British Journal of Pharmacology (1995) 116, 2743-2749

Specificity of different organic nitrates to elicit NO formation in rabbit vascular tissues and organs *in vivo*

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1 In the present study we assessed the formation of nitric oxide (NO) from classical and thiol-containing organic nitrates in vascular tissues and organs of anaesthetized rabbits, and established a relationship between the relaxant response elicited by nitroglycerin (NTG) and NO formation in the rabbit isolated aorta. Furthermore, the effect of isolated cytochrome P450 on NO formation from organic nitrates was investigated.

2 Rabbits received diethyldithiocarbamate (DETC; 200 mg kg^{-1} initial bolus i.p. and 200 mg kg^{-1} during 20 min, i.v.) and either saline, or one of the following organic nitrates: nitroglycerin (NTG, 0.5 mg kg^{-1}), isosorbide dinitrate (ISDN), N-(3-nitratopivaloyl)-L-cysteine ethylester (SPM 3672), S-carboxyethyl-N-(3-nitratopivaloyl)-L-cysteine ethylester (SPM 5185), at 10 mg kg^{-1} each. After 20 min the animals were killed, blood vessels and organs were removed, and subsequently analyzed for spin-trapped NO by cryogenic electron spin resonance (e.s.r.) spectroscopy.

3 In the saline-treated control group, NO remained below the detection limit in all vessels and organs. In contrast, all of the nitrates tested elicited measurable NO formation, which was higher in organs (liver, kidney, heart, lung, spleen) (up to 4.8 nmol g^{-1} 20 min^{-1}) than in blood vessels (vena cava, mesenteric bed, femoral artery, aorta) (up to 0.7 nmol g^{-1} 20 min^{-1}). Classical organic nitrates (NTG, ISDN) formed NO preferentially in the mesenteric bed and the vena cava, while the SPM compounds elicited comparable NO formation in veins and arteries.

4 Using a similar spin trapping technique, NO formation was assessed *in vitro* in phenylephrine-precontracted rabbit aortic rings. The maximal relaxation elicited by a first exposure (10 min) to NTG (0.3 to 10 μM) was positively correlated ($r=0.8$) with the net increase (NTG minus basal) of NO spin-trapped during a second exposure to the same concentration of NTG in the presence of DETC.

5 Cytochrome P450 purified from rabbit liver enhanced NO formation in a NADPH-dependent fashion from NTG, but not from the other nitrates, as assessed by activation of purified soluble guanylyl cyclase.

6 We conclude that the vessel selective action of different organic nitrates *in vivo* reflects differences in vascular NO formation. Thus, efficient preload reduction by classical organic nitrates can be accounted for by higher NO formation in venous capacitance as compared to arterial conductance and resistance vessels. In contrast, NO is released from cysteine-containing nitrates (SPMs) to a similar extent in arteries and veins, presumably independently of an organic nitrate-specific biotransformation. Limited tissue bioavailability of NTG and ISDN might account for low NO formation in the aorta, while true differences in biotransformation seem to account for differences in NO formation in the other vascular tissues.

Keywords: Nitric oxide; nitroglycerin; isosorbide dininitrate; isosorbide 5-mononitrate; N-(3-nitratopivaloyl)-L-cysteine ethylester (SPM 3672); N-(3-nitratopivaloyl)-S-(N'-acetyl-D,L-alanyl)-L-cysteine ethylester (SPM 5185); *in vivo* spin trapping; diethyldithiocarbamate; e.s.r. spectroscopy; cytochrome P450

Introduction

It is now generally accepted that NO accounts for the vasodilator activity of organic nitrates and other so-called nitrovasodilators (NVD) (Ahlner *et al.*, 1991; Moncada *et al.*, 1991; Bennett *et al.*, 1994), but neither the amount of NO generated by NVD in vascular target tissues, nor its quantitative relationship to the vasorelaxant response is known. Recent measurements of NVD-derived NO released into the superfusate of vascular tissues suggest that such a relationship exists (Schrör *et al.*, 1991), but NO released into superfusates represents an unknown fraction of NO generated in the target tissue. Furthermore, classical organic nitrates exhibit a vessel type specificity different from other NVD, in that they preferentially induce dilatation of large veins and the splanchnic and the mesenteric vascular beds (Ferrer *et al.*, 1966; Mackenzie & Parratt, 1977; Imhof *et al.*, 1980; Loos *et al.*, 1983). Although this effect has been ascribed in part to an attenuated NO release in the vascular resistance beds (Kurz *et al.*, 1991), the actual amounts of NO formed in these tissues in an intact organism following administration of therapeutic doses of organic nitrates have not been assessed. Previous reports (Vanin *et al.*, 1984; Kuropteva & Pastushenko, 1985) on nitrosyl-haemoprotein and nitrosyl-iron complex formation in animals after application of toxic (methaemoglobinaemia-inducing) doses (25 to 40 mg kg^{-1}) of nitroglycerin (NTG) fail to provide insight into the therapeutic actions of organic nitrates.

