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Nitric Oxide Inhibits Vascular Bioactivation of Glyceryl Trinitrate: A Novel Mechanism to Explain Preferential Venodilation of Organic Nitrates

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ABSTRACT

Organic nitrates undergo enzymatic metabolization in the vasculature to release the active compound nitric oxide (NO). The resulting preferential venodilation has been suggested to be related to the vascular bioactivation process of organic nitrates because sodium nitroprusside, which is bioactivated differently, is not venoselective. We sought to determine whether NO has an influence on vascular bioconversion of organic nitrates because endogenous endothelial production of NO is smaller in veins than in arteries. Rings of porcine coronary arteries were subjected to radioactive glyceryl trinitrate (GTN) after preincubation with defined amounts of NO. The vascular content of GTN and the dinitrates (GDNs) 1,2-GDN and 1,3-GDN then was quantified. NO (3 μM, 30 min) significantly impaired bioactivation of GTN as indicated by a 30–50% reduction in the accumulation of 1,2-GDN and 1,3-GDN, whereas unchanged GTN was increased. Incubation with NO also reduced the stimulated specific activity of soluble guanylate cyclase isolated from human platelets. Its specific activity was reduced from 2.6 $\pm$ 0.2 to 2.1 $\pm$ 0.13 nmol of cGMP/mg/min. Relaxation studies with rings of porcine coronary arteries showed that NO-induced inhibition of vascular GTN metabolism and cGMP accumulation decreased the vasodilator potency of GTN by 10-fold. Further experiments showed that the duration of NO treatment is more important for this effect than the concentration of NO. We suggest that NO can inhibit vascular bioactivation of organic nitrates and might slightly desensitize soluble guanylate cyclase. The preferential venodilation induced by organic nitrates might be the result of the comparably low production of endogenous NO in veins.

Organic nitrates such as GTN are widely used for the treatment of coronary artery disease and heart failure. It has been shown that organic nitrates are prodrugs that undergo enzymatic bioactivation within the vascular wall to release NO, which is the pharmacologically active compound (Feelisch and Noack, 1987; Chung and Fung, 1990). GTN-induced vasorelaxation is preceded by vascular formation of the 1,2-GDN and 1,3-GDN and by activation of soluble guanylate cyclase producing cGMP (Brien *et al*., 1986). There is a general agreement that other organic nitrates, such as isosorbide dinitrate, isosorbide-5-nitrate, and pentaerythritol tetranitrate, undergo a similar bioactivation process as a prerequisite for their pharmacological activity (Ahlner *et al*., 1991). The second messenger cGMP activates the cGMP-dependent protein kinase and initiates several effects such as phosphorylation of myosin light chain, sequestration of intracellular calcium, reduction of calcium entry from the extracellular space, reduced release of intracellularly stored calcium, and inhibition of formation of inositol-1,4,5-triphosphate (Pfitzer *et al*., 1984; Collins *et al*., 1986; Twort and van Breemen, 1988; Lang and Lewis, 1989).

Among antianginal drugs used for therapy of coronary artery disease, organic nitrates elicit unique and favorable hemodynamic changes. The most striking difference from other antianginal drugs such as β blockers and calcium antagonists is the preferential venodilation causing preload reduction (Bassenge and Stuart, 1986). Selective reduction of preload has several advantages for patients with coronary artery disease; it reduces left ventricular end-diastolic pressure and systolic ventricular wall tension and increases cardiac output. Interestingly, the preferential reduction of preload also distinguishes organic nitrates from other nitrovasodilators such as sodium nitroprusside despite the presumed common generation of NO (Armstrong *et al*., 1975). It has been shown that sodium nitroprusside undergoes a completely different bioactivation process in the vascular wall (Bates *et al*., 1991; Kowaluk *et al*., 1992). Thus, it is

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ABBREVIATIONS: GTN, glyceryl trinitrate; GDN, glyceryl dinitrate; DEA/NO, 2,2-dietyl-1-nitroso-oxihydrazine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SPER/NO, 1,3-propandiamin-*N*-[4[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]*butyl*]; DTT, dithiothreitol; HPLC, high performance liquid chromatography; $PGF_{2\alpha}$; prostaglandin $F_{2\alpha}$; 8-pCPT, 8-(4-chlorophenylthio); KHP, Krebs-Henseleit; DMSO, dimethylsulfoxide.

likely that selective reduction of preload is related to the enzymatic bioactivation of organic nitrates.

One major difference between arteries and veins is the intensity of endogenous NO production in endothelial cells. Stimulation of venous endothelium results in a low production of NO as demonstrated by the weak endothelium-dependent vasorelaxation in veins of different species, including humans (De Mey and Vanhoutte, 1982; Lüscher *et al.*, 1988; Kojda *et al.*, 1994). The different intensity of endogenous NO production in arteries and veins might have an impact on vascular bioactivation of organic nitrates, leading to preferential venodilation. In accordance, it has been shown previously that endogenous NO production by the vascular endothelium reduces the vasodilator potency of organic nitrates such as GTN (Alheid *et al.*, 1987; Moncada *et al.*, 1991; Kojda *et al.*, 1994). In this study, we sought to determine the influence on the activity of GTN of pretreatment of coronary arteries with NO. We measured the kinetics of NO release from the used NO donors, the vascular formation of 1,2-GDN and 1,3-GDN, alterations in the activity of isolated human soluble guanylate cyclase, vascular accumulation of cGMP, and vasorelaxation induced by GTN and by a specific stimulator of cGMP-dependent protein kinase. We demonstrate that continuous subjection of coronary arteries to micromolar concentrations of NO inhibits vascular bioactivation of GTN, accumulation of cGMP, and vasorelaxation. Pretreatment with NO also caused a desensitization of soluble guanylate cyclase.

