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Novel Organic Nitrates are Potent Dilators of Large Coronary Arteries with Reduced Development of Tolerance During Long-Term Infusion in Dogs: Role of the Sulfhydryl Moiety

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Summary: The vasodilator action of organic nitrates can be severely impaired by induction of drug tolerance. A critical depletion of sulfhydryl groups has been proposed to play a key role in impairment of the biotransformation of organic nitrates to nitric oxide (NO). We studied the effects of the new cysteine-containing nitrate SPM-5185 and the corresponding cysteine-free compound SPM-4744 on hemodynamics and large coronary artery dilation in chronically instrumented conscious dogs. Both nitrates caused dose-dependent increases of the diameter of the left circumflex artery (LCX); the cysteine-containing compound SPM-5185 however, caused such increases at ≤30-fold lower doses as compared with SPM-4744. Coinfusion of the cysteine-containing analogue of SPM-5185 lacking the nitrate group (SPM-5267) did not alter the dose–response relationship to SPM-4744. Continuous infusion of SPM-5185 (4 μg/kg/min, n = 6) and SPM-4744 (2.7 μg/kg/min, n = 5) elicited LCX diameter increases of 0.24 ± 0.06 and 0.17 ± 0.07 mm, respectively, representing 60–70% of maximal dilator capacity. In contrast to classic organic nitrates, both SPM-5185 and SPM-4744 caused LCX diameter to decrease only slightly during 5-day infusions. Both compounds elicited sustained dilation even at day 5 ($p \leq 0.05$). SPM-5185 caused an initial decrease in mean arterial pressure (MAP) and evoked sustained increases in heart rate (HR), whereas SPM-4744 had no significant peripheral effects. On withdrawal of SPM-5185, LCX diameter was decreased below pretreatment values for several hours. The dose–response relationship was not altered significantly by chronic administration of either nitrate after 5 days of infusion nor 1 day after discontinuation of the infusion, demonstrating preservation of pharmacologic efficacy. SPM-5185 and SPM-4744 are both effective vasodilators that dilate large coronary arteries without rapid development of drug tolerance. The cysteine moiety probably is not a prerequisite for prevention of tolerance at the level of large coronary arteries but may improve pharmacologic properties of nitrate compounds. Key Words: Conscious dogs—Coronary vasodilation—SPM-5185—SPM-4744—Nitrate tolerance—Sulfhydryl group.

The preferential dilation of large epicardial arteries, an important therapeutic effect of organic nitrates in treatment of coronary artery disease, has been shown to be an early victim of drug tolerance (1,2) when intermittent therapy is not observed. The mechanisms underlying this process are still poorly understood (3,4). Organic nitrates are known to be metabolized to an endothelium-derived relaxing factor (EDRF)-like compound by vascular smooth muscle (5,6) and require thiol (sulfhydryl, SH) groups to become effective vasodilators (7), a phenomenon that appears to be related to release of nitric oxide (NO) from these compounds (5,8). Because spontaneously NO-releasing drugs such as sodium nitroprusside (SNP) or sydnonimines are more resistant to development of tolerance, enzymatic conversion of nitrates to NO may play a key role in this process. Although tolerance to glyceryl trinitrate (GTN) cannot be prevented by a pharmacologic supply of thiols (9,10), beneficial effects of

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thiol-containing compounds have been reported in clinical studies (11). By comparing the effects of the new organic nitrate SPM-4744 (3-nitratopivalic acid) and of SPM-5185, (*N*-(3-nitratopivaloyl)-*S*-(*N'*-acetyl-D,L-alanyl)-L-cysteine-ethylester), the same nitrate combined with a cysteine ethylester moiety, during long-term infusion in chronically instrumented dogs, we studied the possible influence of the molecular structure of organic nitrates and effects of additional thiol groups on the development of nitrate tolerance in vivo.

