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Novel S-nitrosothiols do not engender vascular tolerance and remain effective in glyceryltrinitrate-tolerant rat femoral arteries ☆, ☆☆

Mark R. Miller [a,*], Marc J. Roseberry [a], Francesca A. Mazzei [b], Anthony R. Butler [b], David J. Webb [c], Ian L. Megson [a]

[a] *Clinical Pharmacology Unit, University of Edinburgh, Department of Biomedical Sciences, Western General Hospital, Edinburgh, EH4 2LH, Scotland, UK*

[b] *School of Chemistry, University of St. Andrews, St. Andrews, Fife, KY16 9ST, Scotland, UK*

[c] *Clinical Research Centre, University of Edinburgh, Western General Hospital, Edinburgh, EH4 2XU, Scotland, UK*

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Abstract

Organic nitrates, such as glyceryltrinitrate, are nitric oxide (NO) donor drugs that engender tolerance with long-term use. Here, we tested the hypothesis that our novel S-nitrosothiols, *N*-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-β-D-glucopyranose (RIG200) and S-nitroso-*N*-valeryl-D-penicillamine (D-SNVP), do not induce vascular tolerance ex vivo. Femoral arteries from adult male Wistar rats were preconstricted with phenylephrine and perfused with the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME). Perfusion pressure was measured during 20 h treatment with supramaximal concentrations of NO donor (10 μM). Perfusion with glyceryltrinitrate caused a vasodilatation, which recovered over 2–20 h. In contrast, the S-nitrosothiols caused vasodilatations that were maintained throughout the 20 h perfusion period. Responses to S-nitrosothiols were partially reversed by the NO scavenger ferrohaemoglobin and fully reversed by the soluble guanylate cyclase inhibitor [1H-[1,2,4] oxadiazole [4,3-a]quinoxaline-1-one (ODQ). Glyceryltrinitrate-tolerant vessels were fully responsive to bolus injections of S-nitrosothiols. Resistance to tolerance is an attractive property of our novel compounds, particularly in view of their sustained activity in arteries with damaged endothelium.

Keywords: Nitric oxide (NO); S-Nitrosothiol; Organic nitrate; Tolerance; Blood vessel

1. Introduction

Nitric oxide (NO) is synthesised by endothelial cells in blood vessels (Palmer et al., 1987, 1988; Palmer and Moncada, 1989) and stimulates smooth muscle cell soluble guanylate cyclase, leading to relaxation of vascular tissue (Waldman and Murad, 1987). Synthesis of NO is now recognised to be a major factor in the control of blood pressure and local blood flow in animals (Aisaka et al., 1989; Rees et al., 1989; Gardiner et al., 1990; Chu et al., 1991) and man (Vallance et al., 1989; Haynes et al., 1993). Delivery of exogenous NO to areas of diminished NO activity (Drexler et al., 1991; Calver et al., 1992a,b) is an attractive therapeutic option in the management of many cardiovascular conditions.

Organic nitrates are the most commonly used NO donor drugs in cardiovascular medicine. Glyceryltrinitrate (Fig. 1(a)) is currently used for angina, and for symptomatic relief in severe cardiac ischaemia, myocardial infarction and heart failure. The beneficial action of nitrates is thought to involve NO-mediated systemic venodilatation and dilatation of large arteries, including affected coronary arteries, resulting in reduced venous return and increased blood flow to cardiac tissue (Abrams, 1985). However, the therapeutic use of nitrates is limited by the development of tolerance, where a diminished effectiveness of these drugs is seen after 24 h of continuous therapy (Parker and Fung, 1984). Tolerance can be demonstrated ex vivo, suggesting impairment of a direct vascular mechanism, such as inefficient biotransformation of glyceryltrinitrate (Brien et al., 1986; Slack et al., 1989), or desensitization of the target

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* Corresponding author. University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, EH8 9XD, Midlothian, UK; Tel.: +44-131-651-1703; fax: +44-131-650-3711.

E-mail address: m.r.miller@sms.ed.ac.uk (M.R. Miller).

