Prolonged effect of a novel S-nitrosated glyco-amino acid in endothelium-denuded rat femoral arteries: potential as a slow release nitric oxide donor drug

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1 The vasodilator properties of a novel S-nitrosated glyco-amino acid (RIG200) were investigated in isolated rat femoral arteries and compared with those of the parent S-nitrosothiol compound, S-nitroso-N-acetylpenicillamine (SNAP).

2 Spectrophotometric analysis revealed that 2.5 mM solutions of RIG200 decomposed more slowly (half-life ($t_{1/2}$) = 216.2 ± 26.7 min) than SNAP ($t_{1/2}$ = 37.2 ± 13.8 min) in Krebs buffer at 24°C. Furthermore, the rate of decomposition of SNAP, but not of RIG200, was significantly reduced by the Cu(I) chelator, neocuproine. We concluded that the relative stability of RIG200 is due, at least in part, to its resistance to trace Cu(I)-catalyzed decomposition. Nitric oxide (NO) generation from SNAP and RIG200 was confirmed by use of an NO electrode.

3 Experiments to investigate the vasodilator effects of RIG200 were carried out on isolated femoral arteries taken from adult male Wistar rats (400–550 g). Lengths of artery (7–8 mm long) were cannulated, dissected free and perfused at constant flow rate (0.6 ml min^{-1}) with Krebs buffer. Vessels were precontracted with phenylephrine ($10.2 \pm 0.3 \mu\text{M}$) and developed pressures of $91.8 \pm 4 \text{ mmHg}$, detected upstream by a differential pressure transducer.

4 Concentration-dependent vasodilator responses to bolus injections of SNAP or RIG200 ($10 \mu\text{l}$; 10^{-8} – 10^{-3} M) made into the perfusate of endothelium-intact vessels were transient, recovering the pre-injection pressure in <20 min.

5 Responses to equivalent bolus injections of SNAP in endothelium-denuded vessels were also transient but those in response to concentrations of RIG200 $>10^{-5}$ M were sustained. Responses to 10^{-3} M RIG200 were sustained for periods >4 h. Sustained vasodilatation was reversed by the NO scavenger, ferrohaemoglobin ($10 \mu\text{M}$) but was unaffected by the NO synthase inhibitor, N^ω-nitro-L-arginine methyl ester ($200 \mu\text{M}$), indicating involvement of NO from a source other than NO synthase.

6 We suggest that a possible explanation for the prolonged effect of RIG200 is retention of the compound by the vascular wall, facilitated by endothelial denudation. Slow decomposition of RIG200 *in situ* would release sufficient NO to maintain a 'vasodilator tone' which persists for more than 4 h. Selective retention by damaged vessels could have important therapeutic implications with regard to targeted delivery of NO, restoring protection to areas deprived of endogenous NO, whilst avoiding unwanted hypotension.

Keywords: Nitric oxide; S-nitrosothiols; vasodilatation; RIG200

Introduction

Nitric oxide (NO) synthesized in the endothelium of blood vessels (Palmer *et al.*, 1987; 1988; Palmer & Moncada, 1989) is recognised to be an important factor in control of local blood flow and of blood pressure 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). In addition, NO is known to inhibit platelet aggregation and adhesion (Radomski *et al.*, 1987a,b; Vallance *et al.*, 1989; Haynes *et al.*, 1993), smooth muscle mitogenesis (Garg & Hassid, 1989) and monocyte adhesion (Lefer, 1997). In view of these properties, endothelial dysfunction resulting in reduced NO synthesis is thought to play an important role in atherogenesis (Chappell *et al.*, 1987; Harrison *et al.*, 1987; Forstermann *et al.*, 1988; Guerra *et al.*, 1989). Physical damage to, or removal of, the endothelium during percutaneous transluminal angioplasty (PTA) is a major contributory factor in the high incidence of restenosis following the procedure (Langford *et al.*, 1994).