Materials and Methods

Measurement of NO release. Release of NO by GTN, DEA/NO, SNAP, and SPER/NO was measured at pH 7.4 and 37° in the presence of oxygen with a commercially available NO meter (ISO-NO; World Precision Instruments, Berlin, Germany) that works in a manner similar to that of the well known Clark-type electrode for oxygen. Calibration of the electrode was performed daily before the experiments. Volumes (10 μl) of aqueous KNO_2 solution (2 μM), used as a generator of NO, were added cumulatively (four times) to 300 μl of a mixture of KI and H_2SO_4 (0.1 M each). A typical plot of the measured signal (in pA) versus the NO concentration (in nM), calculated on the basis of a quantitative reaction of KNO_2 to NO according to the equation $2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I_2 + 2H_2O + 2K_2SO_4$, yielded a linear relationship with a correlation coefficient of 0.999 and a slope of 0.87 nM NO/pA.

Preparation of guanylate cyclase. Preparation of human platelet guanylate cyclase was performed as reported previously (Kojda and Noack, 1993). Briefly, 1000 ml of human platelet-rich plasma was mixed with 50 ml of EDTA (0.1 M), and platelets were concentrated by centrifugation (1000 $\times$ *g* for 10 min). The platelets were washed twice with Tris buffer (50 mM, pH 7.6) containing 154 mM NaCl by repeated resuspension and centrifugation (500 $\times$ *g*). Washed platelets were resuspended in the Tris buffer (16 ml) and slowly cooled to 4°, which was the temperature for the next steps. Lysis of platelets was achieved by the addition of 100 ml of hypotonic Tris buffer (5 mM, pH 7.6) containing 0.05% leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 1 mM DTT. During lysis, platelets were sonicated (50 W, 30 sec). The supernatant from centrifugation at 10,000 $\times$ *g* for 10 min was collected and recentrifuged at 105,000 $\times$ *g* for 1 hr. The obtained cytosolic fraction was loaded onto a diethylaminoethanol-Sepharose column (HiLoad 26/10 Q Sepharose HP; Pharmacia, Freiburg, Germany) after preequilibration with Tris buffer containing 1 mM DTT. A linear sodium chloride (0–0.4 M) gradient in the same buffer was started. Active fractions (cGMP accumulation >30% of the maximal value) were identified after stimulation with 500 μM SNAP in the presence of 1 mg/ml bovine serum albumin and were pooled and stored in aliquots at −80°. Protein content was measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

Determination of guanylate cyclase activity. Specific activity of soluble guanylate cyclase was measured on the basis of the formation of [^{32}P]cGMP from [α-^{32}P]GTP as described previously (Schulz and Böhme, 1984). Briefly, soluble guanylate cyclase of the single diethylaminoethanol fractions (20–40 μg of protein) was incubated in a total volume of 100 μl of a triethanolamine HCl buffer (50 mM, pH 7.4, 37°) containing 5 nM [α-^{32}P]GTP (0.4 μCi), 100 μM GTP, 1 mM cGMP, 1 mM 3-isobutyl-1-methylxanthine, 1 mM $MgCl_2$, and 1 mM DTT in the presence of 500 μM SNAP or vehicle (0.25% DMSO). To determine the dose-dependent effects of SNAP, SPER/NO, DEA/NO, and GTN (with or without 5 mM cysteine), the assay volume contained concentrations of these drugs or vehicle as indicated in Results.

Preparation of isolated vessel segments. Right coronary arteries were obtained from the local slaughterhouse and taken from the hearts of freshly slaughtered female pigs (5–7 months old). Coronary arteries were prepared free from the aorta to the ramus interventricularis posterior and perfused with cold KH buffer, pH 7.4, containing 143.07 mM Na^+, 5.87 mM K^+, 1.6 mM Ca^{2+}, 1.18 mM Mg^{2+}, 125.96 mM Cl^-, 25.00 mM HCO_3^-, 1.18 mM $H2PO_4^-$, 1.18 mM SO_4^{2-}, and 5.05 mM glucose. The arteries were cut from their muscle foundation, immediately stored in cooled KH buffer, and transferred into the laboratory, where they were carefully dissected free from all surrounding tissue. The proximal ends were rejected, and the remainder of the arteries was cut into rings (length, 5 mm). Great care was taken to preserve the intimal endothelium. In some cases, its function was controlled in separately performed organ bath studies. Two to four coronary segments were put into a polypropylene vial and equilibrated (37°) in modified and oxygenated (95% O_2/5% CO_2) KH buffer for 90 min. The buffer was exchanged every 15 min.

Aortic segments of rats were prepared similarly. Aortas were excised rapidly from male 3–4-month-old Wistar rats. These segments were used only for organ bath studies.

Determination of vascular metabolites of GTN. The porcine coronary artery rings were incubated in KH buffer after the application of vehicle [0.1% ethanol (v/v) or 0.01 M NaOH], 100 μM DEA/NO (four cumulative applications every 8 min), 100 μM SNAP, 200 μM SPER/NO, and 100 μM GTN. Incubation was terminated by repeated washout (two times at once and two times after 5 and 15 min). Then, [2-^{14}C]GTN (specific activity, 55 mCi/mmol) was added. The amount of the radioactivity was 0.25 μCi (2.28 μM GTN). After 2 min, these coronary rings were flash frozen with liquid nitrogen and stored at −20°.

Porcine coronary rings were thawed, cut, and homogenized in 1 ml of ice-cooled NaCl solution (0.9%) with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After being washed twice in 1 ml of NaCl solution, the 3-ml suspension was extracted three times with dichloromethane (Uvasol; Merck, Darmstadt, Germany) in a ratio of 1:1. Preliminary experiments revealed that one extraction step resulted in an accumulation of 1,3-GDN, 1,2-GDN, and GTN in the dichloromethane phase, amounting to 76.2 ± 1.6%, 82.6 ± 1.9%, and 98.7 ± 4.4% (six experiments), respectively. Thus, triple extraction yielded a recovery rate of GTN and the dinitrate metabolites of ≈99%. The pooled dichloromethane phases (9 ml) were transferred in 20-ml plastic tubes, evaporated to dryness, and stored at −20° for a maximum of 10 days. The extracted buffer phase was centrifuged again, and the pellet was used for protein determination (Bradford, 1976).