MATERIALS AND METHODS

Animals

Adult mongrel dogs of either sex weighing 24–27 kg were chronically instrumented during N_2O-droperidol-fentanyl anesthesia. An aseptic left thoracotomy was performed through the fourth intercostal space under positive pressure respiration. The left circumflex coronary artery (LCX) was isolated 1–4 cm from its origin and instrumented with perivascular piezoelectric crystals for continuous diameter recordings as described previously (1). A polyethylene catheter was implanted in the pulmonary artery for drug application. Wires and catheter were tunneled subcutaneously to the back. The dogs received antibiotics for 1 week postoperatively and were allowed to recover from operation for at least 10 days. During the recovery period, the dogs were trained to rest quietly on the table for experimental periods of 3 h. Before the dogs were chronically instrumented, a common carotid artery was translocated into a cutaneous loop at the ventral surface of the neck. Throughout the study, the dogs were fed standard diet and had free access to tap water. Care of the dogs and execution of the experimental protocol were supervised by an independent veterinarian in accordance with German laws and the animal welfare regulations of the University of Freiburg (corresponding to the guidelines for animal welfare of the American Physiological Society).

Measurements and protocols

When the dogs were lying on the experimental table in a conscious state, indwelling cannulas were inserted in the common carotid artery in the skin loop and into a tibial vein. Systemic blood pressure (BP) was determined by a Statham P 23 pressure transducer connected to the cannula in the carotid artery, and heart rate (HR) was determined from the arterial pressure signal. LCX diameter was measured by ultrasonic transit time crystals. The crystals were triggered at a frequency of 4.43 MHz, providing linear measurements in a range of 1 mm from the lower to the upper calibration limits, which were adjusted individually in each experiment. Phasic and mean recordings of all variables were documented on a Watanabe linear recorder. All measurements were started after a 20-min rest period.

For long-term exposure, 6 dogs were treated with a dose of 4 μg/kg/min SPM-5185, which induced ~60–70% of maximal dilation at the beginning of the chronic treatment. In another group of 5 animals, the initially equieffective dose of SPM-4744 (2.7 μg/kg/min) was administered in the same protocol. The drugs were infused for 5 days through the pulmonary catheter at a flow rate of 2 ml/h by a portable battery-operated infusion pump (Baxter, autosyringe AS 30C). The dogs were evaluated on day 0 under control conditions, on each of the 5 days during long-term infusion, and 24 h after discontinuation of the infusion (day 6). Saline solutions of SPM-5185 and SPM-4744 were prepared fresh every day before the 50-ml drug reservoir was filled.

On day 0 under control conditions, on day 5 after long-term application before infusion was discontinued, and 24 h after infusion was discontinued, dose–response relationships (DRR) for changes in LCX diameter, mean arterial pressure (MAP), and heart rate (HR) induced by increasing equimolar doses of either SPM-5185 or SPM-4744 were determined in each group. The applications were separated by an interval of 15 min. SPM-5185 doses administered as intravenous (i.v.) bolus injections of 0.1, 0.3, 1.0, 3.0 and 10.0 mg in 10 ml saline solution. SPM-4744 doses were 0.04, 0.12, 0.4, 1.2, 4.0, and 12.0 mg. In an additional experiment with the group of animals receiving SPM-4744, dose–response curves for SPM-5185, SPM-4744, SPM-5267 (*N*-(3-hydroxypivaloyl)-*S*-(*N'*acetyl-D,L,-alanoyl)-L-cysteine ethylester), the corresponding cysteine derivative without nitrate group, and for SPM-4744 + SPM-5267 (coadministered at equimolar doses), respectively, were obtained 1 week before continuous infusions were started.

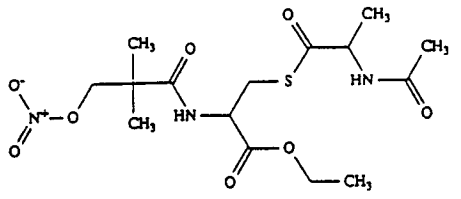
Calculations and drugs

All results are means ± SEM. An analysis of variance (ANOVA) with Tukey's Studentized range test for multiple comparison of means was performed to determine statistical significance. Differences of $p \leq 0.05$ were considered significant. Values for LCX and MAP are expressed in part as changes from individual control values before drug administration (ΔLCX, ΔMAP). SPM-5185, SPM-4744 and SPM-5267 were obtained from Schwarz Pharma AG, Monheim, Germany. The chemical structures are shown in Fig. 1.