Organic nitrates (such as glyceryl trinitrate) have been used for many years in the treatment of angina (Fung & Bauer,

1994). Their actions are mediated by NO generated *in situ* by a mechanism which may involve reduced tissue thiols (Needleman *et al.*, 1973; Ignarro *et al.*, 1981). However, the therapeutic use of organic nitrates is limited by the development of nitrate tolerance, possibly due to the depletion of reduced tissue thiols essential to their metabolism (Fung & Bauer, 1994). Sodium nitroprusside (SNP) is another NO donor drug which can be used as a post-operative anti-hypertensive agent. The mechanism of NO release from SNP is poorly understood but it is known not to decompose spontaneously in solution at physiological pH unless exposed to light (Butler & Glidewell, 1987). It has been suggested that *in vivo* decomposition is tissue-dependent, and may involve specific membrane-bound proteins (Kowaluk *et al.*, 1992). SNP administration requires careful titration and prolonged use can result in cyanide poisoning (Butler & Glidewell, 1987). Neither organic nitrates nor SNP can be targeted at areas of endothelial damage and their use is often associated with systemic hypotension. The possibility of localizing supply of NO to areas of the vasculature devoid of endothelium-derived NO is a more desirable therapeutic option.

S-Nitrosothiols (general formula R-S=NO) decompose spontaneously in solution to their disulphide form, generating

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NO in the process (Williams, 1985), and present a potential alternative to organic nitrates, particularly as they do not appear to engender vascular tolerance (Harowitz *et al.*, 1983; Bauer & Fung, 1991). However, the therapeutic potential of many existing S-nitrosothiols, like S-nitroso-N-acetylpenicillamine (SNAP; Figure 1a) and S-nitrosoglutathione (GSNO) is limited by the rapid and unpredictable nature of their decomposition due to the catalytic effect of trace Cu(I) ions (Dicks *et al.*, 1996; Gordge *et al.*, 1996). Accelerated decomposition in the presence of Cu(II) (De Man *et al.*, 1996) is now thought to be mediated by prior reduction to Cu(I) by thiols. *In vivo*, decomposition may also be accelerated by direct transfer of NO⁺ to reduced tissue thiols (transnitrosation; Askew *et al.*, 1995) and by enzyme-dependent mechanisms (Askew *et al.*, 1995; Gordge *et al.*, 1996). Therapeutic effects of existing S-nitrosothiols are, therefore, potentially variable and short-lived.

Endogenous S-nitrosothiols have been identified in both human plasma (Stamler *et al.*, 1992a,b) and tissue extracts (Gaston *et al.*, 1993) but their role is, as yet, unclear. GSNO has shown a degree of tissue specificity, inhibiting platelet aggregation more readily than causing vasodilatation (Radomski *et al.*, 1992; De Belder *et al.*, 1994) and acting as a more potent vasodilator in arteries than veins (MacAllister *et al.*, 1995). GSNO has also been shown to have potentially beneficial effects with regard to inhibition of platelet aggregation associated with PCTA of coronary arteries (Langford *et al.*, 1994).

Here, we investigate the vasodilator properties of a novel S-nitrosated glyco-amino acid (RIG200; Figure 1b) in rat isolated femoral arteries. RIG200 is SNAP coupled to glucosamine tetra-acetate by an amide bond. The molecule is different from those described recently (Ramirez *et al.*, 1996) in that the glucose moiety is acetylated at the 1, 3, 4 and 6 positions. It was envisaged that non-polar glucosamine tetra-acetate would cause steric hindrance of Cu(I)-catalyzed decomposition and also increase lipid solubility of RIG200. These features would result in slow release of NO from RIG200, independent of Cu(I) concentration, and could facilitate uptake of RIG200 into vascular tissue, altering the mode of NO delivery.

Methods

Decomposition of SNAP and RIG200 *in vitro*

SNAP and RIG200 (2.5 mM) diluted in oxygenated (95% O₂, 5% CO₂) Krebs buffer (composition in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25 and glucose 5.5) were incubated in the dark at 24°C. Care was taken to use the same Krebs solution to dilute SNAP and RIG200 in order to ensure identical copper ion content. The decrease in absorbance at a wavelength (λ) of 341 nm was measured by use of Phillips PU 8720 ultraviolet/visible scanning spectrophotometer (path length = 1 cm).

Experiments at 24°C were repeated in the presence of either an intermediate concentration of CuSO₄ (1 μ M; Askew *et al.*, 1995) or the specific Cu(I) chelator, neocuproine (NCu; 1 μ M; Dicks *et al.*, 1996) in order to establish the effect of Cu(I) on the rate of decomposition of RIG200 and SNAP.

