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Preferential Dilation of Large Coronary Microvessels by the Mononitrates SPM-4744 and SPM-5185

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Summary: A novel aspect of the pharmacodynamic action of nitroglycerin is that it is a potent dilator of larger coronary arteries, yet it dilates smaller coronary microvessels submaximally and only in high concentrations. We sought to determine whether this property was shared by other organic nitrates. The effects of two mononitrates, SPM-4744 and SPM-5185 (the latter of which possesses a thioester in its structure), on coronary microvessels of different sizes were studied. Large (200-μm diameter) and small (<100-μm diameter) porcine coronary microvessels were studied in vitro while pressurized in a no-flow state. After constriction with the thromboxane analogue U46619, maximal dilations (as a percent of preconstricted tone at the highest applied concentration, 10 μ*M*) of small coronary microvessels were 18 ± 3 and 16 ± 2% in response to SPM-4744 and SPM-5185, respectively. The dilations of larger coronary microvessels to SPM-4744 and SPM-5185 were 55 ± 5 and 43 ± 6%, respectively (both $p < 0.001$ vs. the small vessel responses). This pattern of differential vasodilatation of large and small coronary microvessels was similar to that produced by nitroglycerin. In contrast, sodium nitroprusside produced equivalent degrees of vasodilation of small and large coronary microvessels. Additional experiments demonstrated that both SPM compounds produced dilation of the coronary microcirculation in isolated rat heart and relaxed isolated segments of rat aortic rings only in high (≥1 μ*M*) concentrations. These data demonstrate that the organic mononitrates are similar to nitroglycerin in their selectivity for larger coronary microvessels and produce only minimal dilation of coronary microvessels <100 μ*M* in diameter. **Key Words:** Organic nitrates—Sodium nitroprusside—Vasodilatation.

Despite widespread clinical use of nitroglycerin and the related organic nitrates, the mechanisms responsible for their antianginal effects remain imprecisely defined. Nitroglycerin is incapable of producing direct vasodilatation and must be "bioconverted" to a vasoactive metabolite that is probably nitric oxide or an intermediate (such as a nitrosothiol) that releases nitric oxide (1–3). This bioconversion process can occur through enzymatic (2,4, 5) and nonenzymatic means (6) although, in vivo, enzymatic bioconversion is believed to be most important. In contrast, compounds such as sodium nitroprusside (7) and nitrosothiols (8) release nitric oxide predominantly by nonenzymatic means. The differences in the manner in which these nitrovasodilators release nitric oxide remain unclear. One difference relates to the pathway of bioconversion, which in turn may depend on the degree of reduction of the nitrogen released as nitric oxide. In the case of sodium nitroprusside, this can be accomplished by a one-electron reduction (7), whereas in the case of the organic nitrates, a three-electron reduction is required to release nitric oxide (9). Because of the complexity of the latter process, cellular enzymatic processes are probably involved.

An important pharmacological property of nitroglycerin is that it produces prolonged dilation of the larger coronary arteries while causing only brief and submaximal increases in coronary flow (10,11). Recent in vitro and in vivo studies of the coronary microcirculation have provided insight into the mechanisms underlying the vascular selectivity of

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nitroglycerin. In studies in vitro, nitroglycerin produced only slight vasodilatation of coronary microvessels <100 μm in diameter whereas putative metabolites of nitroglycerin such as nitric oxide and *S*-nitrosocysteine caused potent dilation of these vessels (12). These findings suggest that coronary microvessels may be incapable of converting nitroglycerin to its active vasodilator metabolite. Therefore, the need for bioconversion of nitroglycerin appears to target this drug to certain vessels that can accomplish this process. This aspect of the pharmacology of nitroglycerin is probably of extreme importance because it prevents the phenomenon of "coronary steal" that occurs with compounds that produce vasodilatation of smaller coronary arteries.

