Effect of Nitric Oxide Donors on Neointima Formation and Vascular Reactivity in the Collared Carotid Artery of Rabbits

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Summary: Intimal thickening in arteries is considered a site of predilection for atherosclerosis. We investigated whether oral application of the nitric oxide (NO) donors SPM-5185 [N-nitratopivaloyl-S-(N'-acetylalanyl)-cysteine ethylester, 10 mg/kg body weight twice daily (b.i.d.)] and molsidomine (10 mg/kg body weight/day) can retard neointima formation and changes in vascular reactivity induced by a nonocclusive, soft silicone collar positioned around the left carotid artery of rabbits. The contralateral carotid artery was sham operated and served as a control. Drug and placebo (diet without drug) treatments were initiated 7 days before placement of the collar. At the end of the experiments, two segments were cut from each collared and sham-treated artery, one for measurement of the cross-sectional area of intima and media and the other for isometric tension recording. Sham treatment did not result in intimal thickening in either group. In contrast, the intima/media (I/M) ratio was considerably increased after 14 days of collar treatment as a result of neointima formation. Intimal thickening was significantly inhibited by SPM-5185 (I/M ratio 0.05 ± 0.01 vs. 0.11 ±

0.02, p < 0.05), but not by molsidomine (0.06 ± 0.02 vs. 0.08 ± 0.02, p = 0.49), which is a donor of both NO and superoxide anions. Neither collar nor NO donor treatment altered the area of the media. SPM-5185 did not alter the percentage of replicating smooth muscle cells (SMC) in the media after collar treatment, as demonstrated by their immunoreactivity for proliferating cell nuclear antigen (PCNA). Neointima formation was associated with a decreased sensitivity to acetylcholine (ACh), an increased sensitivity to 5-hydroxytryptamine (5-HT), and a decreased maximum force development to 5-HT and KCl. Despite the significant reduction of intimal thickening, SPM-5185 did not antagonize these collar-induced modifications in vascular reactivity, although a tendency toward normalization of the pD_2 value of ACh in collared arteries was observed. Moreover, SPM-5185 did not lead to cross-tolerance towards nitroglycerin (NTG). Development of a neointima can be inhibited by the NO-donor SPM-5185. **Key Words:** Atherosclerosis—Intima—Endothelium—Nitric oxide—5-Hydroxytryptamine—SPM-5185—Molsidomine.

An early and essential step in the development of atherosclerosis is thickening of the intima. At birth, the arterial wall of human arteries is covered with endothelial cells (EC), which rest directly on the internal elastic membrane. This elastic lamina demarcates the EC from the smooth muscle cells (SMC) of the media. Early in life, however, SMC appear between the EC and the internal elastic membrane of large arteries. In contrast to their normal circular orientation in the media, these cells lie in a longitudinal direction, with collagen and elastic

fibers between them. In this way, a thickening of the intima is formed. This so-called neointima is a site of predilection for atherosclerosis (1,2).

In an attempt to develop a suitable animal model that mimics these vascular changes, several methods have been applied to produce neointima formation. They can be divided into two categories: those that use intraluminal and those that use perivascular manipulation. An example of the former is denudation of the EC with a balloon catheter; an example of the latter is the positioning of a nonocclusive,

soft and flexible silicone collar around the rabbit carotid artery, resulting in the generation of a neointima in 1 week (3,4). The perivascular collar placement has the particular advantage of avoiding direct injury to the endothelium (5).

It has been reported (6–8) that chemically dissimilar nitric oxide (NO)-generating (9) drugs concentration-dependently inhibit DNA synthesis and proliferation of rat and rabbit aortic SMC in culture and that their antimitogenic effect was mimicked by 8-bromo-cyclic GMP. These effects were independent of a cellular damage. Therefore, NO was suggested as a modulator of vascular SMC mitogenesis and proliferation, acting through a cyclic GMP-mediated mechanism. In the present study, we used both molsidomine, which releases NO spontaneously (10), and a novel type of NO donor, SPM-5185 (N-nitratopivaloyl)-S-(N'-acetylalanyl)-cysteine ethylester). The advantage of the latter agent is that it contains both a nitrate ester and a thiol, in casu cysteine, in a single molecule (11). In view of these observations, our objectives in the present in vivo study were to determine whether the collar-induced neointima formation can be prevented or retarded by treatment with an NO donor and whether exogenous NO supplementation can overcome the modifications in vascular reactivity associated with neointima formation.