Generation of NO from SNAP and RIG200 *in vitro*

SNAP and RIG200 1 mM in Krebs buffer were stirred in the dark at 24°C. NO generation was measured using an NO probe (ISO-NO World Precision Instruments, Stevenage, Herts, U.K.), calibrated with NaNO₂ (1–25 μ M) acidified with 1 mM ascorbic acid (Gaston *et al.*, 1993). The effect of CuSO₄ (1 μ M) or NCu (100 μ M) on NO generation was also investigated. A higher concentration of NCu was necessary in the electrode experiments to scavenge Cu(I) derived from added (1 μ M) rather than trace Cu(II).

Figure 1 Structural formulae and full generic names for SNAP (a) and RIG200 (b). Note that RIG200 contains a SNAP moiety linked by a peptide bond to glucosamine tetra-acetate.

Biological activity of SNAP and RIG200

Preparation Experiments were carried out on isolated segments of femoral artery from adult male Wistar rats (400–550 g; $n = 36$). The perfusion system used was similar to that described previously for rat tail artery perfusion (Flitney *et al.*, 1992). Briefly, animals were killed by cervical dislocation and both femoral arteries were exposed and cannulated immediately distal to the epigastric arterial branch. Cannulated arterial segments (7–8 mm long) were dissected free and transferred to perspex organ bath chambers (1 ml volume) at 37°C where they were perfused (0.6 ml min⁻¹; Gilson minipuls 3, Anachem, Luton, U.K.) and superfused (1 ml min⁻¹, Watson Marlow 302S; Watson Marlow, Falmouth, U.K.) with fresh oxygenated Krebs buffer solution. Twin vessels were precontracted with phenylephrine (PE) and perfusion pressure was monitored by a differential pressure transducer (T; Sensym SCX 15ANC, Farnell Electronic Components, Leeds, U.K.) located upstream.

The apparatus permits exclusive drug delivery to the luminal surface of the vessel by bolus injection (10 μ l) through a resealable rubber septum into the perfusate immediately upstream of the vessel (transit time to artery ~ 3 s, through lumen ~ 300 ms). Injections of vehicle (Krebs buffer) had no effect on perfusion pressure. Vasodilator responses in control vessels could be compared to those perfused with supra-maximal concentrations of either the recognised NO scavenger, ferrohaemoglobin (Martin *et al.*, 1985; Hb; 10 μ M), or the NO synthase (NOS) inhibitor, N^o-nitro-L-arginine methyl ester (Rees *et al.*, 1990; L-NAME; 200 μ M). Where possible, two vessels from each animal were used in parallel; one receiving SNAP and the other RIG200.

Experimental protocols 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). All drugs were dissolved and diluted in PE-containing Krebs solution and kept on ice before use.

Once precontracted, endothelial function was assessed by use of the endothelium-dependent vasodilator, carbachol (CCh). Bolus injections of supra-maximal concentrations of CCh (10 μ l; 10 mM) into the perfusate of endothelium-intact vessels caused transient vasodilations similar to those described in rat tail arteries (Flitney *et al.*, 1992). In experiments where the endothelium was removed, air was passed through the lumen until such time as the vessel was unresponsive to CCh

(5–10 min). Removal of the endothelium invariably caused an increase in pressure due to loss of endothelium-derived NO synthesis. Pressure was restored to its original level by appropriate reduction in PE concentration ($\sim 0.5 \times$ original concentration). Selected vessels were taken for histological staining to confirm endothelial removal (5 μ m paraffin sections, fixed in formalin (10%; 24 h) and stained with haematoxylin and eosin). Final assessment of the integrity of the endothelium was achieved at the end of experiments where vessels were perfused with ferrohaemoglobin (Hb; 10 μ M). Perfusion pressure in endothelium-intact vessels rose considerably higher than pretreatment pressure, indicating the presence of endothelium-derived NO, whilst endothelium-denuded vessels failed to develop pressures above pretreatment levels.