Another still unresolved issue is the possible role of non-vascular cells in various organs in the formation of NO from organic nitrates. For instance, in addition to cultured vascular endothelial and smooth muscle cells (Mülsch *et al.*, 1989; Feelisch & Kelm, 1991), kidney epithelial cells (Schröder & Schör, 1990) and lung fibroblasts (Schröder, 1992) have been shown to generate NO or NO-like bioactivity from NTG.

The major obstacle to obtaining quantitative data on NO formation from organic nitrates *in vivo* has until now been the lack or unawareness of a suitable detection method. However,

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Vanin and coworkers developed a NO spin-trapping technique that enables detection of NO in tissues by cryogenic electron spin resonance (e.s.r.) spectroscopy (Vanin *et al.*, 1984). We used this technique to assess NO formation in rabbit isolated aortic rings suspended in organ chambers for isometric tension recording in order to establish a relationship between tissue NO and relaxant responses. The same technique was also used in anaesthetized rabbits to compare concomitant NO formation in vascular tissues and organs after infusion of NTG, isosorbide dinitrate (ISDN), and the new organic nitrates, N-(3-nitratopivaloyl)-L-cysteine ethylester (SPM 3672; Kojda & Noack, 1993), and its related thiol-protected prodrug S-carboxyethyl-N-(3-nitratopivaloyl)-L-cysteine (SPM 5185, Noack *et al.*, 1988; Zanzinger *et al.*, 1994). The thiol moiety of SPM 3672 (and of SPM 5185 after cleavage of the thiol-protecting group) is supposed to generate NO from the nitroester group by inter- or intra-molecular attack (Noack *et al.*, 1988). It is expected that these compounds, in contrast to the classical organic nitrates (Brien *et al.*, 1986; Bennett *et al.*, 1994), release NO *in vivo* largely independent of organic nitrate-specific biotransformation. Spontaneous and cytochrome P450-dependent NO release from organic nitrates was assessed using a purified soluble guanylyl cyclase (GC), an NO effector enzyme.

Methods

Assessment of relaxant responses

The experiments described involving the use of animals were performed in accordance with national guidelines on animal welfare. The entire (abdominal and thoracic) aorta was removed from anaesthetized (60 mg kg^{-1} sodium pentobarbitone i.v.) New Zealand white rabbits (2.5 to 3.5 kg; SAVO, Kiessleg, Germany), cleaned of connective tissue and fat, and cut into rings of 6 mm length, four of which were mounted in thermostated (37°C) organ baths (10 ml each; Hugo Sachs Elektronik, March, Germany). The rings were connected to a hook and to a force transducer (K30, Hugo Sachs Elektronik), and equilibrated for 30 min under a resting tension of 3 g in carbonated (95% O_2; 5% CO_2) Krebs-Henseleit solution, pH 7.4 (composition in mM: Na^+ 144.0, K^+ 5.9, Cl^- 126.9, Ca^{2+} 1.6, Mg^{2+} 1.2, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25.0, D-glucose 11.1), in the presence of the cyclo-oxygenase inhibitor, diclofenac (1 μM). The remaining rings were not placed under tension but were immersed in the same organ baths in order to maximise the amount of aortic tissue exposed to the same drug treatment. Rings were subsequently contracted by 1 μM phenylephrine (PE) and the function of the endothelium was tested by recording the relaxant response to acetylcholine (1 μM). Following a 30 min wash period, the rings were contracted to 11 ± 1 g tension by 10 μM PE, and the relaxant response to one predetermined concentration of NTG was recorded on all 4 rings over 10 min (in two organ baths with a lower and in two other with a higher concentration of NTG). After washout and equilibration (45 min), formation of the NO trapping agent was induced. Briefly, a freshly prepared aqueous solution (100 μl) of $FeSO_4$/sodium citrate (0.1 mM/0.3 mM final concentration) was added to the organ baths, followed 3 min later by a solution (100 μl) of sodium diethyldithiocarbamate (DETC; 2 mM final). This protocol generates the NO trapping agent, the $Fe(DETC)_2$ complex, in the lipophilic tissue compartments (Mülsch *et al.*, 1992). After a further 10 min the rings were contracted with PE (10 μM). When a stable contraction developed (10 min) the relaxant response to the previous concentrations of NTG was recorded again for 10 min. Rings exposed to the same NTG concentration were combined, blotted on filter paper, and frozen for cryogenic e.s.r.. spectroscopy (Mülsch *et al.*, 1992), yielding a cylindrical specimen of aortic tissue approximately 4 mm high and 4.5 mm wide. In control experiments the influence of Fe-DETC-treatment on the relaxant response to an NO-independent vasodilator (forskolin, 0.1 to 1 μM) was tested.