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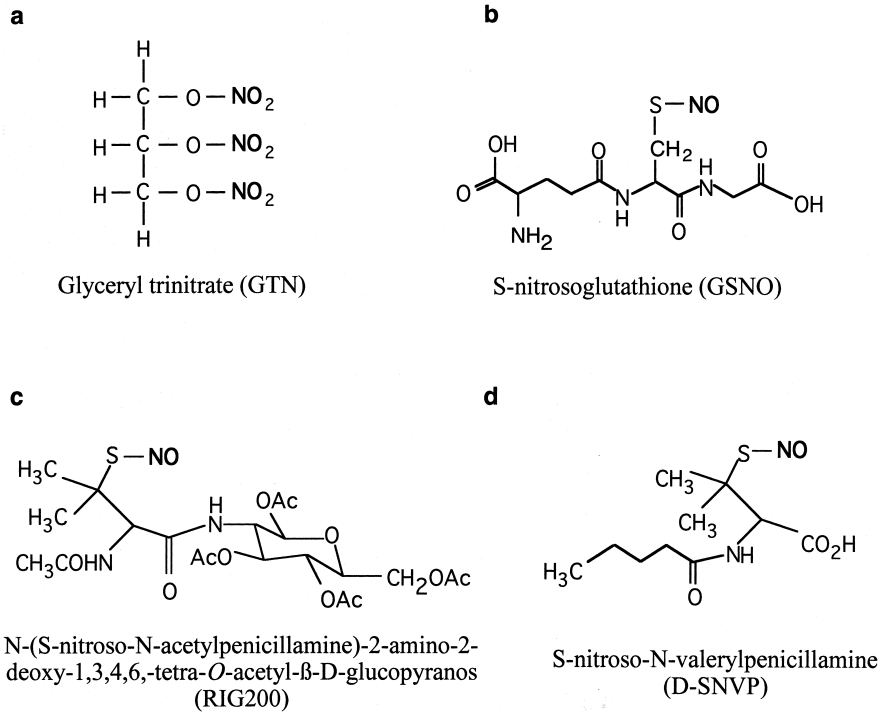

Fig. 1. Structural formulae and full generic names for NO donors used in this study: (a) glyceryltrinitrate (GTN); (b) S-nitrosoglutathione (GSNO); (c) RIG200; (d) D-SNVP.

enzyme, guanylate cyclase (Needleman and Johnson, 1973; Axelsson and Andersson, 1983; Waldman et al., 1986). Recently, in vivo and clinical evidence suggest that continuous nitrate therapy is associated with elevated superoxide production from the endothelium (Munzel et al., 1995, 1996). Superoxide reacts with NO, forming cytotoxic products, such as peroxynitrite, and reducing NO bioavailability (White et al., 1994).

S-Nitrosothiols (general formula R—S—N═O) are nitrosated derivatives of sulphydryl-containing compounds, some of which have been identified as endogenous vasodilators (Stamler et al., 1992a,b). S-Nitrosothiols do not require biotransformation to activate guanylate cyclase, suggesting that S-nitrosothiols may not induce self-tolerance. Indeed, one such agent, S-nitroso-*N*-acetylpenicillamine has been shown to develop less tolerance than glyceryltrinitrate and to remain effective in glyceryltrinitrate-tolerant vessels ex vivo (Kowaluk et al., 1987; Kowaluk and Fung, 1990; Matsumoto et al., 1995) and in vivo (Bauer and Fung, 1991; Shaffer et al., 1992).

Most existing S-nitrosothiols, including S-nitroso-*N*-acetylpenicillamine and S-nitrosoglutathione (Fig. 1(b)), rapidly decompose in an unpredictable manner due to the catalytic effect of trace Cu^+ ions (Dicks et al., 1996; Gordge et al., 1996), thus limiting their therapeutic potential (Megson et al., 1997). We have recently described several novel S-nitrosothiols. *N*-(S-nitroso-*N*-acetylpenicillamine)-2-amino-2-deoxy-1,3,4,6,tetra-*O*-acetyl-ß-D-glucopyranose (RIG200) consists of S-nitroso-*N*-acetylpenicillamine coupled to glucosamine tetra-acetate by an amide bond (Fig. 1(c); Megson et al., 1997) and S-nitroso-*N*-valeryl-D-penicillamine (D-SNVP) which is an *N*-substituted analogue of S-nitroso-*N*-acetylpenicillamine, with a five carbon side-chain (Fig. 1(d); Megson et al., 1999). Both compounds are significantly more stable than S-nitroso-*N*-acetylpenicillamine in solution (half-life; ~40 and ~220 min for S-nitroso-*N*-acetylpenicillamine and RIG200, respectively, D-SNVP exhibits a similar rate of decomposition to RIG200), and are less susceptible to trace Cu^+-catalyzed decomposition (Megson et al., 1997, 1999). Another potential advantage of these compounds over existing NO donors is that they induce sustained vasodilatation in endothelium-denuded rat femoral arteries, suggesting that they may be able to selectively deliver NO to areas of endothelial damage (Megson et al., 1997, 1999).

For these benefits to be maximally exploited therapeutically, it would be valuable if these novel compounds did not engender tolerance with continued use. Here, we used an isolated rat femoral artery model of nitrate tolerance to test the hypothesis that RIG200 and D-SNVP do not induce vascular tolerance or show cross-tolerance with glyceryltrinitrate.