Vasodilator responses to bolus injections of SNAP and RIG200 Bolus injections of increasing concentrations of SNAP or RIG200 (10 μ l; 10^{-8} – 10^{-3} M) were made sequentially into the perfusate of precontracted, endothelium-intact or -denuded vessels. Responses were deemed to have recovered once pressure was maintained for more than 2.5 min, at which time the next concentration was injected. Time intervals between injections of SNAP and RIG200 were matched for each individual experiment. Responses to 10^{-3} M concentrations were then allowed to recover for periods of between 15 min and 4 h, after which vessels were perfused with Hb (10 μ M).

The effect of Hb on vasodilator responses to SNAP and RIG200 In order to assess the role of NO in SNAP- and RIG200-induced vasodilatation, sequential bolus injection experiments were also carried out in endothelium-denuded vessels perfused with Hb (10 μ M).

The effect of L-NAME on sustained vasodilator responses to RIG200 The possible role of the inducible isoform of NOS (iNOS) in sustaining RIG200-induced vasodilatation in endothelium-denuded vessels was assessed by examining the effect of perfusing vessels with the NOS inhibitor, L-NAME (200 μ M; 3 h), on responses to a single bolus injection (10^{-3} M) of RIG200 in endothelium-denuded vessels. Perfusion commenced 1 h after the bolus injection of RIG200.

Drugs and reagents S-Nitrosothiols were synthesized as described below in the School of Chemistry (University of St. Andrews, St. Andrews, Scotland). All other chemicals were obtained from Sigma Ltd. (Poole, Dorset, U.K.) Met-Hb was reduced to the ferro- form with sodium dithionite as described previously (Martin *et al.*, 1985).

SNAP was prepared by an established method (Field *et al.*, 1978). Ultraviolet/visible spectral analysis confirmed an absorption peak at $\lambda = 341$ nm, characteristic for S-nitrosothiols. The disulphide products formed during decomposition of S-nitrosothiols do not absorb at this wavelength. The extinction coefficient (ϵ) at this wavelength was $1168 \text{ M}^{-1} \text{ cm}^{-1}$. SNAP is soluble in Krebs buffer at concentrations < 10 mM.

RIG200 was prepared by mixing equimolar amounts of glucosamine tetra-acetate synthesized by the method previously described (Bergmann & Zervas, 1931) and N-acetyl-D,L-penicillamine in dichloromethane. Addition of an equimolar amount of the coupling agent, 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-p-toluene sulphate, resulted in the formation of a white precipitate. The mixture was stirred for 24 h, filtered, and the filtrate washed (1 M HCl, saturated NaHCO_3 , water), dried (MgSO_4), evaporated and recrystallized from methanol (46% yield). The product was then nitrosated in 50% methanol/water by NaNO_2 acidified with HCl. The green solution formed was extracted into dichloromethane and evaporated in an oil. Trituration with ether gave a dark green solid (88% yield). The identity of the product was confirmed by use of mass spectrometry (EPSRC Mass Spectroscopy Service, University College, Wales, U.K.) and nuclear magnetic resonance (n.m.r.) spectroscopy. Ultra-

violet/visible spectral analysis revealed an absorption peak at $\lambda = 341$ nm where $\epsilon = 640 \text{ M}^{-1} \text{ cm}^{-1}$. RIG200 is soluble in Krebs buffer at concentrations < 3 mM after ultrasonication (1 min).

Analysis of results

Signals from the pressure transducers were processed by a MacLab/4e analogue-digital converter and displayed through 'Chart' software (AD Instruments, Sussex, U.K.) on a Macintosh Performa 630 microcomputer. Vasodilator response amplitude was expressed as a % of PE-induced pressure existing before the first in a series of drug applications (% pressure change; negative values represent relaxation, positive

Figure 2 Mean absorbance (A), expressed as a fraction of A at time 0 (A_0) measured at $\lambda = 341$ nm for 2.5 mM solutions of SNAP (a) and RIG200 (b) in Krebs solution alone, Krebs solution + 1 μ M neocuproine (NCu) and Krebs solution + 1 μ M copper sulphate (CuSO_4) at 24°C plotted against time (all $n = 6$). Half-lives ($t_{1/2}$) for decomposition derived from semi-logarithmic plots of the above data are given in the text. Vertical lines show s.e.mean.

represent constriction). Data are given as % pressure change both at the peak of responses and following response recovery as defined earlier. Mean values are given $\pm$ s.e.mean.