Separation of GTN and the dinitrate metabolites by HPLC. After reconstitution of the evaporated dichloromethane phase (see above) in 55 μl of dichloromethane, 5 μl of a stock solution of GTN, 1,2-GDN, and 1,3-GDN (2 mM each) was added (final concentration, 167 μM), and the mixture was used directly for separation by HPLC. A liquid chromatograph was used (655A-11, LC-controller L 5000,

chromatointegrator D-2000; Merck/Hitachi, Merck, Darmstadt, Germany), including a Li Chro Cart R 250–4 Superspher 100 RP-18 column combined with a Li Chro Cart R 4–4 filled with Li Chrosorb RP-18 (5 μm) as a precolumn. The columns were equilibrated with methanol/phosphate buffer [50 mM, pH 7.4; 4:6 (v/v)] at a flow rate of 0.5 ml/min (pressure, 193 kg/cm^2) and room temperature (20–24°). Analysis was started by the injection of 50 μl of the reconstituted and spiked dichloromethane solution by means of an injection slope. A representative tracing of the separation is shown in Fig. 1. Collection of samples (24-sec steps for dinitrates and 60-sec steps for GTN) was done in 20-ml plastic tubes (Frac 100 fraction collector; Pharmacia) according to the peak pattern registered in parallel by UV detection at 210 nm (UV-Detector, 655 A variable wavelength; Merck Hitachi). After the addition of 10 ml of Rotiszint (eco plus; Carl Roth & Co., Karlsruhe, Germany), scintigraphic determination (in cpm) was done with a Beckman Instruments counter (LS 6500 or LS 5000 TD; Columbia, MD).

Determination of vascular cGMP accumulation. Porcine coronary artery rings were freshly prepared and cut to a length of 1 cm. These rings were equilibrated for 3 hr at 37° in polyethylene vials containing continuously oxygenated KH buffer, which was changed every 30 min. Then, the rings were incubated with GTN (100 μM), SNAP (100 μM), or vehicle (0.9% NaCl and 0.05% DMSO in KH buffer) for 30 min. Incubation was stopped by repeated (three times) washout with KH buffer (within 30 min). Thereafter, the rings were incubated again with GTN (10 μM), and after 5 min, rings were flash-frozen in liquid nitrogen and stored at −80°. Frozen artery rings were homogenized with a Polytron in 1 ml of ice-cold $HClO_4$ (10%) and then centrifuged at 4500 × *g* for 10 min. The pellet was used for protein determination (Bradford, 1976); 900 μl of supernatant was neutralized (pH 7.4) with K_3PO_4, centrifuged again, and used directly for determination of cGMP by radioimmunoassay with ^{125}I-cGMP as radiolabeled antigen. Preliminary experiments with this method yielded recovery rates for cGMP and protein of >90% (Kojda and Noack, 1993).

Organ bath studies. Porcine coronary arteries were cut into ring segments (4 mm) and fixed between stainless-steel hooks in a waterjacketed organ bath (37°) as described previously (Kojda *et al*., 1991). Resting tension was 2 *g*. After equilibration (1 hr), contractile function was tested by the addition of KCl (60 mM) and $PGF_{2\alpha}$ (0.1–100 μM) to reach a maximal tension of ≈5 *g*. The presence of intact endothelium was verified by complete, transient relaxation of $PGF_{2\alpha}$-precontracted (10 μM) segments after the application of 3 nM substance P (Cocks and Angus, 1983). Vasorelaxing activities of GTN (1 nM to 100 μM), DEA/NO (1 nM to 100 μM), and SNAP (1 nM to 100 μM) were evaluated by cumulative application after precontraction with $PGF_{2\alpha}$ (50 μM). To study the influence of NO on relaxant activity of GTN in these arteries, cumulative application of GTN was performed after washout (15 min) of a 30-min preincubation with either vehicle (0.01 M NaOH and 0.9% NaCl), GTN (100 μM), SNAP (100 μM), DEA/NO (100 μM once or 100 μM four times every 8 min), or SPER/NO (200 μM). In some experiments, this preincubation procedure was followed by the cumulative application of 8-pCPT-cGMP, a membrane-permeable activator of cGMP-dependent protein kinase (Sekhar *et al*., 1992).

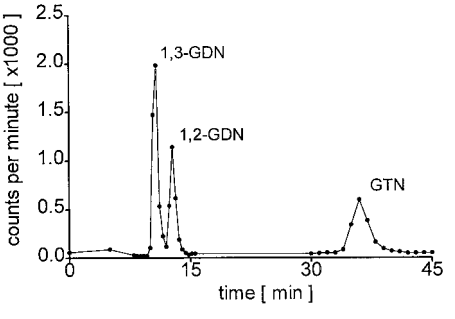

Fig. 1. Separation of 1,2-GDN, 1,3-GDN, and GTN extracted from vascular tissue and reconstituted in dichloromethane. Radioactivity was measured in fractions collected at time intervals of 24 sec (8–16 min) and 60 sec (30–45 min) that eluted from the HPLC column. Incubations of isolated porcine coronary arteries were performed for 2 min with 2.28 μM [2-^{14}C]GTN (specific activity, 55 mCi/mmol). Visualization of the peaks to simplify fraction collection was accomplished by spiking the dichloromethane extract to give concentrations of GTN and its metabolites (167 μM each) that were easily detected with a UV detector.

Organ bath studies with rings of rat aorta were performed in a similar manner as described previously (Kojda and Noack, 1993). Intact endothelium was verified by dose-dependent (1 nM to 1 μM) vasorelaxation in response to acetylcholine after precontraction with phenylephrine (0.2 μM). Vasorelaxation due to cumulative application of 8-pCPT-cGMP (10 nM to 100 μM) was investigated in aortic rings precontracted with 3 μM phenylephrine after a 30-min preincubation period with either vehicle (10 μl of 0.01 M NaOH in 10 ml of buffer) or SPER/NO (200 μM).

Substances and solutions. SNAP was synthesized according to Field *et al*. (1978) as described previously (Kojda *et al*., 1996). GTN (4.404 mM in 154 mM NaCl, used directly as stock solution) was generously provided by Schwarz Pharma AG (Monheim, Germany). DEA/NO and SPER/NO was a gift from Dr. L. Keefer (National Cancer Institute, Frederick, MD). 8-pCPT-cGMP was obtained from Biolog (Bremen, Germany). [2-^{14}C]GTN (specific activity, 55 mCi/mmol) was obtained from Biotrend (Köln, Germany). [α-^{32}P]GTP (specific activity, 800 Ci/mmol) was obtained from Du Pont de Nemours (Bad Homburg, Germany). Leupeptin, phenylephrine, acetylcholine, 3-isobutyl-1-methylxanthine, $PGF_{2\alpha}$, and phenylmethylsulfonyl fluoride were obtained from Sigma Chemie (Deisenhofen, Germany). All other chemicals (analytical grade) were obtained from Merck.