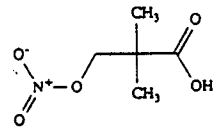
RESULTS

Coronary and systemic effects during acute administration

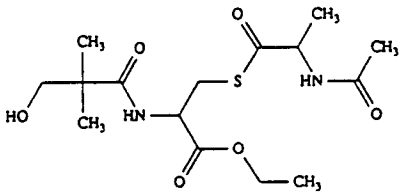
Changes in LCX, MAP, and HR induced by increasing i.v. doses of SPM-5185 before (control), immediately before discontinuation of the 5-day infusion period, and 1 day after discontinuation of infusion summarized in Fig. 2. Control values for LCX and MAP were 2.50 ± 0.23 mm and 94.0 ± 4.1 mm Hg before infusion and 2.49 ± 0.16 mm and 91.3 ± 6.8 mm Hg 1 day after the infusion period, respectively. Increasing dosages of SPM-5185 (0.1, 0.3, 1.0, 3.0, 10.0 mg) caused strong dose-dependent increases in LCX, slight decreases in MAP, and distinct increases in HR. Bolus injection of 3 mg SPM-5185 caused maximal coronary dilation in all experiments. The individual maximum dilator capacity of the dogs was 6.3–16.6% of resting diameter (mean 11.0 ± 3.8%). The acute dilator capacity of the cysteine-free nitrate SPM-4744 as compared with SPM-5185 is shown in Fig. 3. SPM-4744 was much less effective, and even 30-fold higher concentrations failed to induce the maximal



SPM 5185



SPM 4744



SPM 5267

FIG. 1. Chemical structures of compounds administered.

dilation achieved with SPM-5185. Coadministration of the cysteine-containing compound SPM-5267 had no effect on the DRR of SPM-4744 (Fig. 3). SPM-5267 was ineffective when given alone.

Coronary and systemic effects of 5-day continuous i.v. infusion

Data of the changes in MAP and HR during the 5-day continuous i.v. infusion of SPM-5185 as well as 2, 4, and 24 h after discontinuation of the infusions are shown in Table 1. The course of LCX diameter changes throughout the infusion period is shown in Fig. 4. Previously obtained results with GTN under identical experimental conditions (1) are provided for comparison. The LCX reaction to the drug withdrawal is shown in Fig. 5. Continuous exposure to SPM-5185 and SPM-4744 produced strong increases in LCX corresponding to ~60–70% of the maximal dilator response. SPM-5185 induced a decrease in MAP and an increase in HR on the first day after the infusion was started. The increase in HR persisted throughout the infusion period (Table 1 and Fig. 4), whereas MAP returned almost to control values after 3 days. Even on the fifth day of continuous SPM-5185 infusion, LCX diameters were significantly different from preinfusion values. After discontinuation of the SPM-5185 infusion, LCX diameters returned rapidly to control levels and decreased further below preinfusion values ($p \leq 0.1$) with a maximum 4 h after the infusion was discontinued (Fig. 5); 24 h later, all parameters were similar to the preinfusion controls (Table 1). In contrast, SPM-4744 caused sustained coronary dilation for 5 days without significantly affecting MAP and HR. After discontinuation of the SPM-4744 infusion, LCX returned rather slowly to the control level and no rebound constriction was observed (Fig. 5).

Effects of long-term infusion on DRRs

Changes in LCX diameter, MAP, and HR induced by increasing doses of SPM-5185 under control conditions at the fifth day of continuous infusion of 4 μg/kg/min and 24 h thereafter are shown in Fig. 2. DRRs for SPM-4744 and SPM-5267 on LCX diameters are shown in Fig. 3. DRRs were shifted to slightly higher doses both after 5 days during infusion of 4 μg/kg/min SPM-5185 and 1 day after the end of the infusion period, especially for HR response, but the differences between the DRRs did not reach statistical significance. Similarly, there were no significant differences in the dose–response curves for SPM-4744 before and after chronic administration (data not shown). Levels of LCX diameter under control conditions and 24 h after continuous infusion were very similar (described herein), but starting levels for LCX-DRR on the