P values stated in the text were obtained by two-factor, repeated dose ANOVAs except where stated otherwise. Unpaired, two-tail student's *t*-tests and Mann-Whitney U (MWU) non-parametric tests were also used where appropriate. *P* < 0.05 was accepted as statistically significant.

Results

Decomposition of SNAP and RIG200 in vitro

Absorbance (*A*) was plotted as a fraction of *A* at time 0 (*A*₀). Half-lives (*t*_{1/2}) of SNAP and RIG200 were derived from linear plots of log *A*/*A*₀ against time. Figure 2 shows the rate of decomposition of SNAP (a) and RIG200 (b) in oxygenated Krebs buffer at 24°C, as assessed by monitoring absorbance at 341 nm. The rate of SNAP decomposition (*t*_{1/2} = 37.2 $\pm$ 13.8 min) was significantly faster than that of RIG200 (*t*_{1/2} = 216.2 $\pm$ 26.7 min, *P* = 0.006, Mann Whitney U non-parametric test). Decomposition of SNAP was more variable than that of RIG200 in Krebs alone and was significantly reduced in the presence of NCu (1 μ M; *t*_{1/2} = 57 $\pm$ 6.1 min, *P* = 0.04, Mann Whitney U test). NCu did not affect the rate of RIG200 decomposition (*t*_{1/2} = 227 $\pm$ 24.45 min, *P* = 0.68, Mann Whitney U test). In the presence of CuSO₄ (1 μ M), there was accelerated decomposition of both SNAP (*t*_{1/2} = 10.0 $\pm$ 3.2 min, *P* = 0.01, Mann Whitney U test) and RIG200 (*t*_{1/2} = 55.1 $\pm$ 8.9 min, *P* = 0.006, Mann Whitney U test).

Generation of NO from the decomposition of SNAP and RIG200 (1 mM) was confirmed with an isolated NO electrode (Figure 3). In Krebs buffer alone at 24°C, SNAP generated more NO than RIG200, reflected in NO concentrations at equilibrium of 5.9 $\pm$ 0.9 and 3.8 $\pm$ 0.7 μ M, respectively (*P* = 0.028, Student's unpaired *t* test). The amount of NO measured in solution increased to 11.7 $\pm$ 2.7 and 6.2 $\pm$ 1.1 μ M, respectively, on addition of CuSO₄ (1 μ M). The increase was reversed by adding excess NCu (100 μ M), yielding NO concentrations of 3.5 $\pm$ 0.6 and 3.2 $\pm$ 0.8 μ M, respectively (all *n* = 5).

Biological activity of SNAP and RIG200

Vessel parameters Vessels were precontracted with PE (10.2 $\pm$ 0.3 μ M) in order to give pressures of $\sim$ 90 mmHg

(91.8 $\pm$ 4.0 mmHg; *n* = 52). Endothelium intact vessels dilated in response to 10 mM bolus injections of CCh (25.5 $\pm$ 3.3% relaxation; *n* = 22). Pressure in PE-contracted vessels increased by 52.3 $\pm$ 9.3% (*n* = 14) when perfused with 10 μ M Hb, confirming the presence of a functional endothelium.

Endothelium removal by perfusion of air abolished responses to CCh and caused an increase in PE-induced pressure of 75.4 $\pm$ 11.3% (*n* = 30). Pressure in these vessels failed to rise significantly above baseline pressure when perfused with Hb (+4.5 $\pm$ 4.8%; *n* = 14).

Examination of histological sections of vessels confirmed the presence of endothelium in intact vessels and successful removal in those perfused with air.

Vasodilator responses to bolus injections of SNAP and RIG200

Bolus micro-injections of SNAP into the perfusate of endothelium-intact vessels caused concentration-dependent, transient vasodilations (Figures 4a and 5a). Responses to moderate concentrations of SNAP typically recovered to pressures higher than pre-injection pressure. The effect subsided, but pressure often remained slightly elevated (+11.2 $\pm$ 8.1% for 10⁻⁵ M SNAP; *P* = 0.042). Responses to the highest concentration of SNAP (10⁻³ M) caused relaxations of -75.2 $\pm$ 4.7% and generally recovered fully in

Figure 3 Representative recording of NO in a stirred solution of RIG200 or SNAP (1 mM) in Krebs buffer at 24°C, measured with an isolated NO electrode. CuSO₄ (1 μ M) and NCu (100 μ M) were added to the solution as indicated. Baseline recordings at the beginning and end of experiments were made in Krebs buffer alone.