METHODS

Treatment

Experiment 1. After 7 days of quarantine, 24 male New Zealand white rabbits weighing 2.5–3.5 kg were randomly divided into two groups. The treatment group received SPM-5185 at a dose of 10 mg/kg body weight twice daily (b.i.d.) with the drinking water. The drug was freshly dissolved in 150 ml drinking water and was given to the rabbits in the morning and in the evening. A placebo group received the same volume of drinking water without the drug. Previous experiments showed that this volume (300 ml) supplied the daily need of drinking water of our rabbits. Throughout the experiment, rabbits were housed in individual cages and had ad libitum access to food.

Experiment 2. After 7 days of quarantine, 16 male New Zealand white rabbits weighing 2.5–3.5 kg were divided into two groups, which received diets (150 g/day) containing molsidomine 0 or 200 mg/kg chow. This resulted in doses of molsidomine of 0 and 10 mg/kg body weight/day, respectively. Throughout the experiment, rabbits were housed in individual cages and had ad libitum access to water.

The doses of molsidomine and SPM-5185 were selected because they were equivalent on a gram per kilogram of body weight base. Rabbits require ~100 times more molsidomine than humans to induce a decrease in blood pressure (BP) (J. Ostrowski, unpublished observation). Therefore, the 10 mg/kg/day was equivalent to the dose used in the clinic. The half-life (t½) of SPM-5185 in rabbits is not known. The dose of SPM-5185 was chosen in analogy with that of molsidomine.

Induction of neointima

After 7 days of treatment with or without the NO donor, the rabbits were anesthetized with sodium pentobarbital 30 mg/kg intravenously (i.v.). Subsequently, the left carotid artery was freed and surrounded by a nonocclusive, flexible silicone collar 2.2 cm long (3,12–14). The right carotid artery was sham operated, i.e., separated from the surrounding connective tissue and the vagus nerve, receiving a stretch similar to that used for the contralateral collared artery. The carotid arteries were then returned to their original position, and the wounds were sutured. After recovery from anesthesia, the animals received their respective treatment for 1 (experiment 2) or 2 (experiment 1) weeks.

Morphometry

The rabbits were killed with sodium pentobarbital, and two segments (3 mm) were cut from both the collared and sham-operated artery, one for morphometry and the other one for isometric tension recording (described herein). The former was immediately placed in methacarn fixative (methanol:1,1,1-trichloroethane:glacial acetic acid, 60:30:10 by volume) for 24 h, and dehydrated in a graded series of isopropyl alcohol (60–100%) followed by toluol before being embedded in paraffin. Transverse sections were cut and stained with sirius red hematoxylin. The cross-sectional area of lumen, neointima, and media was measured with the Osteomeasure package (Osteo Metrics, Atlanta, GA, U.S.A.) by an independent observer who was unaware of the treatment. Furthermore, the intima/media (I/M) ratio of the artery was calculated.

For the immunohistochemical staining of proliferating cell nuclear antigen (PCNA) a PC10 cyclin monoclonal antibody (MoAb; Dako, Glostrup, Denmark) was used at a 1:10 dilution. The MoAb was diluted in phosphate-buffered saline (PBS). After three washes with PBS, the sections were incubated with rabbit anti-mouse peroxidase for 45 min. For the demonstration of the complex, 3-amino-9-ethylcarbazole was used as a chromogen. For negative controls, the primary antibody was omitted (4). The PCNA-immunoreactive SMC nuclei in the media and the total number of SMC nuclei in the media were counted using a projection microscope. The PCNA activity was expressed as the percentage of PCNA-positive SMC nuclei per total number of medial SMC nuclei (4).

Vascular reactivity

The ring segments were immediately placed in physiological salt solution (PSS) and, after being cleaned of loose connective tissue, were mounted in organ chambers filled with 25 ml PSS maintained at 37°C, and continuously gassed with 95% O_2/5% CO_2 (12–14). Tension was measured isometrically with a Statham UC_2 force transducer. After 15-min equilibration, the preparations were gradually stretched up to a force of 7 g to bring the segments to the optimal point of their length–tension relationship. The rings were then allowed to equilibrate for 45 min more at their optimal length. During this period, the bath solution was changed every 15 min. Indomethacin (3 × 10^{-6} M) was added to block prostaglandin biosynthesis.