Stock solutions (10 mM) of DEA/NO and SPER/NO in 0.01 M NaOH, of SNAP in KH buffer containing 5% DMSO or 5% ethanol, and of 8-pCPT-cGMP in KH buffer were prepared daily and kept, protected from daylight, on ice until use. All concentrations indicated in the text, figures, and tables are expressed as final assay or organ bath concentrations.

Statistics. Vasorelaxation is expressed as remaining percentage of the contractile response achieved with $PGF_{2\alpha}$ (50 μM) at the beginning of the experiment. The concentrations for half-maximal inhibition of precontraction (IC_{50}) were calculated from the individual concentration-effect curves as proposed by Hafner *et al*. (1977). The pD_2 values, representing the negative logarithms of the half-maximal inhibiting concentrations, were taken to test for significant differences. All data were analyzed by one-way analysis of variance with subsequent Student-Newman-Keuls test (SAS PC Software 6.04, PROC ANOVA; SAS Institute, Cary, NC) and are expressed as mean ± standard error values. Significant differences were evaluated by using Student's *t* test, and a value of $p < 0.05$ was considered significant.

Results

Kinetics of NO release by the different nitrovasodilators. Release of NO by GTN, SNAP, DEA/NO, and SPER/NO, which was measured under conditions that were present in the experiments with isolated enzymes and tissues (pH 7.4, 37°, presence of oxygen), showed substantial differences (Fig. 2). DEA/NO rapidly degraded, yielded the highest concentration of NO, and NO release was completed after 6–7 min. SNAP showed a similar time course of NO release but a much lower peak concentration of NO. SPER/NO degraded slowly. Maximal concentrations of NO were similar to those released by SNAP but occurred later. Maximal NO concentrations remained constant for ≈5 min and then slowly declined. Calculation of the area under curve resulted in simi-

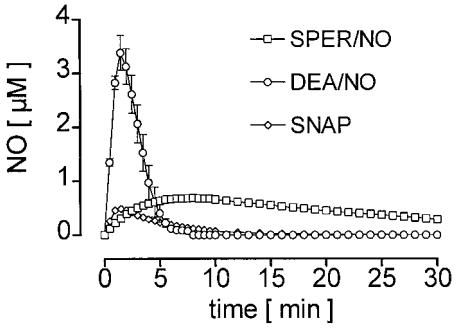

Fig. 2. Kinetics of NO release from 10 µM of the spontaneous NO donors SPER/NO, DEA/NO, and SNAP. The experiments were performed in oxygenated buffer (37°, pH 7.4), and NO was measured polarographically. Plotted are the mean ± standard error values of NO concentration measured at each time point in three different experiments.

lar values for DEA/NO (9,755 ± 1,404 nM × min) and SPER/NO (14,697 ± 1,770 nM × min), whereas the release of NO from SNAP was significantly lower (2,890 ± 311 nM × min, $p < 0.01$). There was no detectable NO release from 10 µM GTN in the absence and presence of 5 mM cysteine.

Activation of soluble guanylate cyclase from human platelets. The basal activity of the enzyme preparation was 70.2 ± 10.3 pmol cGMP/mg/min (16 experiments). The spontaneous NO donors SPER/NO, DEA/NO, and SNAP dose-dependently activated soluble guanylate cyclase partially purified from human platelets. DEA/NO and SPER/NO equieffectively activated the enzyme, but the activity of NO donor SNAP was ≈10-fold lower (data not shown). The specific activity of soluble guanylate cyclase after incubation with a maximally effective concentration of DEA/NO, SPER/NO, and SNAP was 5.18 ± 0.47 (six experiments), 6.78 ± 0.27 (six experiments), and 5.38 ± 0.31 (six experiments) nmol of cGMP/mg/min, respectively. In presence of equimolar concentrations of oxyhemoglobin, a scavenger of NO, the stimulating effect of 10 µM SNAP (382 ± 43 pmol of cGMP/mg/min, six experiments) was abolished completely.

Maximal stimulation of the enzyme preparation by GTN was very low and occurred at a concentration of >100 µM. Thus, formation of NO from GTN within the assay buffer was negligible. In contrast, GTN considerably activated soluble guanylate cyclase in the presence of 5 mM cysteine, although the maximal stimulation still occurred at concentrations of >100 µM and was substantially lower than the effect of the spontaneous NO donors.

Desensitization of soluble guanylate cyclase from human platelets. To determine whether prolonged subjection of soluble guanylate cyclase with NO results in a change of enzyme activity, maximal stimulation of the enzyme with 500 µM SNAP was investigated after preincubation with either vehicle (0.05% DMSO) or 100 µM SNAP for different time periods (Fig. 3). The significantly lower maximal stimulation after preincubation with SNAP for 30 and 45 min indicates a desensitization of the enzyme by NO. A similar preincubation procedure using 10 µM SNAP had no effect on the activity of the enzyme (data not shown).

Generation of cGMP in isolated arteries. Accumulation of cGMP was determined in porcine coronary artery rings stimulated with GTN. To determine whether prolonged subjection of these rings with NO results in a change on cGMP accumulation, stimulation of the arteries with 10 µM

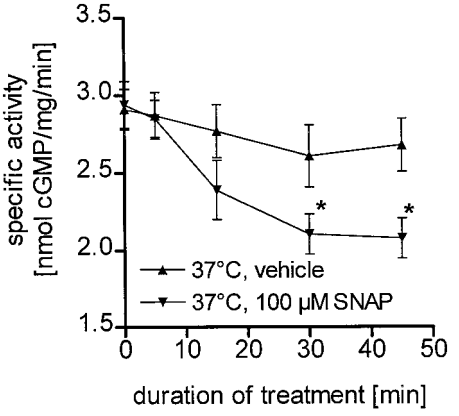

Fig. 3. Influence of a preincubation with either vehicle (0.05% DMSO) or 100 µM SNAP for different time periods on maximal activation of soluble guanylate cyclase isolated from human platelets. Reactions to measure maximal activation were started after the preincubation periods by simultaneous addition of the radioactive substrate (α-^{32}P-GTP) and 500 µM SNAP. Plotted are mean ± standard error values of specific activity measured in four different experiments for each time point. *, Significant difference compared with control conditions, $p < 0.05$.