Figure 4 Pressure recordings showing vasodilator responses to sequential bolus micro-injections (10 μ l) of SNAP (a and b) or RIG200 (c and d; log M concentrations as indicated) into the perfusate of endothelium-intact and endothelium-denuded vessels. Perfusion with Hb (10 μ M) is indicated by the solid bar.

<20 min. However, some vessels ($n=2/7$) failed to recover to pre-injection pressures even after washout periods of up to 1 h. Perfusion with $10\ \mu\text{M}$ Hb invariably caused pressure to rise significantly above pre-injection pressures ($+45.1 \pm 15.1\%$; $P=0.001$).

Endothelium removal did not significantly increase vessel sensitivity to SNAP ($P=0.75$). However, the contractile effect often seen in endothelium-intact vessels following SNAP-induced vasodilations was not seen in denuded vessels (Figures 4b and 5b). Responses to the highest concentration of SNAP ($10^{-3}\ \text{M}$) in some vessels ($n=2/7$) failed to recover to pre-injection pressures even after washout periods of up to 1 h. Perfusion with $10\ \mu\text{M}$ Hb following this concentration of SNAP caused rapid recovery to pre-injection pressures ($+7.9 \pm 3.8\%$) in those vessels which had failed to show full spontaneous recovery (Figure 4b).

Responses to RIG200 in endothelium-intact vessels were transient and similar in nature to those for SNAP (Figures 4c

and 5c). Perfusion of these vessels with Hb invariably caused a rapid rise in pressure to $+74.1 \pm 5.4\%$ ($P=0.001$; two-tailed, unpaired Student's t test) above pre-injection levels. Responses in endothelium-denuded vessels also showed full recovery following 10^{-8} – $10^{-7}\ \text{M}$ microinjections but thereafter failed to recover to pre-injection pressure (Figures 4d and 5d). The sustained vasodilator effect of moderate and high concentrations of RIG200 was concentration-dependent, with perfusion pressure recovering to only $48.0 \pm 5.2\%$ of pre-injection pressure following a $10^{-3}\ \text{M}$ bolus injection. The response persisted for >4 h and was fully reversed on perfusion with Hb, reaching pressures $+1.0 \pm 3.2\%$ of pre-injection levels. The rate of pressure recovery on perfusion with Hb was often noticeably slower in vessels treated with RIG200 than SNAP.

Vessels were less sensitive to RIG200 than SNAP in either endothelium-intact ($P=0.022$) or endothelium-denuded vessels ($P=0.014$), corresponding to greater stability of RIG200 in

Figure 5 Log-dose response curves showing the vasodilator effect (% pressure change) of bolus micro-injections ($10\ \mu\text{l}$) of (a and b) SNAP or (c and d) RIG200 on endothelium-intact (a and c) and -denuded (b and d) rat femoral arteries. Peak amplitude and recovery of responses are illustrated (all $n=8$). Points shown are means and vertical lines indicate s.e.mean.

vitro. Relaxations in response to 10^{-3} M RIG200 were similar in amplitude to those to SNAP ($75.0 \pm 3.0\%$ relaxation). Endothelium-removal did not significantly alter sensitivity to RIG200 ($P=0.66$).

Delivery of maximal and supra-maximal concentrations of both SNAP and RIG200 was limited by their saturation points in Krebs buffer (saturated at $\sim 3 \times 10^{-3}$ M), preventing calculation of EC_{50} values.

The effect of Hb on vasodilator responses to SNAP and RIG200 in denuded vessels The peak amplitude of responses to bolus injections of SNAP in endothelium-denuded vessels was significantly attenuated, but not abolished, in the presence of Hb ($P=0.001$; Figure 6a). Similarly, perfusion of endothelium-denuded vessels with Hb caused inhibition of RIG200-induced

responses ($P=0.019$) and abolished the sustained vasodilator effect of RIG200 (Figure 6b).