In vivo experiments

The rabbits were anaesthetized with sodium pentobarbitone (20 mg kg^{-1} i.v. left ear). Subsequently the animal received a bolus injection (5 ml, i.p.) of 200 mg kg^{-1} diethyldithiocarbamate (DETC), followed by concomitant infusion (i.v. left and right ear; 30 ml h^{-1}) of DETC (200 mg kg^{-1}) and either saline, NTG (0.5 mg kg^{-1}), ISDN, SPM 3672 or SPM 5185 (10 mg kg^{-1} each). After 20 min infusion the animals were killed by an overdose of pentobarbitone (50 mg kg^{-1} i.v.). The abdominal and thoracic aorta, left and right femoral arteries,vena cava, mesenteric bed (connective tissue attached), heart, lung, liver, kidney, and spleen were quickly excised (within 10 min) and frozen for e.s.r. analysis in liquid nitrogen as described in detail previously (Mülsch *et al.*, 1992).

E.s.r. analysis

DETC was applied to generate the NO spin trap in the tissues during application of organic nitrates. DETC chelates intracellular free iron ions to afford the $Fe(DETC)_2$ complex (Vanin *et al.*, 1984). This complex avidly binds NO forming a paramagnetic mononitrosyl-iron complex, $NOFe(DETC)_2$, which can be assessed quantitatively by e.s.r. spectrometry (Vanin *et al.*, 1984). *In vitro* the trapping efficiency is nearly complete (Mülsch *et al.*, 1992), while *in vivo* this is generally assumed, but not proven (Mülsch *et al.*, 1993; 1994; Tominaga *et al.*, 1994). The concentration of NO trapped was calculated from the amplitude of the e.s.r. signal of this complex as described previously (Mülsch *et al.*, 1992). In some cases the underlying e.s.r. signal of the $Cu(DETC)_2$ complex was electronically subtracted for evaluation of the spectra. The detection limit was 0.02 nmol NO per g tissue. For frozen samples smaller than 20 mm in length (blood vessels) a correction factor was applied, which was derived from a calibration curve of sample length versus e.s.r. signal amplitude (data not shown). The e.s.r. spectra were recorded at liquid nitrogen temperature (77 °K) on a Brucker 300E spectrometer at a frequency of 9.33 Ghz, hf-modulation frequency 100 kHz, hf-modulation 0.5 mT, microwave power 10 mW, and time constant 0.05 s.

Detection of NO by activation of guanylyl cylcase (GC)

Release of NO from organic nitrates was assessed by activation of soluble GC purified to apparent homogeneity from bovine lung (Mülsch & Gerzer 1991). Briefly, formation of [^{32}P]-cyclic GMP from [α^{32}P]-GTP catalyzed by GC in the presence of organic nitrates was taken as a semiquantitative measure of NO release. Incubations proceeded for 10 min at 37 °C and contained GC (4 μg protein ml^{-1}), triethanolamine-hydrochloride (30 mM, pH 7.4), [α-^{32}P]-GTP (0.2 μCi; 0.2 mM), cyclic GMP (0.1 mM), $MgCl_2$ (4 mM), erythrocyte superoxide dismutase (0.1 μM; 0.9 units), bovine gammaglobulin (0.1 mg ml^{-1}), creatine phosphate (10 mM), and creatine phosphokinase (1 unit ml^{-1}), in a final volume of 100 μl. In some experiments NADPH (0.2 mM) and purified rabbit liver cytochrome P450 (0.2 nmol) were included. [^{32}P]-cyclic GMP was isolated and determined by liquid scintillation counting to calculate the specific activity of GC (nmol cyclic GMP formed per mg GC per min incubation time; Mülsch *et al.*, 1993). Soluble GC exhibited a basal activity in the absence of NO, which was subtracted from the organic nitrate-elicited activity.

Materials

Cytochrome P450 was purified from rabbit liver (Clement *et al.*, 1992). Sodium pentobarbitone (Nembutal) was from Sanofi Winthrop, München, Germany. NTG was provided as a trituration in lactose (10% NTG) by Pohl-Boskamp, Hohenlockstedt, Germany. ISDN, SPM 3672 and SPM 5185 were provided by Pharma Schwarz, Monheim, Germany. All other

biochemicals were obtained in the highest purity available from Sigma, Deisenhofen, Germany.

Statistics

Data are means ± s.e.mean, unless indicated otherwise. Significance was assessed by Kruskal-Wallis analysis, followed by Wilcoxon-Mann-Whitney-U-test and Bonferroni correction (Wallenstein *et al.*, 1980). $P < 0.05$ was considered significant.