GTN was preceded by preincubation with either SNAP (100 µM) or GTN (100 µM) for 30 min. After preincubation with vehicle, a GTN-induced (10 µM) cGMP accumulation of 8.2 ± 1.4 pmol/mg was observed. Preincubation with both SNAP and GTN significantly diminished GTN-induced (10 µM) accumulation of cGMP to 5.4 ± 1.2 pmol/mg ($p < 0.05$) and 1.3 ± 0.4 pmol/mg ($p < 0.01$), respectively.

Vascular formation of 1,2-GDN and 1,3-GDN. The total radioactivity recovered by extraction of porcine coronary rings after a 2-min incubation period with ^{14}C-GTN was separated into three fractions (^{14}C-1,3-GDN, ^{14}C-1,2-GDN, and ^{14}C-GTN) by HPLC and corrected for protein content (Fig. 1, Table 1). A 5-min incubation period increased these values only slightly for GTN (from 5732 ± 977 to 6779 ± 384 cpm/mg), 1,2-GDN (from 5705 ± 572 to 6761 ± 192 cpm/mg), and 1,3-GDN (from 6535 ± 532 to 7762 ± 256 cpm/mg). Thus, all experiments on vascular metabolization of GTN were done using a 2-min incubation with ^{14}C-GTN.

Any pretreatment of porcine coronary artery rings with either GTN or the spontaneous NO donors resulted in impaired vascular metabolization of GTN, whereas a 30-min preincubation period with either vehicle had no effect. Preincubation with GTN significantly decreased formation of the GDN metabolites (Table 1). As depicted in Fig. 4, formation of 1,2-GDN was significantly more impaired than formation of 1,3-GDN. Preincubation with spontaneous NO donors also decreased formation of the GDN-metabolites (Table 1). In contrast to preincubation with GTN, the impairments of formation of both GDNs were similar.

Relaxation of porcine coronary arteries. The vasorelaxing potency of GTN was determined in isolated ring segments of porcine right coronary artery that had been preincubated for 30 min with vehicle, GTN, or a NO donor (DEA/NO, SPER/NO, or SNAP). The different vasorelaxing potencies of GTN are listed in Table 2. Preincubation with any spontaneous NO donor resulted in a comparable shift to the right of the dose-response curve of GTN (Fig. 5), indicating a desensitization of the vessel segments to the relaxant effects of GTN. The decrease in respective pD_2 values was approximately one order of magnitude (Table 2). Preincuba-

TABLE 1

Contents of GTN and the dinitrates in porcine coronary artery rings pretreated for 30 min with vehicle, different spontaneous NO donors, or GTN

GTN and the metabolites were measured after a 2-min incubation with [2-^{14}C]GTN. Values are mean ± standard error of experiments with two or three rings from four or five different animals (n)

Preincubation	n	Total recovery	Partial recovery	Percent of total recovery
		cpm / mg of protein		
1,3-GDN				
Vehicle 1	4	15,306 ± 4,419	4,810 ± 617	35.6 ± 4.9
DEA/NO	4	25,191 ± 4,728	7,032 ± 1,570	27.3 ± 2.4[a]
SPER/NO	5	19,227 ± 1,669	5,130 ± 525	26.8 ± 2.0[a]
Vehicle 2	4	17,974 ± 2,050	6,535 ± 532	36.7 ± 1.2
SNAP	4	16,350 ± 1,098	4,492 ± 634	27.3 ± 2.1[a]
GTN	5	18,725 ± 1,548	5,472 ± 111	29.9 ± 2.4[a]
1,2-GDN				
Vehicle 1	4	15,306 ± 4,419	4,175 ± 670	30.1 ± 3.4
DEA/NO	4	25,191 ± 4,728	6,002 ± 1,158	23.1 ± 2.1[a]
SPER/NO	5	19,227 ± 1,669	4,165 ± 451	21.9 ± 2.4[a]
Vehicle 2	4	17,974 ± 2,050	5,705 ± 572	31.9 ± 0.9
SNAP	4	16,350 ± 1,098	3,764 ± 52	23.1 ± 1.8[a]
GTN	5	18,725 ± 1,548	3,536 ± 49	19.3 ± 1.4[a]
GTN				
Vehicle 1	4	15,306 ± 4,419	6,320 ± 3,284	34.1 ± 8.2
DEA/NO	4	25,191 ± 4,728	12,156 ± 2,503	48.7 ± 4.3[a]
SPER/NO	5	19,227 ± 1,669	9,930 ± 1,335	51.2 ± 4.1[a]
Vehicle 2	4	17,974 ± 2,050	5,732 ± 977	31.3 ± 1.9
SNAP	4	16,350 ± 1,098	8,093 ± 515	49.5 ± 0.1[a]
GTN	5	18,725 ± 1,548	9,715 ± 1,519	50.6 ± 3.7[a]

[a] Significant difference compared with vehicle conditions.