2. Materials and methods

2.1. Preparation

Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (300–450 g;

$n = 60$) in a perfusion system described previously (Megson et al., 1997). Briefly, animals were killed by cervical dislocation and both femoral arteries were dissected free. Segments of the artery (7–8 mm long) were cannulated immediately distal to the epigastric arterial branch. The vessels were transferred to Perspex organ chambers (1 ml volume) where they were perfused (0.6 ml min^{-1}: Gilson miniplus 3; Anachem, Luton, UK) and superfused (1 ml min^{-1}: Watson Marlow 302S; Watson Marlow, Falmouth, UK) with fresh oxygenated (95% O_2, 5% CO_2) Krebs buffer solution (composition in mM: NaCl 118, $NaHCO_3$ 25, Glucose 5.7, KCl 4.7, $MgSO_4 \cdot 7H_2O$ 0.6, KH_2PO_4 1.2, $CaCl_2$ 2.5; dissolved in distilled and de-ionised water) at 37°C. The contractile state of the vessel was measured by perfusion pressure, monitored by a differential pressure transducer (T; Sensym SCX 15ANC; Farnell Electronic Components, Leeds, UK). The apparatus permits exclusive drug delivery to the luminal surface of the vessel in the

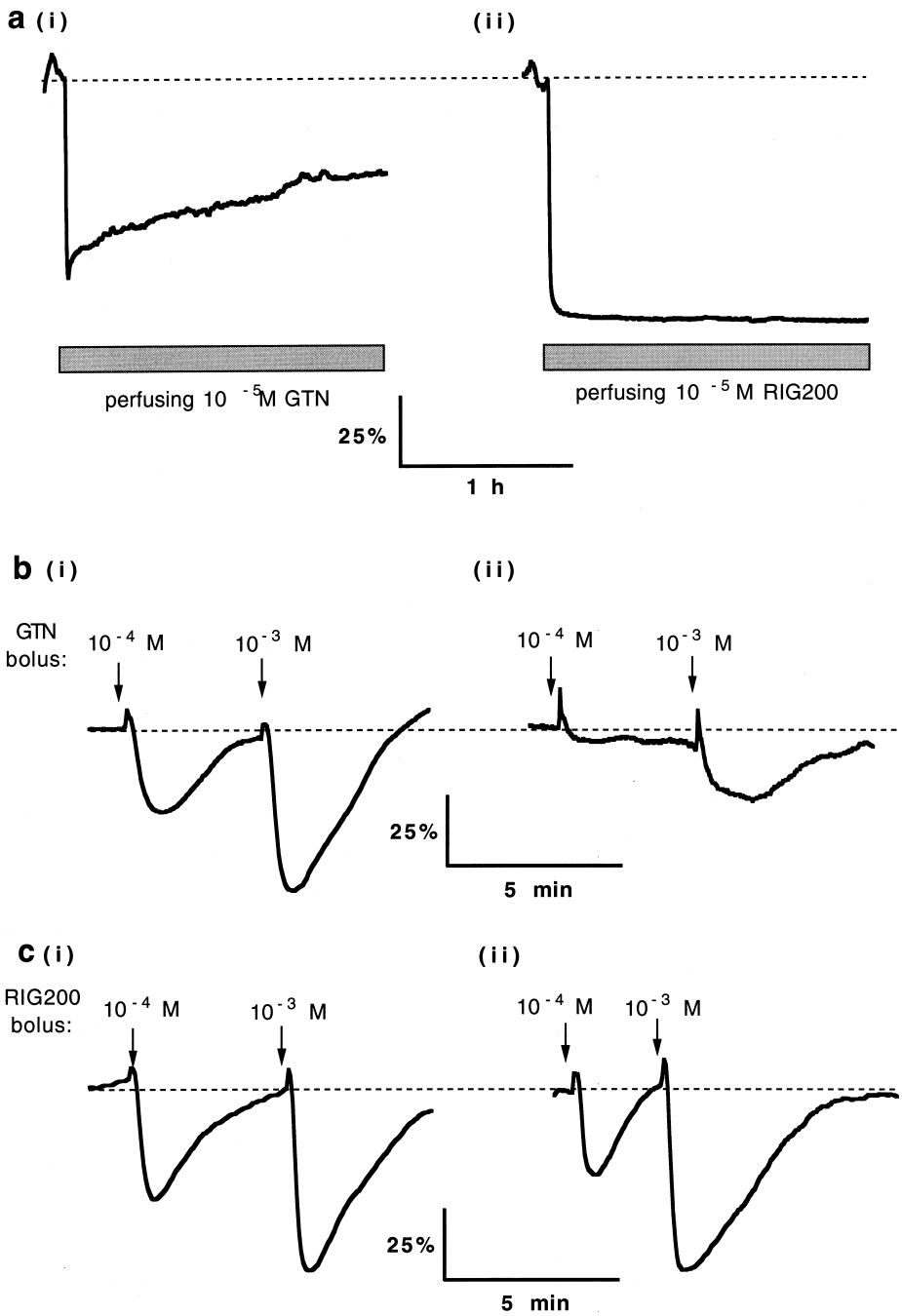

Fig. 2. Pressure recordings showing vasodilator responses. (a) Continuous perfusion (10^{-5} M; 0.6 ml min^{-1}) of (i) GTN or (ii) RIG200. (b) Responses to sequential micro-injections of GTN (10 μl; 10^{-4}, 10^{-3} M) into the perfusate of (i) control and (ii) GTN-tolerant vessels. (c) Responses to sequential micro-injections of RIG200 (10 μl; 10^{-4}, 10^{-3} M) into the perfusate of (i) control and (ii) GTN-tolerant vessels.

internal perfusate or by bolus injection (10 μl) through a resealable rubber septum into the perfusate, immediately upstream of the vessel (transit time to artery ~3 s, through lumen ~300 ms).

All experiments were carried out in a darkened laboratory in order to protect photolabile drugs and to prevent photorelaxation of vessels (Megson et al., 1995).

Vessels were preconstricted with phenylephrine (2–10 μM) in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME; Rees et al., 1990) to exclude endothelial and inducible NO-synthase activation in vasodilator responses. Preliminary experiments were carried out to minimise the concentration of L-NAME (20 μM) used to produce a supramaximal response. An L-NAME-induced increase in pressure of >40% of the existing phenylephrine-induced tone was indicative of an active endothelium.

2.2. Experimental protocols

2.2.1. Induction of tolerance

Vessels were perfused with equivalent concentrations of NO donor (10 μM), or Krebs as a control, and perfusion pressure was monitored for 2 h. Phenylephrine was then removed from the internal perfusate and the perfusion rate lowered to 0.1 ml min^{-1} overnight, at 25°C, to optimise vessel survival. At $t = 20$ h, the original phenylephrine-containing solution was re-perfused at the original flow rate (0.6 ml min^{-1}), at 37°C.