Results

Organic nitrate-derived NO formation in vivo

Figure 1 illustrates representative e.s.r. recordings of frozen tissues (vena cava and liver) obtained from rabbits infused i.v. with either saline (Figure 1a) or organic nitrates (Figure 1, b-e). The $NOFe(DETC)^2$ complex exhibited a typical anisotropic e.s.r. signal with g-factors $g\perp 2.035$, $g \parallel 2.02$ ($\perp$ = perpendicular; $\parallel$ = parallel) and a triplet hyperfine splitting (hfs) at $g\perp$ (splitting constant 1.3 mT) (Vanin *et al.*, 1984; Mülsch *et al.*, 1992; 1993), most clearly seen in livers from organic nitrate-treated animals (Figure 1b-e, right hand recordings). In blood vessels and organs obtained from saline-infused control animals this signal was not detectable (Figure 1a, left recording). Only livers from control animals revealed a faint $NOFe(DETC)_2$ signal (Figure 1a, right recording). Instead, some features (labelled I, II) of the anisotropic e.s.r. signal of the paramagnetic copper(II)-DETC complex (Vanin *et al.*, 1984; Mülsch *et al.*, 1992) were detected in this as well as in all other tissues analyzed. Though this resonance could mask some of the features of the $NOFe(DETC)_2$ complex signal, the copper complex does not directly interfere with trapping of NO (Vanin *et al.*, 1984; Mülsch *et al.*, 1992). At low rates of NO formation (blood vessels) only the third hfs line at $g\perp$ of the $NOFe(DETC)_2$ complex (marked by ↓) was clearly recognized in all spectra and was taken for calculation of the concentration of NO trapped.

Compiled data of NO formation (nmol g^{-1}; mean ± s.d) *in vivo* during the 20 min trapping period are listed in Table 1. From these data it is evident that all organic nitrates generated more NO in organs than in blood vessels, the highest values being found in the liver and kidney. However, each organic nitrate exhibited an individual profile of NO release in different tissues. Most remarkably, classical organic nitrates elicited a significantly higher NO formation in the mesenteric bed (and NTG also in the vena cava), than in the aorta and femoral artery. This vessel type-specific action was not observed with the SPM compounds. Interestingly, the thiol-blocked prodrug SPM 5185 elicited higher NO formation in all tissues than the non-blocked SPM 3672, though the same dose of both was applied. Blood was devoid of e.s.r. detectable $NOFe(DETC)_2$ complexes (data not shown).

The concentration of NO traps, which is equivalent to the concentration of intracellular free non-haeme iron chelated by DETC, was determined in some tissues by titration with an excess of NO gas, as described in detail previously (Mülsch *et al.*, 1992). The NO trap was evenly distributed in all of the blood vessels studied (mean of all vessels 8.8 ± 0.9 nmol g^{-1}; $n = 3$ for each vessel), although markedly less traps were present in vessels than in organs. Similarly, the NO trapping capacity varied little from organ to organ (mean of all organs 36.3 ± 3.8 nmol g^{-1} $n = 3$ for each organ). By comparison with the concentrations of $NOFe(DETC)_2$ achieved in tissues

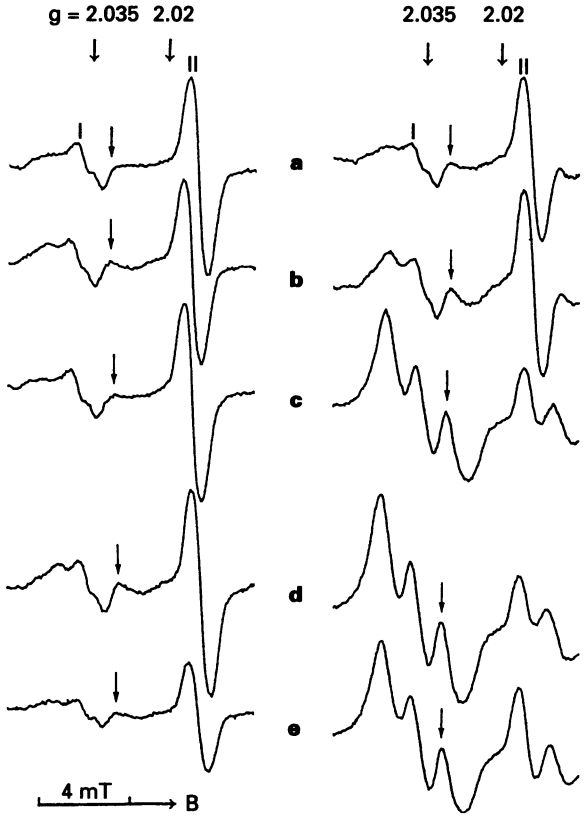

Figure 1 Representative e.s.r. recordings of the vena cava (left side recordings) and the liver (right) of organic nitrate-treated rabbits. (a) Denotes tissues taken from a control rabbit, (b, c, d, e) were taken from NTG, ISDN, SPM 5185 and SPM 3672 infused animals, respectively. (I) and (II) label the position of two hfs lines of the copper-DETC complex signal. ↓ indicates the third hfs line of the $NOFe(DETC)_2$ resonance at $g\perp 2.035$ taken for calculation of NO trapped. Instrument settings were as detailed in Methods. The direction and calibration of the magnetic field is indicated by B and 4 mT (milli Tesla), respectively.