In the first experiment, the rings were exposed to a cumulative (0.5 log unit) concentration range of 5-hydroxytryptamine (5-HT, 3 × 10^{-10}–3 × 10^{-6} M). After

reaching maximum contraction, the rings were consecutively exposed to $3 \times 10^{-9}\,M$, $3 \times 10^{-8}\,M$, and $3 \times 10^{-7}\,M$ nitroglycerin (NTG). After washing, the rings were again contracted with phenylephrine (PE $3.5 \times 10^{-7}\,M$) and cumulative (0.5 log unit) concentration–relaxation curves were performed for acetylcholine (ACh 3×10^{-9}–$10^{-6}\,M$) (12). After final washing, the maximum contractile force was determined at the end of the experiment by exposure of the tissues to 120 mM KCl (15). In the second experiment, the rings were contracted with PE $3.5 \times 10^{-7}\,M$. Cumulative concentration–response curves were constructed for ACh (10^{-9} to $4 \times 10^{-6}\,M$) and SIN-1 (10^{-9} to $1.6 \times 10^{-5}\,M$), the NO-releasing metabolite of molsidomine. The bath medium was exchanged three times between the addition of agonists, and tissues were allowed to equilibrate for 30 min.

BP measurements

Acute drug treatment (intravenous). Rabbits were anesthetized, and the BP was measured. Thereafter, the animals received either an intravenous bolus injection of 1 mg SPM-5185/kg body weight followed by an intravenous infusion of 2 mg SPM-5185/kg body weight/h or were treated with saline. The BP was measured after 30-min treatment.

Long-term drug treatment (oral). The BP of the molsidomine-treated (10 mg/kg body weight/day orally) and the respective control rabbits was measured in the conscious state after 7 day treatment, 3 h after the last administration. In a separate experiment, we measured the BP of 6 conscious rabbits before and after oral treatment with SPM-5185 (10 mg/kg body weight, b.i.d.) for 7 days, 18 h after the last administration.

In all cases, the BP was measured with a Statham P2310 pressure transducer connected to an HP 88058 carrier amplifier. For this purpose, the skin of the ear of the rabbits was disinfected and sprayed with lidocaine (10% solution, Astra Nobelpharma, Brussels, Belgium) and a 22-gauge cannula was inserted in the central ear artery, using translumination.

Materials

The normal rabbit chow (Altromin 2023), and the molsidomine-containing chow (200 mg/kg) were prepared by and obtained from Altromin (Lage, Germany). The PSS contained (in mM): NaCl 118, KCl 4.7, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 25, and glucose 11.1. ACh chloride and 5-HT were obtained from Sigma (St. Louis, MO, U.S.A.). NTG was purchased as commercial aqueous solution from Merck (Darmstadt, Germany), indomethacin sodium tetrahydrate from Merck, Sharp & Dohme (München, Germany), and sodium pentobarbital from Psyphac (Brussels, Belgium). SPM-5185 was obtained from Schwarz Pharma (Monheim, Germany), and SIN-1 and molsidomine were obtained from Therabel Research (Brussels, Belgium). Silicone (Silastic E, Dow Corning) was provided by the Compagnie Commerciale de Matière Premières (Antwerp, Belgium). 5-HT was dissolved in an aqueous solution of ascorbic acid (0.01%) and diluted in distilled water. The other drugs were made up in distilled water.

Data analysis

All data are mean ± SEM. The number of arteries reported (n) equals the number of rabbits used. For the statistical analysis, the SPSS/PC$^+$ package (SPSS, Chicago, IL, U.S.A.) was applied. A 5% level of significance was selected. To compare the I/M ratio, areas of intima and media, and the percentage of PCNA-positive SMC nuclei of the media between placebo and NO donor groups, we used the Mann-Whitney U test. The effect of the collar treatment (i.e., sham vs. collar) was evaluated in each group by the Wilcoxon matched-pairs signed-ranks test. Effects on BP were evaluated by Student's t test.

Relaxations were expressed as percentage of the initial contraction. The negative logarithm of the molar concentration of agonist that produced a half-maximal contraction for that agonist (-log EC$_{50}$ or pD$_2$) was determined for each segment by linear regression. These data were evaluated statistically by a factorial analysis of variance (ANOVA) with drug treatment (two levels, NO donor or placebo) as between-rabbit factor and collar (two levels, present or not) as within-rabbit factor.

RESULTS

Morphometric analysis of neointima formation

Placebo groups. The area of the intima and the I/M ratio were significantly increased after the positioning of the silicone collar as compared with the sham operation. In contrast, the area of the media had not changed after collar application (Figs. 1 and 2).

Treatment groups. A neointima had developed after collar placement in all treatment groups. However, both the intimal area and the I/M ratio were significantly lower in the SPM-5185 group (Fig. 1). Molsidomine tended to decrease cross-sectional area of intima and the I/M ratio, but the effect was not statistically significant (Fig. 2). Neither the collar nor the SPM-5185 or molsidomine treatments significantly altered the area of the media (Figs. 1 and 2).