2.2.2. Cross-tolerance

In glyceryltrinitrate-treated and control vessels ($t = 20$ h), bolus injections of increasing concentrations of NO donor (10 μl; 10^{-8}–10^{-3} M) were made sequentially into the perfusate. Responses were deemed to have recovered once pressure was maintained for more than 2.5 min, at which time the next concentration was injected.

2.2.3. Washout of NO donor

To confirm viability in vessels that did not re-develop tone with phenylephrine following 20 h of S-nitrosothiol perfusion, the S-nitrosothiol was washed out and the time taken for maximum pressure to be restored was measured.

2.2.4. Nature of NO donor vasodilatation

In S-nitrosothiol-treated vessels at $t = 20$ h, the NO scavenger, ferrohaemoglobin (10 μM; Martin et al., 1985) was added to the internal perfusate, and subsequently, to the superfusate to allow ferrohaemoglobin to infiltrate the vascular smooth muscle, as it has been shown previously that the endothelium may act as a barrier to ferrohaemoglobin (Foley et al., 1993). Responses were deemed complete after pressure was maintained for 5 min. A supramaximal concentration of the soluble guanylate cyclase inhibitor [1H-[1,2,4] oxadiazole [4,3-a]quinoxaline-1-one (ODQ; Garthwaite et al., 1995) was added to the internal perfusate and rapidly washed out once pressure had reached plateau.

2.3. Drugs and reagents

RIG200 was synthesised by a published method (Megson et al., 1997). D-SNVP was synthesised by the following procedure. Sodium valerate was synthesised by reacting sodium hydroxide pellets (8 g, 0.2 mol) in distilled water (100 ml) with valeric acid (21.6 ml, 200 mmol). Sodium valerate (3.9 g, 40 mmol) was added to D-penicillamine (3 g, 20 mmol) in a chilled solution of tetrahydrofuran:water, 4:1 (20 ml). Valeric anhydride (4 ml, 20 mmol) was added and the mixture stirred at room temperature overnight. The tetrahydrofuran was evaporated in vacuo and water (30 ml) was added. Concentrated HCl was added dropwise until a white precipitate formed. This was filtered and recrystallised from hexane to yield *N*-valeryl-D-penicillamine, which was then dissolved in a minimum amount of dichloromethane. Concentrated HCl was dropped onto sodium nitrite and the resulting nitrogen dioxide were bubbled into the penicillamine solution to yield *N*-valeryl-S-nitroso-D-penicillamine. Identity of the products was confirmed by mass spectrometry (EPSRC Mass Spectroscopy Service, University College, Wales, UK) and nuclear magnetic resonance spectroscopy. ODQ was obtained from Tocris Cookson (Langford, Bristol, UK). All other chemicals were obtained from Sigma (Poole, Dorset, UK) Methaemoglobin was reduced to the ferro-form with sodium dithionite (fivefold excess; 57.4 μM) as described previously (Martin et al., 1985).