Table 1 Organic nitrate-derived NO formation in rabbit tissues *in vivo*

Drug (*n*)	*NO* (nmol g^{-1} 20 min^{-1})								
	Aorta	*Fa.*	*Vca.*	*Mes.*	*Heart*	*Lung*	*Kidn.*	*Liver*	*Spleen*
NTG	0.08	0.27	0.45*§	0.45*§	0.54	0.79	1.55	0.87	0.33
(6)	±0.03	±0.18	±0.15	±0.25	±0.23	±0.28	±0.60	±0.32	±0.15
ISDN	0.11	0.15	0.14	0.70*§	0.78	2.13	2.40	4.25	0.90
(3)	±0.02	±0.02	±0.02	±0.30	±0.10	±0.25	±1.10	±1.52	±0.10
SPM 5185	0.47	0.60	0.50	0.45	1.20	1.00	4.40	4.80	2.01
(3)	±0.12	±0.02	±0.02	±0.26	±0.20	±0.10	±1.00	±0.24	±0.52
SPM 3672	0.16	0.30	0.30	0.17	0.60	0.46	2.94	4.24	0.68
(4)	±0.07	±0.06	±0.06	±0.05	±0.06	±0.10	±1.24	±1.52	±0.38

Tissue concentrations of NO trapped as $NOFe(DETC)_2$ complex (mean values ± s.d.) derived from the e.s.r. recordings. The liver values are corrected for NVD-independent NO formation (0.2 nmol g^{-1} 20 min^{-1}). Abbreviations: Fa. = femoral artery; Mes. = mesenteric bed; Vca. = vena cava. Significant difference between Vca. and Mes. *vs.* Ao. (*) and *vs.* Fa. (§) ($P < 0.05$). The number of animals (*n*) included in each treatment group is given below the drugs.

(Table 1) it is clear that only a small fraction of the total amount of traps available was required for binding of organic nitrate-derived NO.

Relationship between vasodilator response and NO formation

Endothelium-intact rabbit aortic rings precontracted by 10 μM PE relaxed following administration of NTG (0.3 to 10μM) in a concentration-dependent manner (Figure 2, open circles), maximally to about 70% of PE-induced tone. There was no desensitization in the vasodilator response to a second exposure of NTG following a washout period (45 min)(data not shown). Addition of the NO spin trap, Fe-DETC, to the vessels did not affect resting (passive-stretch) vascular tone (data not shown), but increased contractions to PE (10 μM) by 13 ± 2% (n=24). Relaxations to the cyclic AMP-increasing compound, forskolin (0.1 to 1 μM) were not affected by Fe-DETC-treatment (data not shown). In accordance with its role as an NO trapping agent, addition of Fe-DETC largely attenuated relaxations to NTG (Figure 2, closed circles). Significantly more NO was detected in NTG-exposed than in control aortic rings (NO being increased from 0.10 ± 0.02 nmol g^{-1} tissue in controls to 0.37 ± 0.05 nmol g^{-1} tissue in rings exposed to 10 μM NTG; Figure 2). The net increase in NO formation (NTG minus basal) was closely correlated to the relaxant response elicited by the first exposure to NTG (r = 0.8 in a linear regression analysis plotting individual paired data of net NO (nmol g^{-1}) vs. relaxation (%)). The basal NO formation was abolished by removal of the endothelium or inhibition of endothelial NO synthase by N^G-nitro-L-arginine (data not shown).

Spontaneous and cytochrome P450-catalyzed NO release from organic nitrates

SPM 3672 potently activated soluble GC (EC_{50} 20 ± 2 μM; Figure 3b), whereas GTN (Figure 3a), SPM 5185 (Figure 3b) and ISDN (data not shown) activated marginally in the same concentration range. GC activation by NTG, but not by the other nitrates, was enhanced in the presence of rabbit liver cytochrome P450 and NADPH (Figure 3a). Activation of soluble GC by sodium nitrite, a stable metabolite of organic nitrates, was comparable to that elicited by NTG alone, but was not further increased by cytochrome P450 (data not shown).