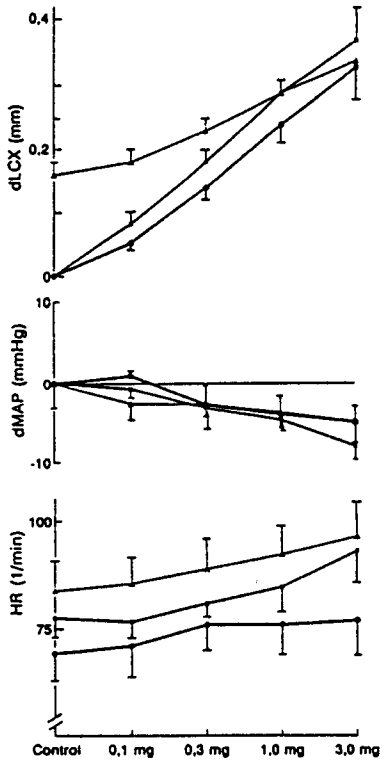


FIG. 2. Dose–response relationships (DRR) of SPM-5185 (bolus injections) before infusion (squares), after 5 days of continuous infusion (triangles), and 24 h after discontinuation of infusion (circles). Data are means ± SEM (n = 6). dLCX, diameter change in left circumflex coronary artery from control; dMAP, change in mean arterial pressure from control; HR, heart rate.

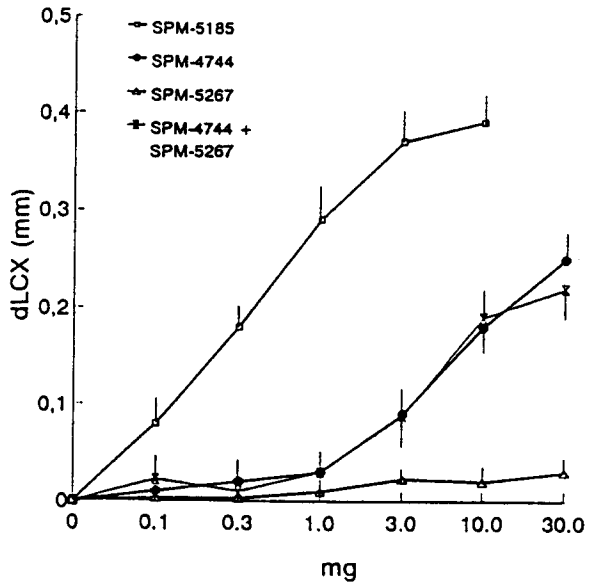


FIG. 3. Comparison of the coronary dilator effects of SPM-5185, SPM-4744, SPM-5267, and SPM-4744 + SPM-5267. Drugs were administered as equimolar concentrations; x-axis shows dosages of SPM-5185. Data are means ± SEM (n = 5).

fifth day of continuous infusion were higher than control values owing to sustained drug action (Fig. 2).

DISCUSSION

Using conscious chronically instrumented dogs, we showed that new organic nitrates produced strong dose-dependent increases in large coronary artery diameter. The coronary vasodilator potency of SPM-5185 was comparable to that of GTN reported previously (1,12). The dilation of LCX in response to 3 mg SPM-5185 (bolus) represents >10% of diameter increase and can thus be considered the maximal dilation that can be achieved in this experimental model (13). Part of the dilator capacity of conduit arteries was probably masked by impairment of full relaxation owing to the surgical procedure performed before the experiments. Attachment of the crystals to the vessel wall inevitably induces perivascular inflammation, with consecutive growth of connective tissue that limits maximal dilation and which may account for the variability in dilator capacity in individual dogs.

The considerably higher efficacy of SPM-5185 as compared with SPM-4744 on acute administration cannot be explained easily as an SH-dependent effect, since coadministration of SPM-5267 + SPM-4744 did not alter the DRR of the latter. Thus, nonenzymatic extracellular NO release, an important pathway for bioconversion of GTN that can be enhanced by administration of cysteine derivatives (14,15) may play only a minor role in the specific mechanism of NO release in these new compounds.