PCNA activity in the media

In the sham-treated carotid arteries, the percentage of replicating medial SMC amounted to 0.18 ± 0.07% in the placebo group, and was increased to 0.54 ± 0.16% in the SPM-5185 group. In the collared arteries, a higher percentage (1.53 ± 0.68%) of the medial SMC was PCNA-positive in the placebo group. The tendency to reduction (1.14 ± 0.40%) in the SPM-5185 group was not statistically significant.

Vascular reactivity

5-HT. 5-HT (3×10^{-10}–$3 \times 10^{-6}\,M$) induced concentration-dependent contractions in sham and collar-treated segments from all groups. The maximum contractile response (E_{max}) to 5-HT in collar-treated rings was always slightly but significantly diminished as compared with that in sham-treated rings. The treatment with SPM-5185 (Table 1), or molsidomine (data not shown) did not affect these maximum contractions in either sham or collared rings.
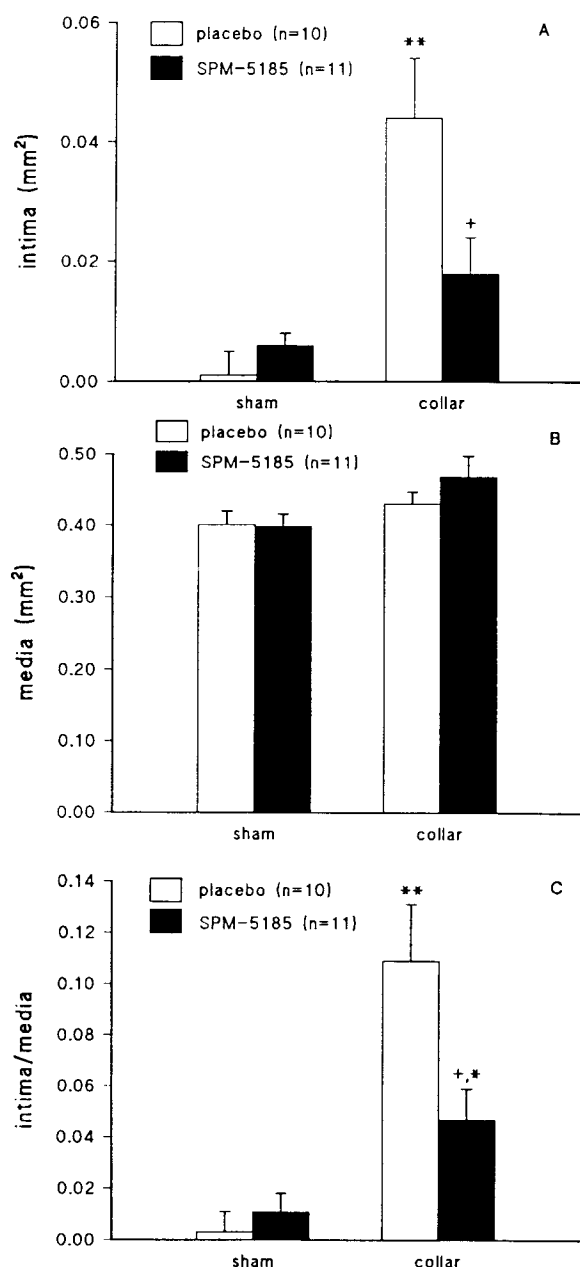
FIG. 1. Effect of the nitric oxide (NO) donor SPM-5185 on neointimal formation in the rabbit carotid artery induced by 14-day collar treatment. SPM-5185 was administered twice daily for 21 days in the drinking water at a dose of 10 mg/kg body weight, beginning 7 days before collar placement. A: Cross-sectional areas of the intima (segments measured in triplicate; distance between sections 250 μm). B: Cross-sectional areas of the media (segments measured in triplicate). C: Intima/media ratio (calculated from measurement in triplicate). Data are mean ± SEM: *p < 0.05, **p < 0.01 sham versus collar (Wilcoxon matched-pairs signed-ranks test), and †p < 0.05 placebo versus SPM-5185 (Mann-Whitney U test).

On the other hand, the presence of the collar led to an increased sensitivity of the artery to 5-HT both in placebo- and SPM-5185-treated groups, as concluded from the increased pD_2 values (from 7.16 ± 0.01 to 7.64 ± 0.12 in the placebo group and from

7.15 ± 0.03 to 7.61 ± 0.12 in the SPM-5185 group, p < 0.001). The treatments with SPM-5185 or molsidomine (data not shown) did not affect the pD_2 values in either sham or collar-treated rings.