Related to these issues is the increasing clinical use of orally bioavailable organic nitrates. These compounds share with nitroglycerin the nitrate ester moiety yet possess markedly different structures with regard to the carbon skeleton. If the explanation for the selective effect of nitroglycerin on various coronary vessels of various size is predominantly related to the chemical nature of the nitrogen ultimately released as nitric oxide, one might hypothesize that other organic nitrates would also exhibit this vascular selectivity. In the present experiments, we examined this hypothesis by investigating the effect on different sizes of porcine coronary microvessels of two mononitrates whose only chemical similarity to nitroglycerin is the presence of an organic nitrate ester group. We compared these responses with those produced by nitroglycerin and sodium nitroprusside. In additional experiments, we compared the effects of these agents on a larger conductance vessel (rat aorta) and the rat coronary microcirculation in isolated perfused heart preparations.

METHODS

Porcine coronary microvessels studies

Yorkshire pigs (n = 7, 18–25 kg) of either sex were premedicated with ketamine (10 mg/kg intravenously, i.v.) and anesthetized with α-chloralose (60 mg/kg i.v.) and urethane (300 mg/kg i.v.). Respiration was maintained by tracheal intubation and mechanical ventilation. Heparin was administered (500 U/kg). After a sternotomy, the heart was rapidly excised and immediately placed in a cold (5°–10°C) Krebs buffer solution of the following composition (in m*M*): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.1.

Subepicardial coronary arterial microvessels were dissected from the left anterior descending artery region of the left ventricle under an ×10–60 dissecting microscope. The vessels were subsequently placed in a Plexiglas microvessel organ chamber, cannulated with dual glass micropipettes 30–80 μm in diameter, and secured with 10-0 nylon monofilament suture. Oxygenated (95% O_2 5% CO_2) Krebs buffer solution, warmed to 37°C, was continuously circulated through the organ chamber and a reservoir (total volume of reservoir and organ chamber = 100 ml). The vessels were pressurized to 40 mm Hg in a no-flow state with a burette manometer filled with Krebs buffer solution. With an inverted microscope (×40–200 Olympus, Japan) connected to a video camera, the vessel image was projected onto a black and white television monitor. An electronic dimension analyzer (Living System Instrumentation, Burlington, VT, U.S.A.) was used to measure internal lumen diameter. Measurements were recorded with a strip chart recorder. The vessels were allowed to equilibrate for 30 min in Krebs buffer solution before drug administration.

Microvessels were categorized into two groups: small (80–93 μm) and large (190–240 μm). We examined relaxations to SPM-4744, SPM-5185, nitroglycerin, and sodium nitroprusside in both small and large microvessels precontracted by 30–50% of their baseline diameter with the thromboxane A_2 analogue U46619. Once steady-state tone was obtained, dose responses to SPM-4744 (10^{-10}–10^{-5}*M*), SPM-5185 (10^{-10}–10^{-5}*M*), nitroglycerin (10^{-9}–10^{-5}*M*), and sodium nitroprusside (10^{-9}–10^{-5}*M*) were examined. Two or three interventions were performed on each vessel. The order of drug administration was random. In preliminary experiments, we noted that acute exposure to the concentrations of the various nitrovasodilators used in these studies had no effect on responses to other vasodilator drugs subsequently administered. All drugs were administrated extraluminally. Measurements were obtained 2–3 min after the drug was administered, after the response had stabilized. Vessels were washed three times with Krebs buffer solution and allowed to equilibrate in a drug-free Krebs buffer solution for at least 15 min between interventions.

Studies of rat aortic rings and isolated perfused hearts

Male Wistar rats weighing 260–340 g (average 301 ± 5 g) were initially anesthetized with diethylether inhalation and killed by a blow to the neck. The chest was opened, and the hearts and thoracic aorta were rapidly excised.

Isolated heart studies

The hearts were perfused with oxygenated (95% 0_2/5% CO_2) Krebs-Henseleit buffer at a constant flow (20 ml/min initially) according to the classic Langendorff technique. Nitrates were applied as a continuous infusion after an equilibration period of 30–45 min, during which time flow was reduced to 14 ml/min. Baseline coronary perfusion pressure after equilibration of the hearts was 106 ± 5 mm Hg (n = 21). The next concentration was applied after the response had stabilized, which usually required 3–10 min. Results were expressed as a percent reduction in the coronary perfusion pressure from baseline values.