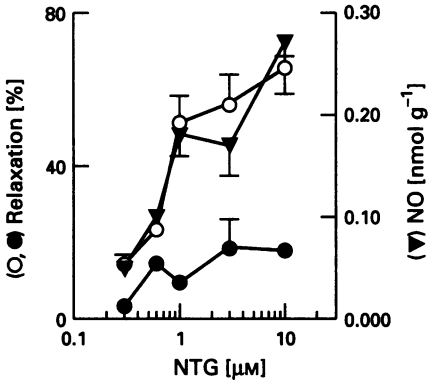

Figure 2 Relationship between NTG-elicited NO formation and relaxation of rabbit isolated aorta. The vasodilator response (% relaxation) of precontracted (PE 10 μM) endothelium-intact rabbit aortic rings to different concentrations of NTG was assessed first in the absence (○) and then in the presence (●) of $FeSO_4$ and DETC. The amount of NO trapped as paramagnetic $NOFe(DETC)_2$ complex (▲) after the second NTG challenge was subsequently determined by e.s.r. spectroscopy of frozen tissues. NO formation in the absence of NTG (0.1 nmol g^{-1} 10 min^{-1}) was subtracted. Mean values ± s.e.mean of 4 to 10 rings for each concentration of NTG tested (2 rings exposed to the same concentration of NTG were combined for e.s.r. recording).

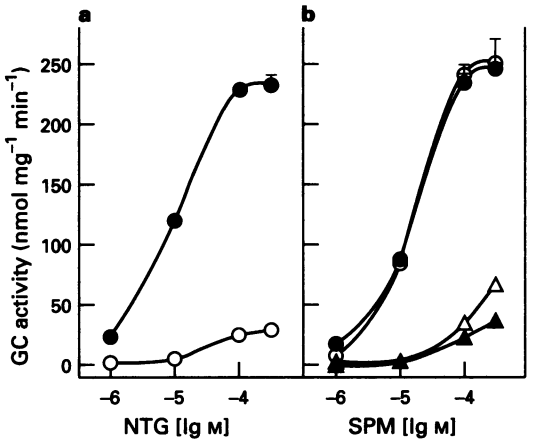

Figure 3 Activation of soluble guanylyl cyclase (GC) by organic nitrates *in vitro*. (a) NTG; (b) SPM 3672 (○, ●) and SPM 5185 (△, ▲). Activation of GC by different concentrations of organic nitrates was assessed in the absence (open) and presence (filled symbols) of cytochrome P450 (200 pmol) and NAPDH (0.2 mM). Specific GC activity (nmol cyclic GMP mg^{-1} enzyme min^{-1}) was calculated from the amount of [^{32}P] -cyclic GMP formed from [α^{32}P]-GTP during 10 min at 37°C. Data show the mean increase ± s.e.mean of basal GC activity from one of two similar experiments performed in triplicate. In most cases s.e.mean fell within symbol size.

Discussion

One objective of this study was to assess whether or not a quantative relationship exists between NVD-derived NO formation in vascular tissue and the concomitant vasodilatation. By applying an NO-spin trapping technique and cryogenic e.s.r. spectroscopy we were able to measure NO formation in isolated endothelium-intact rings of rabbit aorta. A basal (endothelium- and L-arginine-derived) NO formation was detectable in the absence of NTG. Scavenging of this basal NO by spin traps most probably accounts for the increase in contractile force after addition of $FeSO_4$ and DETC to the precontracted rings. The magnitude of NTG-elicited relaxations assessed in the absence of spin traps was correlated (r = 0.8) with the net increase in NO formed (NTG minus basal) during a second exposure to NTG in the presence of spin traps (Figure 2). Efficient trapping of NO was indicated by the marked attenuation of the NTG-elicited relaxations in the presence of spin traps (Figure 2). Since cyclic AMP-mediated relaxations to forskolin were not influenced by spin traps, an unspecific interference of Fe-DETC-treatment with vasodilatation was excluded. Our present findings should be extrapolated to the *in vivo* situation with some caution, in light of the rather high concentrations of NTG applied to maximally contracted vessels in order to facilitate detection of NO by the e.s.r. method. Nevertheless, it is more likely that a qualitatively similar relationship between NO formation and vasodilatation is also apparent under more physiological conditions. These data now fill in the missing link in the previously established relationship between the NVD dose applied, and the resulting increase in cyclic GMP and vasodilatation (Kukovetz & Holzmann, 1989).