KCl. KCl (120 m*M*) contracted sham and collared rings from both treatment groups, but the response was significantly diminished in collar-treated rings.
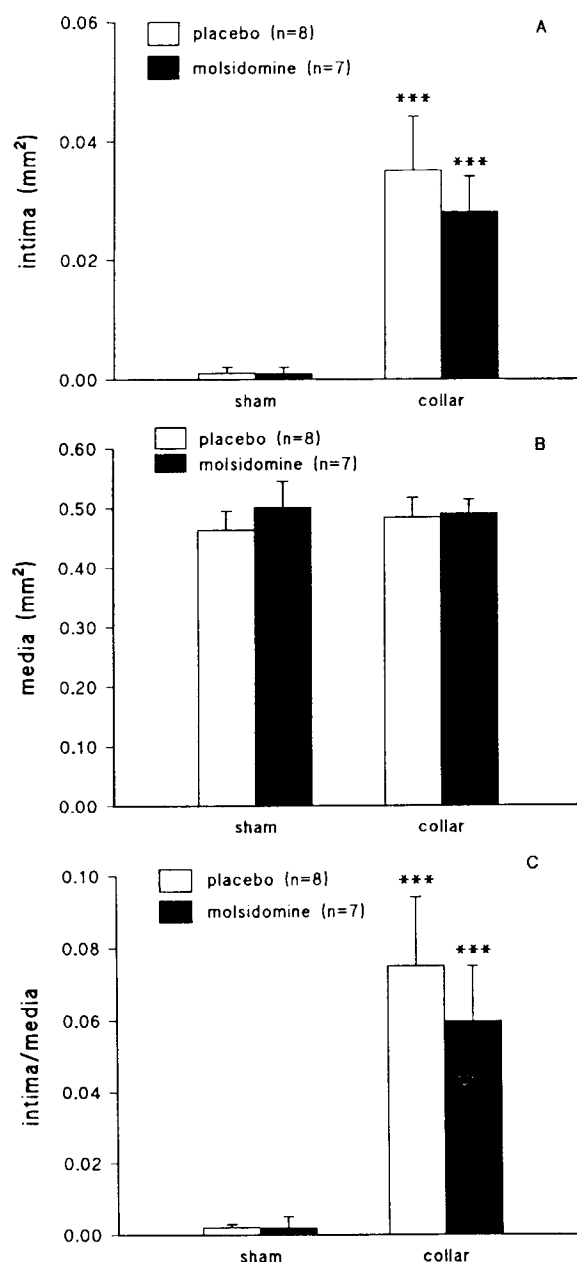
FIG. 2. Effect of molsidomine on neointimal formation in the rabbit carotid artery induced by 7-day collar treatment. Molsidomine was administered for 14 days in the diet at a dose of 10 mg/kg body weight/day, beginning 7 days before collar placement. A: Cross-sectional areas of the intima (segments measured in quintuplicate). B: Cross-sectional areas of the media (segments measured in quintuplicate). C: Intima/media ratio (calculated from measurement in quintuplicate). Data are mean ± SEM: ***p < 0.001 sham versus collar (Wilcoxon matched-pairs signed-ranks test).

TABLE 1. *5-HT-, KCl-, and PE-induced contraction (g) of rabbit carotid arteries without and with a silicone collar positioned for 14 days in placebo- and SPM-5185–treated rabbits*

Condition	5-HT $3 \times 10^{-6}M$		KCl 120 mM		PE $3.5 \times 10^{-7}M$	
	Sham	Collar	Sham	Collar	Sham	Collar
Placebo (n = 8)	9.1 ± 0.5	7.3 ± 0.8^a	11.0 ± 0.9	5.7 ± 0.9^b	9.9 ± 0.5	7.6 ± 0.9^a
SPM-5185 (n = 7)	9.5 ± 0.6	8.1 ± 0.6^a	11.1 ± 1.0	6.1 ± 0.8^b	10.1 ± 0.8	7.9 ± 0.6^a

5-HT, 5-hydroxytryptamine; PE, phenylephrine.
Data are mean $\pm$ SEM.
^{a} $p < 0.01$ versus sham; ^{b} $p < 0.05$ versus sham.

SPM-5185 did not affect force development to KCl in either sham or collar-treated rings (Table 1).

Initial contraction to PE

The contractile response to PE ($3.5 \times 10^{-7}M$) in collar-treated rings was significantly diminished as compared with that of sham-treated rings (Table 1). The treatment with SPM-5185 or molsidomine (results not shown) did not affect the contraction to PE in either sham or collar-treated rings.