Isolated aortic rings

Excess fat and adventitial tissue was removed from the aorta, and the vessel was cut into 4 to 5-mm rings. The rings were mounted in organ baths containing oxygenated (95% O_2/5% CO_2) Krebs-Henseleit buffer (pH = 7.4). A passive stretch of 2 g was applied, and the vessels were allowed to equilibrate for 90 min at 37°C. After equilibration, tissues were submaximally precontracted with 200 n*M* phenylephrine. In this preparation, the contractions are stable for as long as 5 h. Complete concentration–

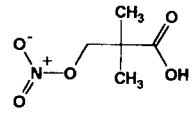
response curves were obtained by continuous recording of changes in isometric tension after cumulative addition of test compounds.

Drugs

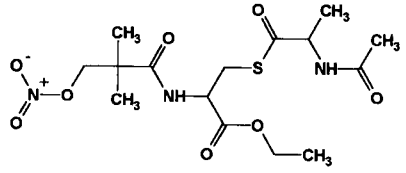
SPM-4744 (3-nitratopivalic acid) and SPM-5185 (*N*-(3-nitratopivaloyl)-*S*-(*N'*-acetyl-D,L-alanyl)-L-cysteine ethyl ester) were obtained from Schwarz Pharma (Monheim, Germany). The respective structures of each are shown in Fig. 1. Sodium nitroprusside and the thromboxane A_2 analogue U46619 were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Nitroglycerin was obtained from Du Pont Pharmaceuticals (Manati, Puerto Rico). For the porcine coronary microvessels studies, all drugs were dissolved or diluted in ultrapure distilled water except for the thromboxane A_2 analogue U46619, which was dissolved in ethanol to make a stock solution and stored at 20°C. All solutions were prepared on the day of the study. For the studies in rat hearts and isolated aortic rings, all nitrates were dissolved in 10% ethanol/90% saline, except sodium nitroprusside and nitroglycerin, which were dissolved in saline. The final concentration of the vehicle in these studies was kept constant at 0.1% under all conditions, and this concentration was shown to have no effect on either the coronary circulation of isolated hearts or the aortic rings in organ baths (n = 3 for each, data not shown).

Data analysis

Relaxations are expressed as percent relaxation of the U46619-induced precontraction of the vessel diameter, the data are mean ± SEM. The concentrations of each drug necessary to produce 25% relaxations (EC_{25}) were calculated after log conversion of the individual concentration–response curves. EC_{50} values for the drug effects in the aortic rings were computed after logit transformation of the data after complete concentration–response curves were constructed. Comparisons of relaxations between large and small vessels and comparisons of the



SPM-4744



SPM-5185

FIG. 1. Respective structures of SPM-4744 (3-nitratopivalic acid) and SPM-5185 (*N*-(3-nitratopivaloyl)-*S*-(N'-acetyl-D,L-alanyl)-L-cysteine ethyl ester).

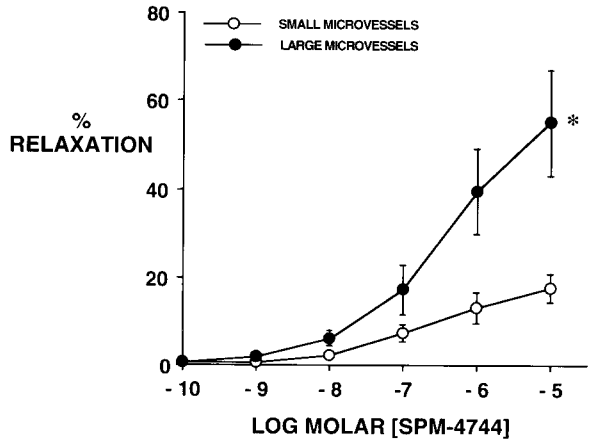


FIG. 2. Relaxations of large (200 μm diameter) and small (<100 μm diameter) coronary microvessels to SPM-4744. The vessels were studied in a pressurized state (40 mm Hg) in vitro. After constriction with U46619 (degree of constriction 33 ± 3 and 67 ± 4 μm in a small and large vessels, respectively), SPM-4744 was administered in increasing concentrations. Responses are expressed as percent relaxation of the tone achieved by the thromboxane analogue U46619. *$p < 0.05$ versus small coronary microvessels.