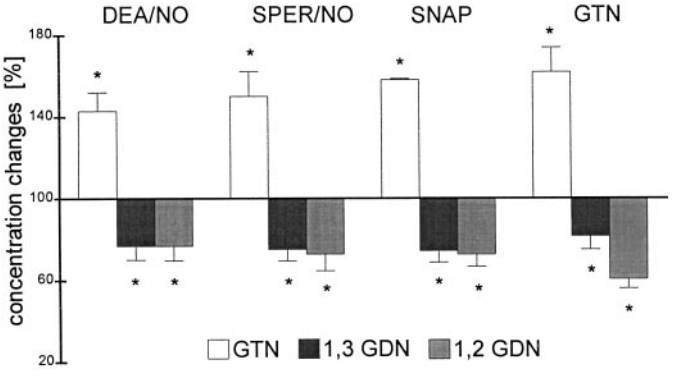

Fig. 4. Inhibition of GTN metabolization in the vascular wall of porcine coronary arteries after pretreatment with the different spontaneous NO donors SNAP, DEA/NO, and SPER/NO and by GTN itself compared with controls (preincubation with vehicle, set to 100%). Plotted are mean ± standard error values of the percentage changes in 1,2-GDN, 1,3-GDN, and GTN that were detected in one or two coronary rings of four or five different animals (for absolute values, see Table 1). The 30-min pretreatment period was followed by a 15-min washout period and subsequent subjection of the rings with 2.28 μM [2-^{14}C]GTN for 2 min. GTN and the GDN metabolites were extracted and separated by HPLC as shown in Fig. 1. *, Significant difference compared with control conditions, $p < 0.05$. Preincubation with any of the NO donors substantially decreased bioactivation of GTN as indicated by the reduced vascular formation of the GDNs.

tion with GTN, which was performed as a control experiment, resulted in the most pronounced rightward shift of the concentration-response curve of GTN (Fig. 5, Table 2). Interestingly, the time of subjection of the arteries to NO is most likely more important than the concentration of NO itself. Preincubation with a single application of 100 μM of DEA/NO, which liberated almost 10 times more NO compared with SPER/NO (Fig. 2), did not change the pD_2 value of GTN (6.51 ± 0.12, six experiments), whereas a single application of SPER/NO or a repetitive application of DEA/NO did (100 μM, every 8 min within 30 min) (Table 2). In contrast, pretreatment with GTN did not decrease the vasorelaxing potency of the NO donor SNAP (Table 2), indicating that under

TABLE 2

Vasodilator potencies of GTN, SNAP, and DEA/NO as evaluated by cumulative application to ring segments of porcine coronary arteries precontracted with 50 μM $PGF_{2\alpha}$

In some experiments, preincubation with GTN (100 μM), SNAP (100 μM), DEA/NO (four times 100 μM every 8 min), or SPER/NO (200 μM) preceded cumulative applications. Given are mean pD_2- values (in −log M) and standard error values for n individual experiments.

Drug	Preincubation *with*	pD_2	n
GTN	Vehicle	6.32 ± 0.10	7
	GTN	5.16 ± 0.12[a]	7
	SNAP	5.59 ± 0.14[a]	6
DEA/NO	5.43 ± 0.12[a]	5	
	SPER/NO	5.54 ± 0.10[a]	5
SNAP	Vehicle	7.55 ± 0.06	9
	GTN	7.35 ± 0.16	6
DEA/NO	Vehicle	7.27 ± 0.09	8

[a] Significant difference compared with vehicle conditions.

these conditions, the capacity to produce cGMP by NO or the efficacy of cGMP itself is not affected.

Activation of cGMP-dependent protein kinase in intact arteries. To study the sensitivity of vascular cGMP-dependent protein kinase, cumulative applications of 8-pCPT-cGMP, a congener of cGMP, were performed in porcine coronary arteries pretreated with vehicle, SPER/NO, or GTN. None of these pretreatments altered the vasodilator activity of 8-pCPT-cGMP indicating an unchanged activity of vascular cGMP-dependent protein kinase (Table 3). However, the very low vasorelaxing potency of 8-pCPT-cGMP in porcine coronary arteries might have masked any potential variation of the activity of cGMP-dependent protein kinase. Thus, the experiments were repeated using rat aorta. In this vessel type, 8-pCPT-cGMP is a much more potent vasodilator (Fig. 6). Nevertheless, preincubation of rat aorta with 200 μM SPER/NO for 30 min had no effect on relaxations in response to 8-pCPT-cGMP. These results indicate that prolonged subjection of vascular smooth muscle to either NO or GTN does not change the activity of cGMP-dependent protein kinase.

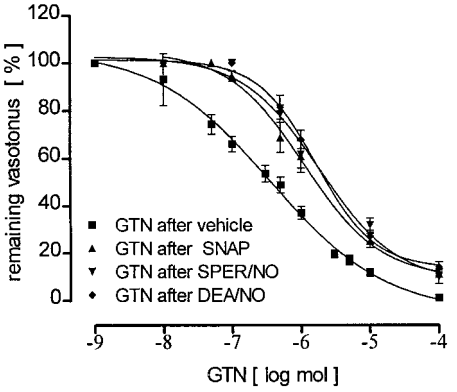

Fig. 5. Inhibition of GTN-induced vasorelaxation of isolated porcine coronary arteries after pretreatment with the different spontaneous NO donors SNAP, DEA/NO, and SPER/NO compared with controls (preincubation with vehicle). The vasodilatory response is expressed as percentage of precontraction induced by 50 μM $PGF_{2\alpha}$, and each dose-response curve was plotted by taking the respective mean values of one or two ring preparations from coronary arteries of 6–10 different animals [mean ± standard error (*bars*)]. The respective concentrations for half-maximal vasodilation are given in Table 2. It is evident that preincubation with any of the NO donors substantially decreased the vasorelaxing potency of GTN.

TABLE 3

Vasodilator potency of 8-pCPT-cGMP in isolated ring segments of porcine coronary artery precontracted with 50 μM $PGF_{2\alpha}$

In these experiments, preincubation with vehicle (0.01 M NaOH and 0.9% NaCl), GTN (100 μM), or SPER/NO (200 μM) preceded cumulative application of 8-pCPT-cGMP. Given are mean pD_2 values (in −log M) and standard error value of n individual experiments. No significant differences were observed.