ACh. ACh (3×10^{-9}–10^{-6} M) induced concentration-dependent relaxations of sham and collar-treated segments of the rabbit carotid artery after contraction with phenylephrine ($3.5 \times 10^{-7}M$). The maximum extent of relaxation was decreased in the collared segments of both treatment groups (from 76 ± 4 to $60 \pm 4\%$ in the placebo group and from 76 ± 5 to $70 \pm 8\%$ in the SPM-5185 group). SPM-5185 and molsidomine (data not shown) did not alter the maximum relaxation in response to ACh in sham or collared arteries.

Furthermore, the presence of the collar led to a decreased sensitivity of the arterial segment to ACh both in placebo and SPM-5185– or molsidomine-treated groups, as concluded from the decrease in pD_2 values. SPM-5185, but not molsidomine, tended to normalize pD_2 values in collared rings (Table 2).

NTG and SIN-1. NTG (3×10^{-9}, 3×10^{-8}, and 3×10^{-7} M) induced concentration-dependent relaxations in sham and collar-treated segments precontracted with 5-HT 3×10^{-6} M. The percentage of relaxation to increasing concentrations of NTG was significantly reduced in collar-treated rings as

TABLE 2. *pD_2 values of ACh-induced relaxation of PE ($3.5 \times 10^{-7}M$)-contracted rabbit carotid arteries without and with a silicone collar in placebo- or NO-donor–treated rabbits*

Condition	Sham	Collar
Placebo (n = 8)	7.07 ± 0.04	6.64 ± 0.18^a
SPM-5185 (n = 7)	7.17 ± 0.07	6.97 ± 0.10^a
Placebo (n = 6)	7.19 ± 0.06	6.76 ± 0.12^b
Molsidomine (n = 7)	7.16 ± 0.10	6.71 ± 0.10^b

ACh, acetylcholine; PE, phenylephrine.
Data are mean $\pm$ SEM.
^{a} $p < 0.01$ and ^{b} $p < 0.001$ versus sham.

compared with sham rings (Table 3). The treatment with SPM-5185 did not influence the extent of these relaxations in either sham or collared rings (Table 3).

SIN-1 evoked complete relaxation of the sham-operated segments contracted with PE. However, in collared segments, the maximum percentage of relaxation was slightly diminished (from 98 ± 1 to $95 \pm 3\%$ in the placebo group and from 97 ± 2 to $90 \pm 4\%$ in the molsidomine group, $p = 0.026$). The pD_2 was not significantly altered by either the collar or the molsidomine treatment (6.46 ± 0.09 in sham and 6.35 ± 0.11 in collared vessels of the placebo group and 6.19 ± 0.12 in sham and 6.26 ± 0.21 in collared segments of the molsidomine group).

BP

When SPM-5185 was administered acutely to rabbits (1 mg/kg i.v. bolus injection + 2 mg/kg/h i.v. infusion) mean arterial BP (MAP) decreased from 100 ± 8 to 82 ± 7 mm Hg after 30 min, whereas BP did not change in saline-treated rabbits. Similar results were obtained with molsidomine (data not shown).

SPM-5185 administered orally to rabbits, induced a paradoxical increase in MAP after 1-week treatment (80 ± 1 mm Hg before and 90 ± 2 mm Hg after SPM-5185, $p < 0.01$). Molsidomine did not affect MAP significantly (97 ± 4 mm Hg in the placebo group vs. 90 ± 3 mm Hg in the molsidomine-treated group).

DISCUSSION

Classic organic nitrates have been reported to promote as well as to inhibit cell proliferation (6,16). Therefore, we wished to investigate the effect of two structurally unrelated NO donor compounds, molsidomine and SPM-5185, on the development of a neointima in vivo. SPM-5185 is a novel, nitrate-based NO donor that liberates NO indirectly after cleavage of its thioester function and reduction of the nitrate group. Depletion of reduced thiols has been implicated in the development of tolerance to classic organic nitrates such as NTG and isosorbide dinitrate (17). Because SPM-5185 contains a cysteine moiety in the same molecule, thiol depletion and development of nitrate tolerance are circum-

TABLE 3. *NTG-induced relaxation (%) of 5-HT (3 × 10^{-6}M)-contracted rabbit carotid arteries without and with a silicone collar positioned for 14 days in placebo- and SPM-5185–treated rabbits*

Condition	NTG $3 \times 10^{-9}M$		NTG $3 \times 10^{-8}M$		NTG $3 \times 10^{-7}M$	
	Sham	Collar	Sham	Collar	Sham	Collar
Placebo (n = 8)	4 ± 1	3 ± 1	18 ± 2	15 ± 4[a]	57 ± 2	50 ± 7[a]
SPM-5185 (n = 7)	4 ± 1	2 ± 1	19 ± 4	10 ± 3[a]	56 ± 6	50 ± 7[a]