EC_{25} or EC_{50} values of the various nitrovasodilators were performed by two-way analysis of variance (ANOVA) and a Scheffé's post hoc test; $p < 0.05$ was considered significant.

RESULTS

Characteristics of the porcine coronary microvessels

The small coronary microvessels ranged from 80 to 93 μm in internal diameter, averaging 86 ± 1 μm. Large coronary microvessels ranged from 190 to 240 μm, averaging 201 ± 2 μm. The degree of contraction after application of the thromboxane A_2 analogue U46619 was 39 ± 1 and 37 ± 1% of the baseline diameter in the small and large microvessel groups, respectively. The average concentration of U46619 required to achieve this degree of contraction was 0.8 μ*M* in each group.

Porcine coronary microvascular responses to SPM-4744 and SPM-5185

The effects of SPM-4744 and SPM-5185 on small and large coronary microvessels are shown in Figs. 2 and 3. At the highest concentration administered (10 μ*M*), SPM-4744 and SPM-5185 produced only slight relaxations of small microvessels (18 ± 3 and 16 ± 2% of the U46619-induced contraction, respectively). In contrast, 10 μ*M* of either SPM-4744 or SPM-5185 produced significantly greater relaxation of large microvessels [56 ± 5% ($p < 0.001$) and 43 ± 6% ($p < 0.001$) of the U46619-induced contraction, respectively] as compared with the respective small microvessel response (Figs. 2 and 3) ($p < 0.001$ for each). The EC_{25} value for nitroglycerin-, SPM-4744-, and SPM-5185-induced relaxations could not be determined in the small coronary mi-

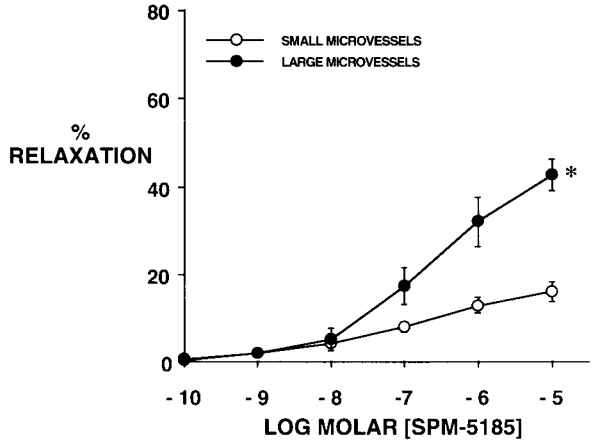


FIG. 3. Responses of coronary microvessels to SPM-5185. The vessels were studied in a pressurized state (40 mm Hg) in vitro. After constriction with U46619 (degree of constriction 35 ± 2 and 80 ± 6 μm in small and large vessels, respectively), SPM-5185 was administered in increasing concentrations. Responses are expressed as percent relaxation of the tone achieved by the thromboxane analogue U46619. *p < 0.05 versus small coronary microvessels.

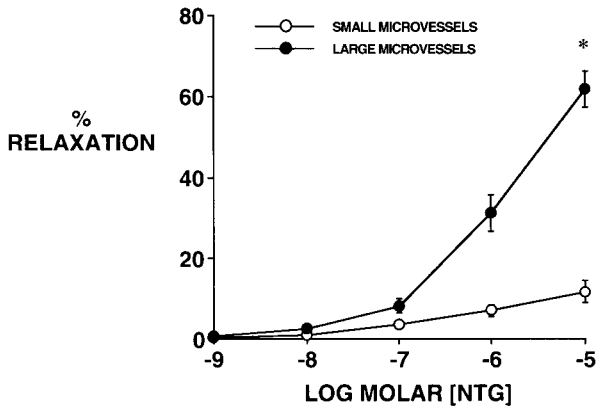


FIG. 4. Responses of large and small coronary microvessels to nitroglycerin. Studies were performed as described for SPM-4744 and SPM-5185, and nitroglycerin was administered in increasing concentrations. The degree of constriction was 38 ± 3 and 85 ± 7 μm in small and large vessels, respectively. Responses are expressed as percent relaxation of the tone achieved by the thromboxane analogue U46619. *p < 0.05 versus small coronary microvessels.