All drugs were stored as solids and dissolved on the day of use with the exception of ferrohaemoglobin, aliquots of which were stored at −70°C and used within 1 month. All

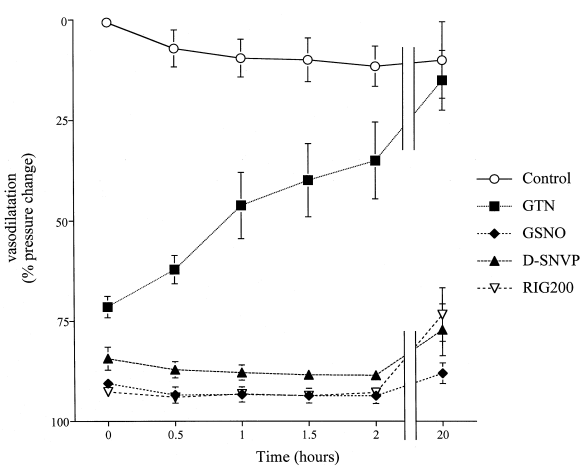

Fig. 3. Effect of perfusing NO donors (10 μM) on perfusion pressure in preconstricted rat femoral arteries. Vasodilatation at 100% represents abolition of tone, with NO donor perfusion beginning at $t = 0$ h. Points shown are means with vertical lines indicating S.E.M. ($n = 45$, 33, 15, 12, 12 for control, GTN, GSNO, RIG200 and D-SNVP respectively).

drugs were diluted in Krebs buffer or saline with the exception of ODQ, which was dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO in the perfusate was $< 0.1\%$ and preliminary experiments showed that this concentration of DMSO does not affect vessel tone.

2.4. Analysis of results

Signals from pressure transducers were processed by a MacLab/4e analogue-digital converter and displayed through Chart software (AD Instruments, Sussex, UK) on a Macintosh Performa 630 microcomputer.

Vasodilator response amplitude is the decrease in pressure, expressed as a percentage of preconstriction pressure existing before the application of each drug concentration (percent pressure change; positive values represent vasodilatation, where 100% represents maximum possible vasodilatation). Mean values are given $\pm$ standard error of the mean (S.E.M.).

P-values in the text were obtained by two-factor, unrelated analysis of variance (ANOVA). Paired and unpaired, two-tailed Student's *t*-tests were also used where appropriate. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Preconstriction of femoral arteries

Vessels were preconstricted with phenylephrine (6.7 ± 0.3 μM) to give pressures of ~ 50 mm Hg (49 ± 3 mm Hg; $n = 60$). L-NAME (20 μM) led to a $151 \pm 13\%$ increase of pre-existing phenylephrine-induced pressure (110 ± 5 mm Hg; $n = 60$). Bolus injections of drug vehicle (Krebs buffer or saline) had no effect on perfusion pressure.