NTG, nitroglycerine; 5-HT, 5-hydroxytryptamine.
Data are mean ± SEM.
[a] $p < 0.05$ versus sham.

vented with this NO donor. SIN-1, the active metabolite of molsidomine, belongs to the class of sydnonimines, compounds that liberate NO spontaneously, i.e., independent of the presence of thiol groups. However, with each molecule of NO released, one molecule of oxygen is reduced to superoxide anion (10). Therefore, SIN-1 is not only a donor of NO, but of superoxide as well. Both types of NO donors were tested in a rabbit model in which intimal thickening is induced by the positioning of a silicone collar around the carotid artery.

The positioning of the collar led to neointima formation, as previously described (3,4,13). SPM-5185 profoundly decreased intimal thickening, as characterized by a significant reduction in both cross-sectional area of intima and I/M ratio. This result is consistent with the observation that chronic inhibition of NO production accelerates neointima formation in hypercholesterolemic rabbits (18). Therefore, the effect of SPM-5185 is likely due to its NO-gener-ating capacity.

To evaluate whether mere hemodynamic effects on the vascular wall may have influenced intimal thickening (19), we measured systemic arterial BP in rabbits from both placebo and treatment groups. The main conclusion that can be drawn from these BP measurements is that the inhibition of intimal thickening is not due to BP-lowering effects of the NO donors. Whereas both NO donors decreased MAP when administered intravenously to anesthetized rabbits, no such effect was noted during long-term oral treatment with the compounds in conscious rabbits. After 7 days of oral treatment, molsidomine had no significant effect on MAP of conscious rabbits, whereas SPM-5185 slightly increased rather than decreased MAP. The lack of a decrease in BP may be explained by the fact that the last dose of molsidomine and of SPM-5185 were given 3 and 18 h, respectively, before BP was measured, by which time the maximal depressor effects may well have disappeared.

The increase in arterial BP observed 18 h after the last treatment with SPM-5185 may be due to (a) a type I error of the statistical test of the small samples, or (b) a rebound effect (20–22) due to repeated activation of neurohumoral systems [sympathoadrenal axis, renin–angiotensin system (RAS)] (23),

and/or intravascular volume expansion. Indeed, NO donors may not only produce direct peripheral arterial and venous dilatation but also activate compensatory mechanisms that cause arterial and venous peripheral vasoconstriction. The hemodynamic effects of an NO donor are therefore the net result of two interacting factors: the rapid but short-lived direct vasodilating effects and the secondary activation of counteractive mechanisms provoked by the reduction in arterial BP. With abrupt discontinuation of an NO donor, the vasodilator component rapidly disappears, leaving the vasoconstrictive mechanisms unopposed. A rebound phenomenon has been described for SPM-5185 in dog coronary circulation (24). Abukhres and colleagues (23) demonstrated that BP rebounded to hypertensive levels after the intravenous infusion of sodium nitroprusside in conscious rats and that this rebound appeared to be mediated by the RAS. Moreover, we recently demonstrated that although three different ACE-inhibitors all significantly decreased MAP in conscious rabbits, none of them altered collar-induced intimal thickening (G. R. Y. De Meyer, unpublished observations). Clearly, the inhibition of intimal thickening observed with SPM-5185 cannot be explained by a reduction in the BP.

Although SPM-5185 significantly decreased the collar-induced neointima, it did not reduce SMC replication in the media. In the media of sham-operated arteries, SPM-5185 instead increased the percentage of PCNA-positive SMC. Therefore, the reduction in neointima formation induced by SPM-5185 may be due to inhibition of migration rather than proliferation of SMC. Indeed, induction of SMC replication and migration are not necessarily causally related events in the vessel wall (4,25). Migration appears to be the initial event in the neointima formation in the collar model. The other possibility, that neointimal cells are descendants of pre-existing intimal cells is unlikely, since there are very few resident subendothelial SMC in control carotid arteries (4).