crovessels because these agents produced vasodilations <25% of the preconstricted tension. The EC_{25} value and maximal relaxations for nitroglycerin, SPM-4744, SPM-5185, and sodium nitroprusside in larger coronary microvessels were similar (Table 1).

Porcine coronary microvascular responses to nitroglycerin and sodium nitroprusside

Nitroglycerin dilated larger coronary microvessels 62 ± 5% of the U46619-induced contraction at the highest concentration administered (10 μ*M*). In contrast, nitroglycerin caused only slight dilation of small coronary microvessels (12 ± 3% at 10 μ*M*, p < 0.0001 vs. large microvessel response) (Fig. 4). Sodium nitroprusside produced similar relaxations of small and large coronary microvessels (65 ± 3 and 72 ± 4%, respectively) (Fig. 5). The EC_{25} value for nitroglycerin and sodium nitroprusside in the larger coronary microvessels was similar (Table 1). In small coronary microvessels, the EC_{25} value for sodium nitroprusside was similar to that observed in the large vessels. The EC_{25} value for nitroglycerin in small coronary microvessels was not determined because the agent did not produce 25% relaxation of these vessels.

Effect of SPM-4744, SPM-5185, nitroglycerin, and sodium nitroprusside in intact hearts

In isolated perfused rat hearts, a complete concentration–response curve was obtained only for sodium nitroprusside, whereas in the concentration range tested (10 n*M* to 1 m*M*) none of the organic nitrates produced a maximum decrease in coronary perfusion pressure. In contrast to sodium nitroprusside, the concentration–response relation for the nitrates was biphasic rather than sigmoid, with an effect amounting to only ~5% reduction in coronary perfusion pressure at threshold (Table 2) and a steep increase in slope at nitrate concentrations

TABLE 1. *Comparison of the effect of sodium nitroprusside, nitroglycerin, SPM-4744, and SPM-5185 on percent relaxations and EC_{25} values in large and small coronary microvessels*

	Large microvessels			Small microvessels		
No donor	Constriction (μm)	Maximal relaxation (%)	EC_{25} (log *M*)	Constriction (μm)	Maximal relaxation (%)	EC_{25} (log *M*)
Sodium nitroprusside	75 ± 5	65 ± 3	−6.4 ± 0.2	32 ± 2.4	72 ± 4	−6.1 ± 0.2
Nitroglycerin	85 ± 7.1	62 ± 5	−6.1 ± 0.1	38 ± 3	12 ± 3[a]	>−5
SPM-4744	67 ± 3.7	55 ± 5	−6.5 ± 0.1	33 ± 2.7	18 ± 3.3[a]	>−5
SPM-5185	80 ± 5.8	43 ± 6	−6.3 ± 0.2	35 ± 1.7	16 ± 2[a]	>−5

NO, nitric oxide, EC_{25}, effective concentration that produced 25% relaxation; large microvessels, >100 μm in diameter; small microvessels, <100 μm in diameter.

Data are mean ± SEM; n = seven for each.

[a] p < 0.05 versus response in large microvessels to the same drug.