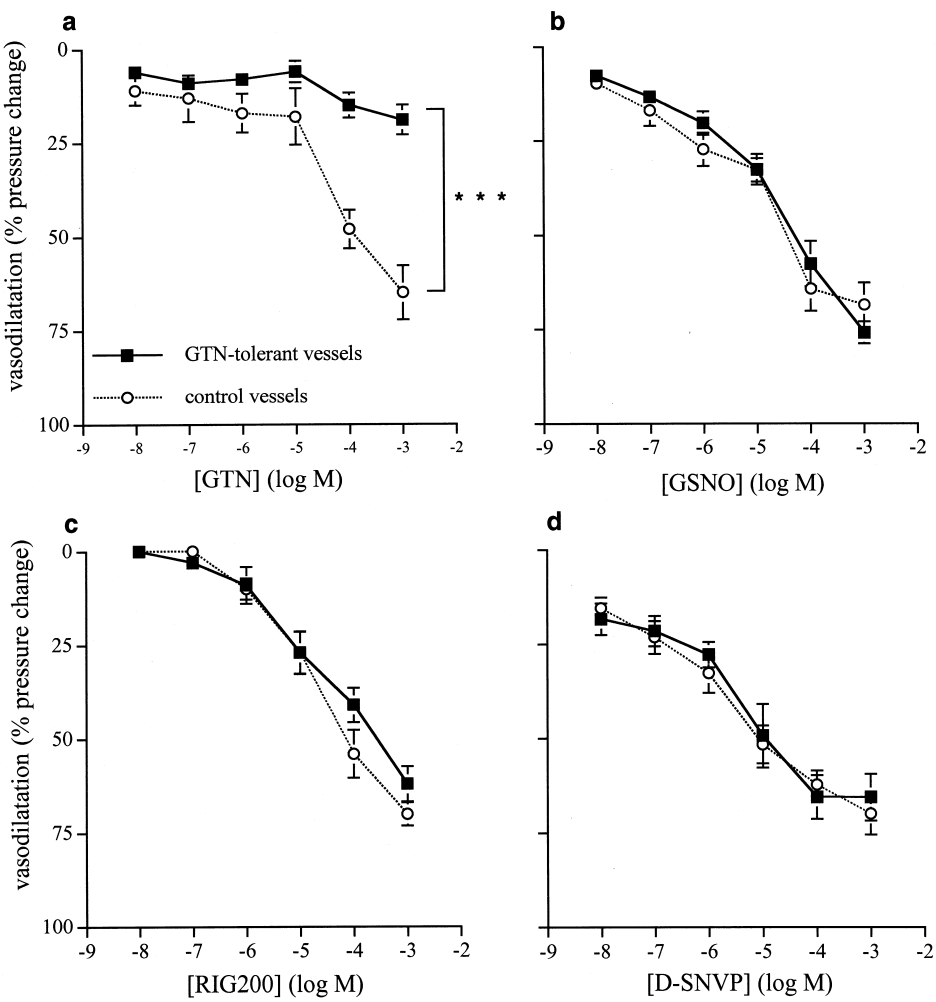

Fig. 4. Log concentration response curves showing the vasodilator effect (% pressure change) of bolus microinjections (10 μl) of (a) GTN, (b) GSNO, (c) RIG200, (d) D-SNVP in control (open circles) and GTN-tolerant (filled squares) vessels. Points shown are means with vertical lines indicating S.E.M. ($n = 6$–9). * * * represents statistical significance at $P < 0.001$ (two-factor, unrelated ANOVA).

3.2. Vasodilator responses to continuous NO donor perfusion

Perfusion of glyceryltrinitrate (10 μM) caused an initial vasodilatation of 72 ± 3% ($n = 33$). Pressure gradually recovered to 35 ± 10% vasodilatation remaining at $t = 2$ h (Figs. 2(a) and 3). After overnight incubation with glyceryltrinitrate ($t = 20$ h), pressure was not significantly different from control (10 ± 10% below preconstriction; $P = 0.64$; 2-tailed, unpaired Student's t-test; $n = 45$).

Perfusion of supramaximal concentrations of S-nitrosothiols (10 μM) produced greater vasodilatation (91 ± 2%, 93 ± 1%, 84 ± 3% for S-nitrosoglutathione, RIG200 and D-SNVP, respectively; $n = 12–15$), which were maintained throughout the 20 h period of perfusion (Figs. 2(a) and 3).

3.3. Vasodilator responses to bolus injections of NO donors in control and glyceryltrinitrate-tolerant vessels

Bolus injections of glyceryltrinitrate (10 μl; 10^{-8}–10^{-3} M) produced transient vasodilatations which recovered within 5 min. In vessels perfused overnight in the absence of glyceryltrinitrate (control), the highest concentration of glyceryltrinitrate tested (10^{-3} M) produced a vasodilatation of 65 ± 7% ($n = 6$). In vessels perfused with glyceryltrinitrate for 20 h (glyceryltrinitrate-tolerant vessels) the response to 10^{-3} M GTN bolus was markedly attenuated (19 ± 4%; $P < 0.001$; two-factor, unrelated ANOVA; $n = 7$; Figs. 2(b) and 4(a)).

Equivalent injections of S-nitrosothiols also produced transient vasodilatations of a similar amplitude to glyceryltrinitrate (69 ± 6%, 70 ± 3% and 70 ± 6% for S-nitrosoglutathione, RIG200 and D-SNVP, respectively; Figs. 2(c) and 4(b), (c), (d)). However, the concentration response curves for the S-nitrosothiols in glyceryltrinitrate-tolerant vessels were not significantly different from those in control vessels ($P > 0.21$; two-factor, unrelated ANOVA; $n = 6–9$).

3.4. Washout of S-nitrosothiols

At $t = 20$ h, the internal perfusate was replaced with Krebs solution containing PE and L-NAME, but without NO donor. On washout of S-nitrosoglutathione, RIG200 or D-SNVP, pressure recovered to levels that were not significantly different from the preconstriction pressure before the perfusion of NO donor ($P = 0.15$; 2-tailed, paired Student's t-test; $n = 18$). Pressure rapidly recovered in 5.5 ± 0.9, 6.5 ± 0.8 and 11.1 ± 4.5 min, respectively ($n = 6$ for all). The time course of D-SNVP washout was significantly slower than S-nitrosoglutathione and RIG200 ($P = 0.001$; two-way, unrelated ANOVA).