Because molsidomine did not have a significant effect on either BP or neointima formation, plasma levels of this drug, owing to pharmacokinetic factors, may not have been sufficiently high to provide continuous NO generation in the vasculature. How-

ever, in a parallel experiment with the thoracic aorta of the same rabbits, molsidomine slightly decreased the maximum amplitude of ACh- and calcium ionophore (A23187)-induced relaxations and rendered the SMC less sensitive to SIN-1 without influencing maximum relaxation. Although the treatment period of the present investigation was only 2 weeks, our findings for that period are in agreement with the outcome of a previous experiment that lasted 16 weeks (26,27). Therefore, molsidomine appeared to be active. The lack of significant effect of molsidomine on intimal thickening may be explained by the fact that NO release from molsidomine is accompanied by the production of superoxide anion, which can react with NO to yield peroxynitrite, which decomposes to form the highly reactive hydroxyl radical (9). We previously demonstrated increased contents of 13-L-hydroxyoctadecadienoic acid and 15-L-hydroxyeicosatetraenoic acid, metabolites of the peroxides of linoleic acid and arachidonic acid, in the thoracic aorta of molsidomine-treated rabbits (28).

In accordance with results of previous studies in our laboratory, collaring decreased the E_{max} of 5-HT and KCl (15) and increased the sensitivity to 5-HT (12,15). Despite a significant reduction in intimal thickening, SPM-5185 could not overcome these changes in vascular reactivity. Two possible explanations are conceivable for this apparent discrepancy: (a) The reduction of the development of a neointima by SPM-5185 was not sufficient to alter the 5-HT–related modifications in vascular reactivity; and (b) the modifications in vascular reactivity in response to 5-HT are not dependent on the extent of neointima formation. Indeed, the sensitization to 5-HT after 1-week treatment is of the same magnitude as that after 2 weeks, although the development of the neointima is still incomplete (4). Therefore, the sensitization to 5-HT apparently is independent of the extent of intimal thickening.

Tolerance to organic nitrates in humans has been recognized for >100 years as a pharmacological phenomenon (29). Several reports showed the existence of tolerance in experimental animals in vivo (30–32). Furthermore, tolerance in isolated vascular SMC from animals made tolerant to organic nitrate esters has been reported (33,34). To prove that SPM-5185 did not induce cross-tolerance toward NTG, carotid arterial segments were exposed to three concentrations of the latter drug. The results confirmed that the semichronic treatment with the long acting nitrate SPM-5185 induced no sign of cross-tolerance toward NTG in either sham or collar-treated rings. Similarly, semichronic treatment with molsidomine did not induce tolerance toward SIN-1 in either sham or collared carotid arteries.

ACh was used to induce relaxation of the segments precontracted with PE $3.5 \times 10^{-7}M$ by stimulation of the endothelial arginine-NO system. The collar decreased the sensitivity of the artery to ACh, a finding consistent with previous reports (13, 14,35). However, neither the pD_2 value of ACh nor the maximum percentage relaxation was significantly affected by the SPM-5185 or molsidomine treatment in normal or collared carotid arteries, suggesting that semichronic treatment with these NO donors did not alter the capacity of the endothelium of the carotid artery to generate NO in response to muscarinic receptor stimulation. However, there was a tendency toward normalization of the pD_2 value of ACh in the collared arteries of the SPM-5185–treated group, which is in accord with the data of Schwarz and co-workers (36). The contractile response to PE $(3.5 \times 10^{-7}M)$ in collar-treated rings was significantly diminished as compared with that in sham-treated rings. A decrease in initial contraction might lead to an enhanced sensitivity to vasodilators (37). However, intima-bearing arteries were less sensitive to ACh. Moreover, treatment with the NO donors did not affect the contraction to PE in either sham or collar-treated rings. Therefore, the tendency toward normalization of the pD_2 value of ACh in the collared arteries of the SPM-5185–treated group as compared with the placebo group cannot be explained by a decrease in the initial contraction to PE.

Our results demonstrate that the administration of an NO-donor can inhibit neointima formation in vivo, confirming the results of Guo and associates (38), who demonstrated a reduction of intimal thickening by SPM-5185 in a rat carotid artery injury model. Furthermore, our results demonstrate that SPM-5185 is active when administered orally, which is an advantage over most of the newer NO-donors so far reported in the literature. Moreover, the drug is not prone to rapid tolerance development and did not lead to cross-tolerance toward NTG. Despite the significant reduction observed in the development of a neointima, SPM-5185 did not significantly antagonize the collar-induced reduction of the maximum contractile response to 5-HT and KCl. Therefore, the maximal contractile response of collared vessels is not dependent on the thickness of the neointima. In contrast, the pD_2 value of ACh tended to normalize in collared arteries of the SPM-5185–treated group. That exogenous NO can modulate the formation of a neointima in vivo may have important implications not only for our understanding of the pathophysiology of atherosclerosis but possibly also for future antiatherosclerotic therapy.

Bossche, Nancy Van Osselaer, and Ludo Zonnekeyn for technical assistance. G.R.Y.D.M. is a Senior Research Assistant of the Belgian National Fund for Scientific Research.

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