In the *in vivo* study we assessed NO formation elicited by two different classes of organic nitrates in vascular tissues and organs of anaesthetized rabbits; the classical nitrates NTG and ISDN and the new mononitrates N-(3-nitratopivaloyl)-L-cysteine ethylester (SPM 3672) and its thiol-protected prodrug SPM 5185. Since the SPM compounds are assumed to release NO independently of an organic nitrate specific bio-

transformation (Noack *et al.*, 1988; Kojda & Noack, 1993), we expected SPMs and classical organic nitrates to produce different tissue profiles of NO formation. Similar to the aforementioned *in vitro* studies NO was trapped by $Fe(DETC)_2$, which was formed and enriched in lipophilic tissue compartments by reaction of systemically applied (i.v. and i.p.) DETC with endogenous free Fe^{2+} ions (Vanin *et al.*, 1984; Mülsch *et al.*, 1992). The level of traps achieved by our protocol in the different tissues was sufficiently high not to be exhausted by NVD-derived NO. NVD-independent NO formation could not be detected in the vascular tissue *in vivo*, unlike the situation *in vitro*. This could be due either to lower endogenous NO formation or to less efficient trapping of NO *in vivo*. The trapping conditions applied *in vitro* (addition of Fe^{2+}) and *in vivo* (no extra Fe^{2+}) were not strictly identical.

All NVD generated measurable amounts of $NOFe(DETC)_2$ in nearly all tissues analyzed. Since we do not know the NO trapping efficiency *in vivo*, data shown in Table 1 should be regarded as relative, not as absolute values of NVD-derived NO formation. NTG was the most effective NO donor with respect to the dose applied and the rate (nmol 20 min^{-1}) of NO formation achieved. This was not surprising, since NTG is also the most potent vasodilator and the most efficient antianginal drug among the organic nitrates (Ahlner *et al.*, 1991). Furthermore, the nitrates exhibited individual profiles of NO formation achieved in the different tissues. Thus, the classical organic nitrates generated NO at a significantly higher rate in the mesenteric bed (NTG, ISDN) and the vena cava (NTG) than in arterial conductance vessels. This finding explains the preferential venodilator activity and efficient preload reduction achieved by these drugs (Ferrer *et al.*, 1966; Mackenzie & Parratt, 1977; Loos *et al.*, 1983). In contrast, SPMs elicited comparable NO formation in all vascular tissues analyzed, confirming our expectations. This finding implies that SPMs should also reduce mean arterial blood pressure (MAP) and afterload. Indeed, in chronically instrumented dogs infused i.v. with SPM 5185 at a 10 fold lower bolus dose than applied in our study (10 mg per dog weighing 25 kg), a transient decrease in MAP and a sustained elevation in heart rate was observed (Zanzinger *et al.*, 1994), indictive of a decrease in peripheral resistance and humoral counter-regulation. Surprisingly, the thiol-blocked prodrug, SPM 5185, generated more NO in most tissues (with the exception of liver and kidney) than its non-blocked supposed metabolite, SPM 3672. This could be a consequence of different pharmacokinetics, for instance a higher clearance of SPM 3672 by the liver and the kidney (which explains high NO values in these tissues), and a less rapid uptake into the vascular walls, as compared to SPM 5185. This remains to be investigated. The finding that NO formation from the SPMs was similar in the arterial and venous tissues might be taken as an indication that metabolism and oxidative inactivation of NO is not different in vascular tissues. This strengthens our consideration that different rates of formation, not degradation, of NO account for different amounts of NTG-and ISDN-derived NO detected in vascular tissues.

It is difficult to identify the relative importance of biodistribution, biotransformation and pharmacokinetics of organic nitrates on vascular NO formation *in vivo*. NTG and ISDN exhibit unusual pharmacokinetics, e.g., extensive and rapid first pass elimination during passage through most vascular beds. For instance, in a previous study the bioavailability of NTG and ISDN was assessed in rats infused intravascularly at different sites (Fung *et al.*, 1984). Steady state plasma concentrations of NTG were observed within 20 min and a rapid and extensive clearance of the organic nitrates from the circulation by different vascular beds (equalling or exceeding cardiac output or organ flow, respectively). This was in accordance with a previous study in human subjects demonstrating extensive clearance of NTG by the forearm vascular bed (Armstrong *et al.*, 1982). In the rat vascular tissue NTG and ISDN were enriched 400 fold and 17 fold *vs.* plasma at the site of infusion. Tissue concentrations of the nitrates decreased in proportion to the distance of the vascular segment downstream from the infusion site. However, extraction of organic nitrates by the aorta was extremely low, and aortic tissue concentrations were always lower than in other vascular segments proximal or distal to the aorta, independent of the site of infusion. It was concluded that the aortic tissue exhibits unusually low affinity for organic nitrates as compared to other blood vessels. In the study by Cossum and coworkers (1986) NTG was continuously infused into the right femoral vein of sheep at a rate (22.5 $\mu g\ min^{-1}\ kg^{-1}$) similar to that used in our study. Organ bioavailability was calculated from the ratio of steady state concentrations of NTG entering and leaving the vascular beds, respectively, multiplied by blood flow. It amounted to 0.99 in the aorta, 0.36 to 0.5 in the liver, 0.39 in the mesentery, 0.53 in the lung, and 0.26 to 0.29 in the hindlimb. Again, NTG was only marginally extracted by the aorta, but to a comparable high extent by the other vascular beds. Therefore, differences in extraction of NTG and ISDN across vascular beds other than the aorta apparently do not explain differences in NO formation. Only in the case of the aorta will uptake of NTG and ISDN limit tissue availability of organic nitrates and this mechanism might explain lower NO formation in the aorta vs. other vessels. However, higher NO formation in the vena cava and mesenteric bed than in the femoral atery is not explained by biodistribution mechanisms, but may be based on differences in biotransformation. Therefore, we conclude that the capability of vascular tissues to generate NO from NTG and ISDN and the dependency of the vasodilator activity of these organic nitrates on such a biotransformation determines the vasodilator sensitivity of a vascular bed to these drugs, hence the type of haemodynamic changes observed *in vivo*. At present a similar conclusion cannot be derived with certainty for the SPMs, since their pharmacokinetics and biodistribution are unknown.