The effect of L-NAME on sustained vasodilator responses to RIG200 Single bolus micro-injections of RIG200 (10^{-3} M) in endothelium-intact vessels caused transient vasodilations which recovered to $95.3 \pm 4.2\%$ in <30 min (Figure 7a; $n=8$). Equivalent micro-injections of RIG200 in endothelium-denuded vessels caused relaxations which only recovered to $48.2 \pm 7.9\%$ of pre-injection pressure 4 h after injection (Figure 7b; $n=6$). Sustained vasodilation was inhibited by Hb, as described previously, and the effect was reversed on washout (Figure 7b; $n=6$). However, sustained vasodilation in endothelium-denuded vessels was not significantly affected by L-NAME (Figure 7c), with perfusion pressure recovering to $45.3 \pm 3.2\%$ of pre-injection pressure 4 h after bolus washout ($n=6$; $P=0.47$ compared to untreated vessels; unpaired Student's *t* test).

Discussion

Our results show that the novel S-nitrosated glyco-amino acid, RIG200, decomposes spontaneously in solution to release NO *in vitro* and is capable of causing NO-mediated vasodilatation in rat isolated femoral arteries. Decomposition was found to be slower than that of the parent compound, SNAP, but was not susceptible to trace Cu(I) catalysis *in vitro*. Bolus injections of both SNAP and RIG200 produced transient responses in endothelium-intact, rat isolated femoral arteries. SNAP-induced vasodilations in endothelium-denuded vessels were transient whilst those induced by RIG200 were sustained for up to 4 h.

Spectrophotometric analysis showed RIG200 to be 5 fold more stable than SNAP in Krebs buffer solution (Figure 2). The rate of SNAP decomposition *in vitro* is notoriously variable, a feature which is now understood to be due to differences in the amounts of trace Cu(I) present in solutions (Dicks *et al.*, 1996). It has been suggested that a small proportion of Cu(II) is reduced to Cu(I) by thiols present as impurities in solutions of S-nitrosothiols and that it is Cu(I) which catalyzes S-nitrosothiol decomposition (Dicks *et al.*, 1996). Our results show that SNAP decomposition was significantly slower in the presence of the Cu(I)-chelator, NCu, confirming the involvement of Cu(I)-catalysis. In contrast, the rate of RIG200 decomposition was unaffected

Figure 6 Log-dose response curves showing the vasodilator effect (% pressure change) of bolus micro-injections ($10 \mu\text{l}$) of (a) SNAP or (b) RIG200 in rat endothelium-denuded femoral arteries whilst being perfused with $10 \mu\text{M}$ Hb. Peak amplitude and recovery of responses is illustrated (all $n=8$). Points show means and vertical lines indicate s.e.mean.

Figure 7 Pressure recordings showing vasodilator responses to single bolus micro-injections of 10^{-3} M RIG200 in (a) an endothelium-intact vessel in the absence of L-NAME (b) an endothelium-denuded vessel in the absence of L-NAME but perfused with Hb ($10 \mu\text{M}$) for the period indicated and (c) an endothelium-denuded vessel perfused with L-NAME for the period indicated (3 h).

by NCu, indicating that it was resistant to trace Cu(I)-catalyzed decomposition. However, addition of a relatively high concentration of Cu(II) (1 μ M) accelerated decomposition of both compounds.

NO generation from both SNAP and RIG200 *in vitro* was confirmed by use of an isolated NO electrode. RIG200 generated less NO than SNAP in Krebs buffer alone, confirming its comparative stability under these conditions (Figure 3). Both SNAP and RIG200 decomposition was accelerated in the presence of Cu(II), an effect that was reversed by addition of the Cu(I) chelator, NCu to the buffer. This result confirms that Cu(I) derived from added Cu(II) is the catalyst for decomposition of both SNAP and RIG200. In addition, NCu caused a considerable reduction of NO generation from SNAP, but not RIG200, in Krebs buffer, further evidence supporting the proposal that trace Cu(I), which may be derived from de-ionized water, glassware and salts, is sufficient to accelerate decomposition of SNAP but not RIG200. It is apparent, therefore, that RIG200 is inherently more stable than SNAP in Krebs buffer solution, due in part to its resistance to trace Cu(I) catalysis.