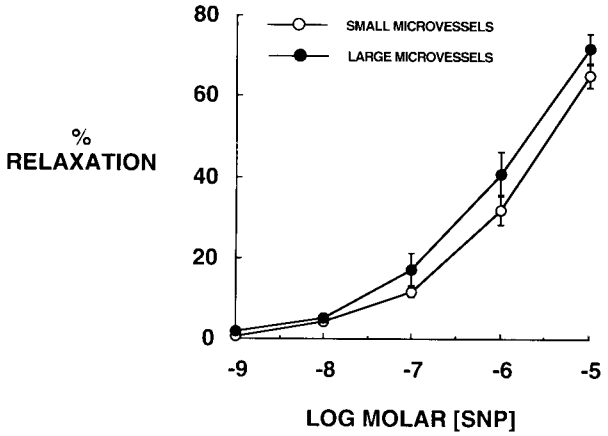


FIG. 5. Responses of coronary microvessels to sodium nitroprusside. Responses are expressed as percent relaxation of the tone achieved by the thromboxane analogue U46619. The degree of preconstriction was 32 ± 2 and 67 ± 4 μm in small and large vessels, respectively.

>1.0 μ*M* for nitroglycerin and 10 μ*M* for the mononitrates respectively. The order of potency of the individual compounds in reducing coronary perfusion pressure at a concentration of 100 μ*M* (Table 2) was sodium nitroprusside>nitroglycerin>SPM-5185>SPM-4744.

Effect of SPM-4744, SPM-5185, nitroglycerin, and sodium nitroprusside on rat aortic segments

All four nitric oxide donor compounds examined in the present study produced a concentration-dependent relaxation of precontracted rat aortic rings. With these vasodilators, full relaxation (≥95%) was achieved with a sensitivity order (EC_{50} value) of sodium nitroprusside = nitroglycerin >SPM-5185>>SPM-4744 (Table 2). Although in this model SPM-4744 was only marginally more potent than isosorbide-5-mononitrate (EC_{50} 4.9 × 10^{-4} *M*, data not shown), substitution of the former with a thioester moiety resulted in a considerable increase in sensitivity (~2 orders of magnitude).

DISCUSSION

In the present experiments, we examined the effect of several nitrovasodilators on both large and small coronary microvessels. The findings with nitroglycerin confirmed previous research (12–15) and allowed us to compare the effect of nitroglycerin and two mononitrates directly. The effect of these organic nitrates with rather diverse structures on large and small coronary microvessels was quite similar and suggests that the mononitrates are, like nitroglycerin, selective vasodilators of larger coronary microvessels. The approximate concentration of organic nitrate that can be achieved in vivo ranges from 1 to 100 n*M* depending on the dose applied and the route of administration. In view of this and our present data, the concentrations of these compounds that can be achieved under normal therapeutic conditions probably would cause preferential dilation of larger coronary microvessels and have little effect on smaller coronary microvessels and systemic conductance vessels.

In contrast to nitroglycerin and the mononitrates, sodium nitroprusside produced equivalent degrees of vasodilatation of coronary microvessels > < 100 μm in diameter. The release of nitric oxide from sodium nitroprusside occurs either through one-electron reduction or on exposure to light (7) and differs substantially from that of nitroglycerin. Cellular membranes, thiols, and various enzyme systems could have been the source of electrons in the present experiments to release nitric oxide from sodium nitroprusside. In addition to nonenzymatic processes, there are probably enzymatic pathways that reduce sodium nitroprusside to nitric oxide (16). The present findings suggest that these pathways leading to sodium nitroprusside bioactivation are similar in large vessels and small coronary microvessels. The mononitrates studied were quite ineffective vasodilators of rings of rat aorta and yet produced rather marked vasodilatation of larger (>100-μm diameter) coronary microvessels. Although the applied methodology, species, and contractile agents were different in the different sets of experiments and therefore cannot be directly compared, the difference between the vascular responses produced by the mononitrates is noteworthy. Mononitrates generally displayed a clearly weaker potency than nitroglycerin (2 log orders) when tested in isolated conductance vessels, whereas in large coronary microvessels both SPM compounds displayed a potency similar to that of nitroglycerin, which was not expected in a mononitrate.