TABLE 1. *Changes in MAP and HR during and after continuous infusion of SPM-5185 (4 μg/kg/min)*

Parameter	Baseline	Days of infusion					Hours after infusion				
		1	2	3	4	5	0.5	1	2	4	24
MAP (mm Hg)	91.3 ± 6.8	−5.3 ± 1.6	−6.0 ± 1.9	−3.3 ± 1.2	−0.6 ± 2.3	+1.0 ± 3.4	+5.0 ± 2.2	+10.3[a] ± 3.0	+11.4[a] ± 3.2	+6.3 ± 3.6	−2.7 ± 2.6
HR (beats/min)	75 ± 8	+18[a] ± 3	+17[a] ± 5	+20[a] ± 5	+19[a] ± 4	+16[a] ± 3	+14 ± 2	+8 ± 1	+5 ± 2	+5 ± 2	+6 ± 3

MAP, mean arterial pressure; HR, heart rate.
Values are means ± SE (n = 6).
[a] Differences from control values at $p \leq 0.05$.

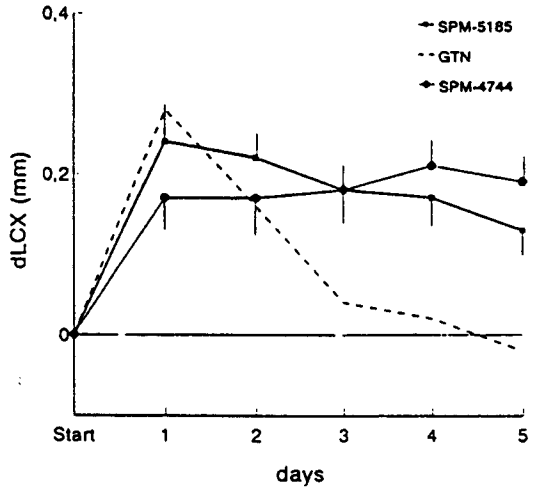


FIG. 4. Dilator effects of continuous 5-day infusion of SPM-5185 (4 μg/kg/min) and SPM-4744 (2.7 μg/kg/min). Data are means ± SEM (n = 6). dLCX, diameter change in left circumflex coronary artery from control. LCX diameters changes in response to infusion of either compound were significant at $p \leq 0.05$ throughout the infusion period. Dashed line represents mean LCX changes to glyceryl trinitrate GTN (1.5 μg/kg/min), as reported previously (1).

Continuous infusion of either nitrate compound resulted in pronounced coronary artery dilation (Fig. 4). The LCX dilator response to SPM-5185 but not to SPM-4744 diminished slightly during the infusion period. Nevertheless, LCX diameter remained significantly increased even at day 5. The DRR for both compounds obtained immediately after discontinuation of the long-term infusion and 1 day thereafter showed only a slight nonsignificant

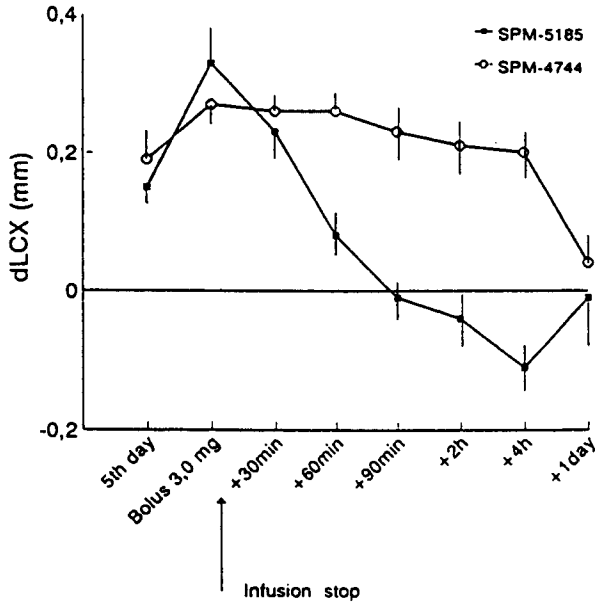


FIG. 5. Withdrawal effects of SPM-5185 and SPM-4744. Data are means ± SEM (n = 6). dLCX, diameter change in left circumflex coronary artery from control; *x*-axis is not time-proportional.