Preincubation condition	Half-maximal vasorelaxing potency of 8-pCPT-cGMP	
	SPER/NO	GTN
	−log M	
Vehicle	4.74 ± 0.09 (n = 9)	4.66 ± 0.08 (n = 7)
Nitrate	4.52 ± 0.10 (n = 10)	4.82 ± 0.09 (n = 7)

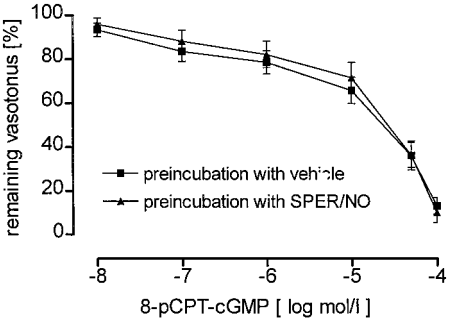

Fig. 6. Lack of effect of pretreatment with the spontaneous NO donor SPER/NO compared with controls (preincubation with vehicle) on the vasodilator potency of 8-pCPT-cGMP, a cGMP analog that penetrates cells, does not undergo hydrolysis by phosphodiesterases, and specifically activates cGMP-dependent protein kinase. The vasodilatory response is expressed as percentage of precontraction induced by 3 μM phenylephrine, and each dose-response curve was plotted by taking the respective mean values of one or two ring preparations from rat aorta of nine different animals [mean ± standard error (*bars*)]. It is evident that preincubation with SPER/NO had no effect on activation of cGMP-dependent protein kinase.

Discussion

We studied the effect of NO pretreatment on vascular bioactivation of GTN, the activity of soluble guanylate cyclase, the activity of cGMP-dependent protein kinase, and the vasorelaxing potency of GTN. Our main finding was that NO can reduce vascular formation of dinitrate metabolites from GTN and its vasorelaxing activity as well. For this effect, a continuous subjection of blood vessels to NO is more important than the concentration of NO itself. Our results suggest that the preferential venodilation, which is typical for organic nitrates such as GTN, is at least in part the result of the low endogenous production of NO by the vascular endothelium in veins.

Inhibition of vascular bioactivation of GTN by NO. The inhibitory effect of NO on vascular bioactivation of GTN is a new observation (Fig. 4, Table 1). The NO-induced impairment of formation of GDN-metabolites occurs in parallel with a substantially decreased vasodilator activity of GTN (Fig. 5, Table 2). A decreased vasodilator activity of GTN also occurred after pretreatment with NO of bovine coronary arteries (Zhang *et al.*, 1994) and porcine vena cordis magna (Kojda *et al.*, 1994). Generation of NO from GTN in tissues is most likely an enzymatic process, but a nonenzymatic cleavage of organic nitrates in the presence of thiols such as cysteine also occurs (Feelisch and Noack, 1987; Chung and Fung, 1990). It has been shown earlier that GTN-induced vasorelaxation is preceded by vascular formation of 1,2-GDN and 1,3-GDN (Brien *et al.*, 1986). In our study, the formation of the GDNs was almost completed after 2 min, and a 1:1 ratio of 1,2-GDN to 1,3-GDN of was observed. Similar results were obtained previously (Fung *et al.*, 1984). Theoretically, formation of 1,2-GDN should be twice as great as formation of 1,3-GDN. Thus, our results and those of others suggest that enzymatic denitration of GTN in the vascular wall preferentially occurs at C2 of the molecule.

The pretreatment of isolated coronary arteries with GTN reduced both formation of the GDNs and vasorelaxation (Figs. 4 and 5). We demonstrate that not only GTN but also NO itself limits vascular bioactivation of GTN and its vasorelaxing potency. Interestingly, a high peak concentration of ≈30 μM NO during a 7-min period as generated by DEA/NO (Fig. 2) is not effective, whereas a 10 times lower concentration of NO during a 30-min period as generated by SPER/NO (Fig. 2) effectively diminished GTN-induced vascular bioactivation and vasorelaxation (see Results; Figs. 4 and 5). These results suggest that the duration of NO exposure is more important than the concentration of NO. In accordance, repeated application of DEA/NO had the same effect as a single application of SPER/NO (Figs. 4 and 5).

The identity of the enzyme mediating vascular bioactivation of GTN remains unknown. Preliminary evidence indicates an involvement of cytochrome P450 enzymes in the bioactivation process of GTN (Schröder and Schrör, 1990); it is interesting to speculate that the inhibitory effect of NO on enzymatic bioactivation of GTN (Fig. 4) might be mediated by binding of NO to the heme moiety of hemoproteins such as cytochrome P450 reductases. Further investigations are needed to elucidate the mechanism of action of NO-induced inhibition of GTN bioactivation.

Effects of NO pretreatment on soluble guanylate cyclase and cGMP-dependent protein kinase. The reduction in the vasodilator potency of GTN induced by pretreatment with SNAP and GTN correlated with a reduction in vascular cGMP accumulation. Preincubation with GTN showed the strongest effect on both GTN-induced vasodilation and GTN-induced vascular cGMP accumulation (see Results). Previous studies have provided evidence that a desen-

sitization of soluble guanylate cyclase occurs after pretreatment with GTN (Axelsson and Andersson, 1983) or as a consequence of endogenous NO production (Moncada *et al*., 1991). As shown in Fig. 3, our results not only confirm the results of previous studies but also suggest that desensitization of soluble guanylate cyclase might be the result of a direct interaction between NO and the enzyme (Schulz and Böhme, 1984). It seems conceivable to suggest nitrosation and transnitrosylation reactions as underlying mechanisms (Barnett *et al*., 1994). Soluble guanylate cyclase is known to contain free sulfhydryl groups that are essential for activation of the enzyme. This is consistent with the control of its activity in mammalian cells by redox mechanisms (Goldberg and Haddox, 1977) and suggests that nitrosation of free sulfhydryl groups of the enzyme might occur.

In contrast, our results do not support an involvement of cGMP-dependent protein kinase in the attenuation of GTN-induced vasodilation. Pretreatment with NO had no effect on the vasodilator potency of 8-pCPT-cGMP (Fig. 6, Table 3), which is a cGMP-analog that penetrates into cells, does not undergo hydrolysis by phosphodiesterases, and specifically activates cGMP-dependent protein kinase (Sekhar *et al*., 1992).

Effects on the hemodynamic profile of organic nitrates. It has been shown that the production of NO by the vascular endothelium also suppresses the vasodilator activity of GTN and that desensitization of soluble guanylate cyclase within the arterial smooth muscle probably is involved (Alheid *et al*., 1987; Moncada *et al*., 1991; Kojda *et al*., 1994). In these studies, inhibitors of NO synthase or endothelial denudation increased the vasodilator potencies of organic nitrates such as GTN both *in vitro* and *in vivo*. Recently, we were able to demonstrate that disruption of the endothelial NO synthase gene in mice increases the relaxant potency of GTN in mouse aorta (Kojda *et al*., 1997). Thus, endogenous production of NO by endothelial NO synthase most likely decreases the vasodilator potency of GTN.