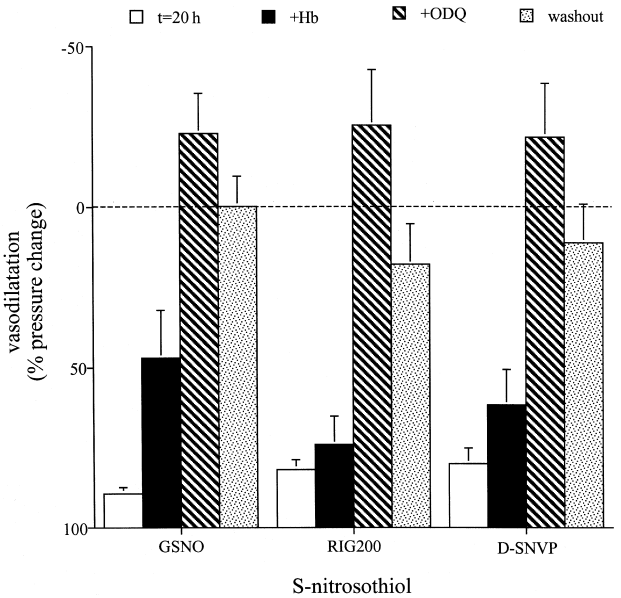

Fig. 5. The effect of ferrohaemoglobin (ferrohaemoglobin; 10 μM), ODQ (20 μM) and S-nitrosothiol washout on the vasodilatation produced by perfusing S-nitrosothiols (10 μM) for 20 h. Points shown are means with vertical lines indicating S.E.M. ($n = 6$ for all). Preconstriction pressure is represented by the dotted line.

3.5. Reversal of S-nitrosothiol vasodilatation with ferrohaemoglobin and ODQ

Following perfusion of S-nitrosoglutathione for 20 h, addition of ferrohaemoglobin (10 μM) to the internal perfusate caused a significant increase in pressure of ~ 40% ($P = 0.005$, 2-tailed, paired Student's t-test; $n = 6$). ferrohaemoglobin had no effect on the vasodilatation produced by RIG200 or D-SNVP ($P > 0.15$ for both; $n = 6$; Fig. 5). Addition of ferrohaemoglobin (10 μM) to the external perfusate caused no additional effect ($n = 6$).

Full restoration of pressure could be achieved by the addition of the soluble guanylate cyclase inhibitor, ODQ (20 μM), to the internal perfusate (Fig. 5). The perfusion pressure in the presence of these compounds was not significantly different from the preconstriction pressure, before addition of NO donor ($P = 0.73$; 2-tailed, paired Student's t-test; $n = 18$).

Treatment of control vessels with ferrohaemoglobin and ODQ had no significant effect on perfusion pressure ($P > 0.05$; paired Student's t-test; $n = 6$).

4. Discussion

Our results show that tolerance to glyceryltrinitrate develops rapidly in rat femoral arteries, within 20 h of continuous exposure, resulting in a marked attenuation of the responses to additional bolus concentrations of glyceryltrinitrate. The endogenous S-nitrosothiol, S-nitrosoglutathione, and novel S-nitrosothiols, RIG200 and D-

SNVP, did not induce tolerance within 20 h or exhibit cross-tolerance in vessels made tolerant to glyceryltrinitrate.

The amplitude of glyceryltrinitrate-induced vasodilatation diminished despite continued perfusion of the drug, and was not evident after 20 h. Down-regulation of NO synthase by NO from glyceryltrinitrate (Moncada et al., 1991) could be excluded as a possible explanation for the results because the NO synthase inhibitor, L-NAME, was present throughout. Given the long duration of the experiments it was necessary to continuously perfuse L-NAME to prevent NO generation from the inducible form, as well as the constitutive, NO synthase. The vasodilator response to boluses of high concentrations of glyceryltrinitrate (10^{-4}, 10^{-3} M) were attenuated in the presence of perfusing glyceryltrinitrate (10^{-5} M), confirming nitrate tolerance in these vessels. Perfused isolated femoral arteries are, therefore, an effective ex vivo model for the investigation of nitrate tolerance and cross-tolerance to NO donor drugs. In addition, tolerance to glyceryltrinitrate can be induced rapidly, facilitating studies investigating the prevention and reversal of nitrate tolerance.