Decomposition of both SNAP and RIG200 would be expected to be greater *in vivo* since Cu(II) levels in human serum are 12–24 μ M of which 1–2 μ M is unbound (Lenter, 1984). The relatively slow, trace Cu(I)-resistant decomposition of RIG200 *in vitro* is advantageous in limiting the undesirable variability in NO generation and the resultant handling difficulties experienced with SNAP, and may influence its biological activity.

Bolus injections of SNAP or RIG200 into the perfusate of precontracted, endothelium-intact rat isolated femoral arteries induced transient vasodilatations (Figures 4 and 5). Vessels were significantly less sensitive to RIG200 than to SNAP, perhaps reflecting the relative stability of RIG200 in solution. NO is known to inhibit synthesis of endogenous NO by endothelial NOS and this process may be responsible for the modest vasoconstriction seen following responses to bolus injections of intermediate concentrations (10^{-5} – 10^{-4} M) of either SNAP or RIG200 in endothelium-intact vessels (Figures 4a,c and 5a,c). Absence of equivalent vasoconstriction in endothelium-denuded vessels (Figures 4b,d and 5b,d) is further evidence for NO-induced inhibition of NOS by NO derived from S-nitrosothiols.

Endothelium-removal did not significantly affect vasodilatations induced by bolus injections of SNAP or RIG200 (Figures 4a,c and 5a,c) and responses to both compounds were significantly inhibited by Hb (Figure 6a,b). However, responses to intermediate and high concentrations of RIG200 in endothelium-denuded vessels recovered at a slower rate (Figure 4d) and caused a sustained depression of tone (Figures 4d and 5d). Pre-injection pressure was restored by perfusing with Hb, confirming that sustained vasodilatation is entirely NO-mediated. The slow rate of recovery observed in

RIG200-treated vessels (Figure 4d) may indicate that NO generated by RIG200 is less accessible to Hb than that derived either from the endothelium or from SNAP. The inhibitory effect of Hb on sustained vasodilatation was reversible on washout (Figure 7b), indicating continuing NO generation after Hb treatment.

Vasodilatation to a single bolus injection of 10^{-3} M RIG200 in endothelium-intact vessels typically recovered to pre-injection pressure within 30 min (Figure 7a). Equivalent responses in endothelium-denuded vessels caused sustained vasodilatation which showed only partial recovery after 4 h washout (Figure 7b). Sustained responses were unaffected by L-NAME treatment (Figure 7c), excluding induction of iNOS as a possible mechanism for the effect.

A possible explanation for our results is that SNAP-induced, transient vasodilatations are caused primarily by spontaneous release of NO in the perfusate, irrespective of the presence of endothelium. Similarly, transient responses to RIG200 in endothelium-intact vessels are due to spontaneous release of NO in the perfusate. However, responses to RIG200 in endothelium-denuded vessels appeared to comprise two elements; a transient component due to spontaneous NO release in the perfusate and a sustained component due to release of NO from RIG200, retained within the tissue long after the bolus has passed through the lumen. It is apparent that the sustained component of vasodilator responses to RIG200 is specific to endothelium-denuded vessels. We suggest that endothelial cells present a barrier to RIG200 retention but that in endothelium-denuded vessels, RIG200 may be retained in the tissue underlying the endothelium (the internal elastic lamina and/or the media). Possible retention of RIG200 by the sub-endothelium may be particularly important in view of emerging evidence that the barrier function of endothelium is altered by oxidized low density lipoprotein (Rangaswamy *et al.*, 1997), which is known to accumulate in atherosclerotic lesions (Haberland *et al.*, 1988).

The stability of RIG200 *in vitro* is a distinct advantage over existing S-nitrosothiols with regard to its handling, and may be a factor in increasing the duration of vasodilator responses *in vivo*. In addition, the ability of RIG200 to cause selectively sustained vasodilatation in vessels where the endothelium has been removed could have important implications in cardiovascular diseases, where endothelial damage is a contributory factor, and may be useful in preventing restenosis of vessels following surgical procedures like PCTA and CABG. The unique properties of RIG200 require confirmation in human vessels and ultimately *in vivo* but RIG200 potentially presents new therapeutic opportunities for S-nitrosothiols as slow-release NO donor drugs.

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