rightward shift. This finding is in accordance with results of recently reported in vitro studies of the effects of SPM-5185 on human arterial and venous strips (16). Continuous application of nitrates such as GTN for several days causes a gradual decrease in their coronary arterial vasodilator capacity as a consequence of development of tolerance (4). Because the new nitrate compounds investigated in this study are believed to dilate large coronary arteries preferentially, long-term application might result in such reduction of their dilator effectiveness. In the present experiments, however, dilation of these large conductance vessels was largely retained throughout the infusion period. The dilation was accompanied initially by a weak decrease in MAP and an increase in HR during SPM-5185 infusion, whereas cysteine-free SPM-4744, which is a weaker dilator when administered acutely, had no systemic effects, possibly indicating an additional dilator effect of SPM-5185 on peripheral resistance vessels caused by the cysteine moiety of the compound. Cysteine enhances dilation to GTN selectively in resistance vessels <100 μm (17), an effect that could likewise be effective for SPM-5185. Because MAP returned to normal values after several days, tolerance to SPM-5185 may occur at the level of these small vessels. On the other hand, the nitrate-free cysteine compound SPM-5267 neither elicited any significant effects when given alone nor altered the DRRs for SPM-4744 during short-term administration (Fig. 3). Further investigations with SPM-5185 are needed to explain increase in HR sustained after normalization of MAP during long-term infusion.

The DRRs for dilation of LCX were almost unaffected by continuous infusion of both compounds for 5 days. In contrast, during previous studies using an identical experimental design, we noted a rapid decrease in LCX diameters after 2 days of GTN infusion (1) (Fig. 3). In the same study in GTN-tolerant dogs, the DRR for GTN was shifted to 17- to 20-fold higher doses after the same infusion period.

The mechanisms leading to induction of tolerance during long-term treatment with organic nitrates remain controversial. They comprise biologic counterregulation (18), impairment of endothelium-independent NO release from the organic nitrate molecule (5,19) and, eventually, desensitization of the key enzyme soluble guanylyl cyclase (sGC) (20,21). Possible involvement of thiol depletion in onset of true tolerance was proposed by several groups of investigators (7,8,11,22), but because the cystein-free compound SPM-4744 is at least similarly resistant toward induction of tolerance as compared with SPM-5185, intracellular thiol depletion as a cause for nitrate tolerance is unlikely. Moreover, other studies showed that induction of tolerance both in vitro (23) and in vivo (24) is not nec-

essarily associated with reduced SH levels. Coadministration of thiols has not been consistently successful in reversing nitrate tolerance (9,10). Therefore, other mechanisms might be primarily involved. In particular, the in vivo metabolism of the applied nitrate compounds may differ from that of classic organic nitrates with respect to specific cytochrome P-450–mediated reactions (25–27) and other enzymatic pathways that participate in biotransformation of organic nitrates (6,28,29). Finally, differences may exist between the action of these new nitrates and classic organic nitrates in the central nervous system, the role of which in development of tolerance is not yet clear.

The slight rightward shift of the DRR and the constrictions of LCX in response to withdrawal of SPM-5185 may represent effects of biologic (neurohormonal) counterregulation that occur during long-term exposure to vasodilators (18), since physiologic readjustments probably take several hours after the drug is withdrawn. Recently we noted that GTN-tolerant dogs reacted similarly in response to drug withdrawal after 5 days (30). The absence of this constriction phenomenon after withdrawal of SPM-4744 may indicate that this apparently less potent compound consequently evoked less pronounced counteregulation. The effects of nitrates on central regulation of vascular tone and BP could have substantial influences on the pharmacologic profile of these drugs and may play a key role in onset of tolerance. Differences in the short- and long-term dilator potencies of SPM-5185 and SPM-4744 may therefore be related as well to their particular pharmacokinetics and metabolism in vivo.

Sudden withdrawal of GTN deteriorates the condition of GTN-treated patients with coronary artery disease (31). Thus, rebound of the LCX vasomotor responses after chronic SPM-5185 treatment could be clinically relevant if this phenomenon occurs at lower therapeutical dosages as well; this should be clarified in further investigations.

Our results suggest that in conscious dogs both SPM-5185 and SPM-4744 act as potent dilators of large coronary arteries, their 5-day continuous administration does not induce significant tolerance. An NO release mechanism distinct from that of other nitrates may account for the advantageous pharmacologic properties of these new compounds. An improved local supply of SH-groups provided by the cysteine moiety of SPM-5185 may in turn improve the dilator efficacy of organic nitrates but is probably not a prerequisite for maintenance of dilator capacity during chronic administration.

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