All the S-nitrosothiols (10 μM) that were investigated relaxed arteries to a similar extent, producing ~90% vasodilatation. Vasodilatation to S-nitrosothiols was maintained throughout the 20 h perfusion period despite the slow decomposition of the compounds in the perfusate reservoir. Our results demonstrate that, despite structural modifications, RIG200 and D-SNVP retain the characteristics of existing S-nitrosothiols in that they do not engender tolerance (Kowaluk et al., 1987; Kowaluk and Fung, 1990; Bauer and Fung, 1991). Following 20 h S-nitrosothiol perfusion, pressure was rapidly restored by washing out the S-nitrosothiol, confirming the reversibility of the effect and indicating that the vessel was still viable. RIG200 and D-SNVP have previously been demonstrated to induce a vasodilatation which persists after washout in endothelium-denuded vessels (Megson et al., 1997, 1999). Therefore, the rapid restoration of pressure following S-nitrosothiol washout suggests that the endothelium was functionally intact after 20 h, consistent with the vasoconstrictor effect of L-NAME at the beginning of the experiment. D-SNVP took significantly longer to wash out than S-nitrosoglutathione and RIG200, perhaps reflecting its greater lipophilicity (Megson et al, 1999).

S-Nitrosoglutathione-induced vasodilatation was partially inhibited by perfusion with ferrohaemoglobin, suggesting that extracellular decomposition of S-nitrosoglutathione to release NO contributes to the vasodilatation in response to this compound. This reflects the sensitivity of S-nitrosoglutathione to catalyzed decomposition by metal ions in Krebs solution (Dicks et al., 1996; Gordge et al., 1996), or by elements of the vascular cell surface (Kowaluk and Fung, 1990; Al-Sa'doni et al., 1997). The vasodilatation produced by RIG200 and D-SNVP was unaffected by ferrohaemoglobin perfusion, suggesting that ferrohaemoglobin does not have access to the site where RIG200 and D-SNVP exert their bioactivity. S-Nitrosothiol-induced vasodilatation could be completely reversed by addition of the soluble guanylate cyclase inhibitor, ODQ, suggesting that in this vascular tissue, unlike in platelets (Gordge et al., 1998), the action of S-nitrosothiols is entirely mediated by activation of this enzyme.

Our finding that S-nitrosothiols do not induce tolerance implies that the underlying cause of nitrate tolerance ex vivo is upstream of NO release. Desensitization of the target enzyme, guanylate cyclase (Needleman and Johnson, 1973; Waldman et al., 1986), or upregulation of cGMP-phosphodiesterase activity (Axelsson and Andersson, 1983) have been suggested as potential mechanisms in tolerance development. However, our results show that S-nitrosothiols remain fully active in glyceryltrinitrate-tolerant vessels through a mechanism entirely mediated by guanylate cyclase. Our results also question the involvement of superoxide generation in tolerance development ex vivo, because S-nitrosothiols retained full activity in glyceryltrinitrate-tolerant vessels. S-Nitrosothiols may release NO at a site inaccessible to scavenging by superoxide or possibly stimulate guanylate cyclase directly. However, elevated superoxide levels would at least be able to inactivate extracellular NO from S-nitrosoglutathione in tolerant vessels. The role of superoxide production may, however, be more prominent in vivo, where neurohormonal mechanisms including the renin–angiotensin and endothelin systems may exacerbate oxidative stress (Munzel and Bassenge, 1996).

All the S-nitrosothiols tested were as effective in glyceryltrinitrate-tolerant vessels as in control vessels. This reinforces our conclusion that events prior to NO release or S-nitrosothiol formation (Ignarro et al., 1981) limit the effectiveness of glyceryltrinitrate in tolerance. S-Nitrosothiols decompose spontaneously in solution at varying rates to generate NO (Williams, 1985) and therefore, may not be dependent on the same co-factors needed to release NO from glyceryltrinitrate. In addition, the ability of S-nitrosothiols to directly transfer NO to reduced tissue thiols without the release of free NO (Askew et al., 1995) could lead to activation of guanylate cyclase through nitrosation of cysteine residues in the enzyme (Ignarro et al., 1981). This property could be the underlying reason why S-nitrosothiols do not induce tolerance.

In summary, we have shown that two novel NO donor drugs, RIG200 and D-SNVP do not induce tolerance with 20 h of continuous perfusion, in an ex vivo model of tolerance. In addition, they retain full vasodilator potency in vessels made tolerant to glyceryltrinitrate, despite its continued presence. Our results lend weight to the argument that RIG200 and D-SNVP may be viable clinical alternatives to organic nitrates and existing S-nitrosothiols, because, added to their previously described increased stability and selectivity for areas of endothelial damage, they do not appear to engender tolerance. These features

suggest that RIG200 and D-SNVP could have potential benefits in the treatment of a number of cardiovascular diseases including angina, atherosclerosis, cardiac ischaemia, heart failure, and other conditions where long-term and high dose vasodilator therapy is required.

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