Previous results obtained with blood vessels from porcine coronary circulation further suggest that this effect is more pronounced in arteries than in veins (Kojda *et al*., 1994). In accordance, stimulated endogenous NO production measured as endothelium-dependent vasorelaxation was substantially greater in arteries than in veins. Comparable weak endothelium-dependent vasorelaxations in veins of different species, including humans, have been reported by others (De Mey and Vanhoutte, 1982; Lüscher *et al*., 1988). Furthermore, it is known that shear stress, which is much less in veins than in arteries, mainly determines endothelial NO production (Pohl *et al*., 1986).

Thus, the results of the current study suggest that inhibition of vascular bioactivation of organic nitrates by endogenous NO predominantly occurs in arteries. The preferential reduction of preload (Ahlner *et al*., 1991) may be a consequence of a less pronounced inhibition of the bioactivation process by endogenous NO in veins (Fig. 7). Our hypothesis is consistent with a recent report demonstrating a higher production of NO from GTN in veins than in arteries *in vivo* (Mülsch *et al*., 1995). It also is consistent with the well known lack of venoselectivity of sodium nitroprusside (Ahlner *et al*., 1991). Sodium nitroprusside is a nitrovasodilator that undergoes a completely different bioactivation process; a difference that is considered to be important for the striking hemodynamic differences between this drug and organic nitrates despite the presumed common generation of NO and cGMP being the mechanism of action responsible for vasorelaxation (Kowaluk *et al*., 1992).

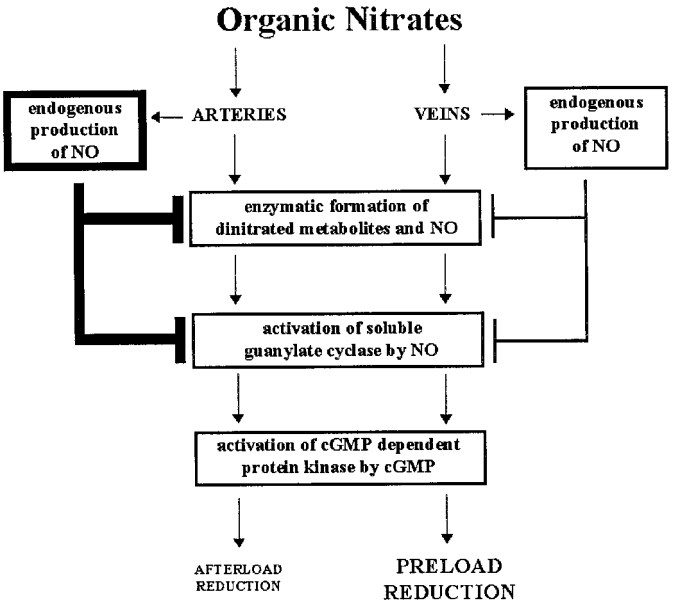

Fig. 7. Suggested mechanism underlying the preferential venodilation elicited by organic nitrates. Prolonged treatment with exogenous NO in concentrations of <3 μM can diminish both vascular bioactivation of GTN and activation of soluble guanylate cyclase. Because production of endogenous NO in the vascular endothelium is substantially lower in veins than in arteries, it is likely that NO-induced inhibition of soluble guanylate cyclase (see Fig. 3) and of bioactivation of organic nitrates (see Fig. 4) also is lower in venous blood vessels. The inhibitory effect of NO on the enzymatic bioactivation seems to be more important for the preferential venodilation induced by organic nitrates than NO-induced desensitization of guanylate cyclase. The nitrovasodilator sodium nitroprusside, which undergoes a different bioactivation process but also acts via NO, does not show a venoselective action.

Mechanism and kinetics of NO release by spontaneous NO donors. In this study, spontaneous NO donors were used instead of NO. NO rapidly reacts with superoxide anions present in all oxygenated physiological buffers to form peroxynitrite, which is pharmacologically active (Beckman and Crow, 1993). The rapid generation of peroxynitrite from NO and superoxide (Goldstein and Czapski, 1995) implies that a delayed release of NO from NO donors prevents a rapid increase in the peroxynitrite concentration. Furthermore, a considerable portion of the NO donor molecules diffuse next to target cells before NO is released, which reduces the probability of oxidation of NO before induction of pharmacological actions such as vasodilation.

DEA/NO, SPER/NO, and SNAP show very different time courses of NO release in our buffer system (Fig. 2). Most notably, calculation of the area under curve revealed that DEA/NO and SPER/NO produce almost similar amounts of NO, whereas spontaneous NO release from SNAP is approximately four to five times less. This probably is the result of different mechanisms of NO release. DEA/NO and SPER/NO are stable in alkaline solutions but spontaneously degrade with different half-lives at physiological pH, for a total of 2 mol of NO/mol of compound (Keefer *et al*., 1996). In contrast, the mechanism of NO release from SNAP is not fully understood. It has been shown that spontaneous release of NO from nitrosothiols is catalyzed by metal ions (McAninly *et al*.,

1993) and might involve a homolytic cleavage of the S—N bond (Barnett *et al.*, 1994). Furthermore, transnitrosylation reactions that provide more rapidly degrading nitrosothiols are likely (Barnett *et al.*, 1994). The occurrence of these reactions in tissues also might explain the similar pD_2 values for the vasorelaxing activities observed with SNAP and DEA/NO despite striking differences in spontaneous NO release. Similar observations have been reported by Kowaluk and Fung (1990).

In summary, our results provide evidence for inhibition of vascular bioactivation of organic nitrates such as GTN initiated by NO. For this effect, the duration of treatment is more important than the concentration of NO. A slight NO-induced desensitization also was observed. Both effects were associated with a decreased vasodilator potency of GTN. Our results suggest that preferential venodilation is caused by the lower production of endogenous NO in veins, which leads to less pronounced inhibition of vascular GTN-bioactivation.

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