The biological implications of the unexpectedly high NO formation by organic nitrates observed in some organs, especially liver, lung and kidney (Table 1), and the cells responsible for this biotransformation, remain to be investigated. With regard to the reportedly low sensitivity of hepatic (Shepard *et al.*, 1985) and cardiac (Kurz *et al.*, 1991) resistance beds to the vasodilator action of classical organic nitrates; it is concievable that these vascular beds are not preeminent sources and targets of NTG-and ISDN-derived NO. Since cultured non-vascular cells have been shown to generate NO from organic nitrates (Schröder & Schrör, 1990; Schröder, 1992), it is conceivable that NO formation in organ tissues is mainly accomplished by nonvascular cells. In the liver, hepatocytes may be the principal source of NTG-derived NO. These cells express high cytochrome P450 activity, which we (Figure 2) and others (Servant *et al.*, 1989; McDonald & Bennett, 1993) have shown to catalyze NO formation from NTG. It is unknown at present whether NVD-derived NO generated in nonvascular cells affects other biological targets besides soluble GC, and whether this interaction has pharmacological consequences. For example, NO can inhibit cytochrome P450 (Stadler *et al.*, 1994), cytochrome c oxidase (Cleeter *et al.*, 1994) and non-haeme iron-dependent cell functions (Hibbs *et al.*, 1990).

SPM 3672, but not the other organic nitrates, activated purified soluble GC at reasonably low concentrations (EC_{50} 20 μM). This indicates a spontaneous NO release, in accordance with the considerations that led to the design of the SPMs (Noack *et al.*, 1988). However, our finding is somewhat in conflict with a more recent one of Kojda & Noack (1993), who reported half-maximal activation of a partially purified solubel GC from human platelets at 10 fold higher concentrations of SPM 3672, and only low spontaneous NO formation, comparable to NTG. We have no ready explanation for this discrepancy. It may be that their GC preparation was not as sensitive to NO as ours.

In contrast, NTG potently activated GC only in the presence of cytochrome P450 and NADPH, similar to previous reports (Servant *et al.*, 1989; McDonald & Bennett, 1993).

Surprisingly, cytochrome P450 did not catalyze NO formation by the other organic nitrates, even if these were tested in relatively high concentrations (0.3 mM). This may be related to the higher lipophilicity and smaller size of NTG as compared to the other organic nitrates, which favours easy access of NTG to the hydrophobic haeme pocket of cytochrome P450. The significance of the cytochrome P450 pathway for bioactivation of nitroglycerin in blood vessels remains controversial (Bennett *et al.*, 1992; McDonald & Bennett, 1993; Liu *et al.*, 1993; Seth & Fung, 1993).

In summary, we provide direct evidence of a close relationship between NTG-elicited NO formation and relaxation of rabbit isolated aorta. We have demonstrated for the first time that organic nitrates applied i.v. in a high but clinically relevant dose generate NO in vascular tissues *in vivo*. Classical organic nitrates, but not the spontaneously NO releasing mononitrate SPM 3672 and its parent compound SPM 5185, generated higher NO levels in the mesenteric bed and NTG also in the vena cava, than in the aorta and femoral artery. This finding probably accounts for the different haemodynamic responses seen with both groups of organic nitrates *in vivo*.

We gratefully acknowledge the generous supply of purified cytochrome P450 by Dr F. Jung and B. Clement, Department of Pharmaceutical Chemistry, University of Kiel, Germany. We also appreciate the kind donation of ISDN and SPMs by Dr M. Feelisch, Schwarz Pharma, Monheim, Germany, and the editorial help of Dr Ingrid Fleming from our laboratory. This study was supported in part by the Land Hessen, FG 1530 Biomembranen, and by the Deutsche Forschungsgemeinschaft.

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(Received May 15, 1995
Revised July 14, 1995
Accepted July 20, 1995)