In the current studies, neither of the organic nitrates produced appreciable vasodilation in the intact rat hearts until very high concentrations (near-micromolar) had been administered. This finding may seem at odds with the findings in the isolated pig coronary microvessels, in which the organic nitrates produced vasodilation of the larger coronary microvessels, which are also resistance vessels and should, if dilated, lead to increased flow in the intact heart (17,18). The explanation for this apparent paradox may be related to the segments of the coronary circulation involved in regulation of myocardial perfusion. In larger hearts (like those of pigs and dogs), pressure gradients exist across vessels <300 μm in diameter, with ~50% of the pressure loss occurring in vessels with diameters ranging from 100 to 300 μm in diameter (17). Therefore, dilation of these larger vessels by an organic nitrate probably would increase perfusion. However, coronary autoregulation and metabolic regulation occurs entirely in vessels <100 μm in diameter (19,20). Therefore, if an organic nitrate were to di-

TABLE 2. *Comparison of the pharmacological effects of nitroglycerin, sodium nitroprusside, SPM-4744, and SPM-5185 in isolated working rat heart and isolated rat aortic rings*

	Heart			Aorta		
NO donor	Baseline perfusion pressure (mm Hg)	Threshold concentration (log *M*)	Effect at 100 μ*M* (% maximal CPP)	Preconstriction (g)	Maximum relaxation (%)	EC_{50} (log *M*)
Sodium nitroprusside	97.8 ± 5.7	−8	45.3 ± 4.0	2.0	99.4 ± 0.5	−7.55
Nitroglycerin	113.7 ± 8.4	−6.4	32.4 ± 2.0	2.0	97.5 ± 0.5	−7.54
SPM-4744	102.8 ± 6.7	−4.4	8.5 ± 4.1	2.0	97.5 ± 0.9	−4.04
SPM-5185	112.8 ± 4.5	−5.2	18.3 ± 2.9	2.0	95.4 ± 1.7	−6.15

CPP, coronary perfusion pressure; other abbreviations as in Table 1.
Threshold concentrations was defined as the lowest concentration at which a detectable response was observed. Data are mean ± SEM; n = five to six for each.

late the larger coronary microvessels, pressure would be transferred to the smaller coronary microvessels, which through autoregulatory vasoconstriction would maintain perfusion constant. Indeed, even in human hearts, concentrations of nitroglycerin that produce near-maximal vasodilation of the conduit coronary arteries have little effect on myocardial perfusion.

A noteworthy aspect of the present experiments is the observation that the incorporation of a cysteine moiety in the structure of SPM-5185 did not alter its vascular selectivity. This modification of the compound has been made in an effort to prevent the development of nitrate tolerance. Previously, we demonstrated that administration of L-cysteine markedly enhanced nitroglycerin-induced vasodilatation in coronary microvessels <100 μm in diameter (13–15). More recently, we showed that this effect of L-cysteine is entirely dependent on L-cysteine acting as a source of glutathione and that glutathione probably participates in the intracellular enzymatic bioconversion of nitroglycerin (14). Compounds that cannot act as a precursor to intracellular glutathione, such as D-cysteine or methionine, do not augment the coronary microvascular response to nitroglycerin. Because the thioester in SPM-5185 probably is not available as a precursor to glutathione, SPM-5185 remains a selective dilator of conductance vessels such as rat aorta and the larger coronary microvessels, while not affecting the smaller coronary microvessels.

SPM 5185 was considerably more effective than SPM 4744 in producing relaxation of larger rings of aorta. The mechanism for this remains obscure. Potentially, cleavage of the *S*-acyl function in SPM 5185 could result in a spontaneous nitric oxide donor, whereas SPM 4744 would probably require enzymatic biotransformation like other organic nitrates. Why a similar difference in reactivity for the two compounds was not observed in the larger coronary microvessels is not clear. Comparison between different vascular beds and between species makes interpretation of these differences difficult.

The phenomenon of coronary steal is believed to be related to inappropriate vasodilatation of smaller coronary arteries in nonischemic regions of the myocardium (21–23). The relative selectivity of nitroglycerin and, as demonstrated in the present work, the mononitrates, probably decreases their potential for the development of coronary steal (22). These properties of the mononitrates and other organic nitrates probably are advantageous in the treatment of angina pectoris.

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