflammatory response including platelet-activating factor, interleukin-1β, superoxide radicals, and proteases (eg, elastase). Moreover, NO gas and NO donors have been shown to significantly retard polymorphonuclear leukocyte adherence to the vascular endothelium and to inhibit neutrophil activation.19,20,32 Thus, SPM-5185 may have protected this model of vascular injury by an antineutrophil action. We have no direct information on this mechanism of action from our present study, and we saw very few adherent leukocytes on the injured intimal surface.

Fifth, NO donors like SPM-5185 could protect against injury by virtue of the fact that NO quenches superoxide radicals,33 and SPM-5185 could reduce the effective local concentration of superoxide radicals. We tested this possibility by using recombinant hSOD, which works well in rats34 but did not protect in this model of vascular injury. However, it is likely that the hSOD could not penetrate the vascular wall, where the generation of superoxide radicals would most likely occur. Thus, we cannot eliminate the superoxide radical-scavenging activity of SPM-5185 as a primary mechanism of protection in this model of vascular injury. It is possible that quenching of oxygen-derived free radicals may contribute to the overall cytoprotective effect of NO in this model of intimal injury.

In summary, we have demonstrated a significant effect of accelerating recovery from intimal injury after a constant intravenous infusion of an NO donor, SPM-5185, in a rat carotid arterial injury model. The intimal thickening was reduced by 82% compared with treatment with an inactive NO donor. Basal NO release and vasodilator-stimulated NO release were significantly increased by the regenerated endothelial cells, as evidenced by a significant increase in the ability to respond to the NO synthase inhibitor L-NAME and to endothelium-dependent vasodilators (eg, ACh and A23187). The data in the present study suggest that NO may be another intervention for preventing or ameliorating the vasospasm and restenosis occurring after angioplasty by attenuating vascular smooth muscle growth and promoting the regeneration of endothelial cells. These findings are consistent with those of Garg and Hassid,12 who showed that S-nitroso-N-acetylpenicillamine, another NO donor, inhibited cultured rat vascular smooth muscle cell proliferation. Thus, endogenous NO may play an important role in preventing or attenuating neointimal formation, as suggested by Scott-Burden et al.35
Acknowledgments

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