British Journal of Pharmacology (1996) 117, 1549–1557

Vasodilator responses of rat isolated tail artery enhanced by oxygen-dependent, photochemical release of nitric oxide from iron-sulphur-nitrosyls

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1 The vasodilator properties and photochemical decomposition of two synthetic iron-sulphur-nitrosyl clusters (cluster *A*: $[Fe_4S_4(NO)_4]$, tetranitrosyl-tetra-μ_3-sulphido-tetrahedro-tetrairon; and *B*:$[Fe_4S_3(NO)_7]^{-1}$, heptanitrosyl-tri-μ_3-thioxotetraferrate(−1)) have been investigated. Experiments were carried out on isolated, internally-perfused segments of rat tail artery.

2 Bolus injections (10 μl) of *A* or *B* (>0.25 mM) delivered into the internal perfusate generated sustained (or *S*-type) vasodilator responses, characterized by a persistent plateau of reduced tone due to NO released from clusters which enter and become trapped within endothelial cells. Clusters were therefore irradiated with visible laser light (λ=457.9 or 514.5 nm) either (a) in solution, while passing through a glass tube *en route* to the artery; or (b) when retained within the endothelium, by illuminating the artery directly during the plateau of an *S*-type response. Irradiation produced an additional vasodilator response, the magnitude of which depended upon wavelength and laser beam energy.

3 The nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (100 μM), had no effect on light-induced vasodilator responses. However, they were (a) blocked entirely by adding oxyhaemoglobin (5 μM) to the internal perfusate; and (b) greatly enhanced by the enzyme superoxide dismutase (150 u ml^{-1}).

4 Photolysis of cluster *B* was measured by absorption spectroscopy and by detecting NO released with an electrochemical sensor. The photochemical reaction was found to be oxygen-dependent. The half-time for inactivation of cluster-derived NO was measured by interposing different lengths of tubing (i.e time delays) between the photolysis tube and NO sensor. The steady-state probe current decayed exponentially with increasing delay time, with a $t_{1/2}$ of 21 s. The amplitudes of vasodilator responses of the tail artery also decreased exponentially by increasing the time delay ($t_{1/2}$=58 s). Superoxide dismutase (150 u ml^{-1}) prevented this from happening, showing that 'inactivation' of cluster-derived NO was caused by reaction with superoxide anions formed during photolysis.

5 We conclude that potentiation of vasodilator responses to iron-sulphur-nitrosyl clusters by visible light is due to an oxygen-dependent photochemical reaction which accelerates the release of ligated nitrosyl groups as free NO. Based on our measurements, we estimate that *ca* 100 pM NO is sufficient to produce a just-detectable additional vasodilatation and that the ED_{50} dose is *ca* 3.7 nM.

Keywords: Nitric oxide; vascular smooth muscle; photodecomposition; iron-sulphur-nitrosyl clusters; vasodilator

Introduction

Endothelium-dependent relaxation (EDR) of vascular smooth muscle (Furchgott & Zawadaski, 1980) is mediated by nitric oxide (NO), synthesized in endothelial cells from L-arginine and dioxygen (Palmer *et al.*, 1987; 1988a,b) by a Ca^{2+}-calmodulin- (Bredt & Snyder, 1990) and NADPH-dependent (Palmer & Moncada, 1989) constitutive enzyme, called endothelial nitric oxide synthase (ecNOS). Endothelium-derived NO (EDNO) stimulates smooth muscle soluble guanylate cyclase and elevates intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels, causing relaxation (Waldman & Murad, 1987).

The 'nitrovasodilators' also relax vascular smooth muscle via NO released *in vivo*, either spontaneously, by thermal decomposition, or after metabolic transformation (Feelisch & Noak, 1987), in effect by-passing the endogenous biosynthetic pathway. We earlier reported that two iron-sulphur-nitrosyl clusters (cluster *A*: $[Fe_4S_4(NO)_4]$, tetranitrosyl-tetra-μ_3-sulphido-tetrahedro-tetrairon; and *B*:$[Fe_4S_3(NO)_7]^{-1}$, heptanitrosyl-tri-μ_3-thioxotetraferrate(−1); Roussin, 1858) are potent nitrovasodilators with unusual pharmacological profiles (Flitney *et al.*, 1990; 1992a,b; 1993a,b,c). Bolus injections of cluster *A* or *B* into pre-contracted rat tail arteries generated two qualitatively distinct types of response. Doses below a critical threshold concentration (<D_T) evoked fully-reversible (*transient* or *T*-type) responses, as seen with conventional nitrovasodilators, for example nitroprusside or S-nitroso-*N*-acetylpenicillamine (SNAP; Askew *et al.*, 1995). However, doses >D_T produced extraordinarily long-lasting (*sustained* or *S*-type) responses, comprising an initial, rapid drop of pressure, which then either failed to recover or showed partial recovery only. *S*-type responses were characterized by a 'plateau' of profoundly reduced vessel tone which persisted for several hours. Cytochemical and X-ray electron microprobe analysis of snap-frozen arteries led us to conclude that the plateau phase was caused by the spontaneous release of NO from clusters trapped within the endothelium.

Here we show that exposure to light greatly enhances the vasodilator actions of iron-sulphur-nitrosyl clusters and conclude that this is due to an oxygen-dependent photochemical reaction which accelerates the release of ligated nitrosyl groups

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as free NO. The potential therapeutic and investigative applications of photodegradable, high-capacity NO generators is discussed briefly.

Preliminary accounts relating to the photosensitive nature of iron-sulphur-cluster nitrosyls were presented to the Physiological Society (June, 1992) and to a NATO-ASI Conference (June, 1992) on Vascular Endothelium (Flitney *et al.*, 1993b,c).

Methods

Preparation

Experiments were carried out on isolated segments of rat tail artery from male Wistar rats. Animals were killed by cervical dislocation. Arterial segments (7–12 mm long) were dissected free and perfused internally with Krebs solution (composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6; gassed with 95%O_2/5%CO_2 to maintain pH 7.4). Vessels were pre-contracted with phenylephrine (2–15 μM), as described previously (Flitney *et al.*, 1992b).

Apparatus

The apparatus is shown in Figure 1. The internal perfusate was pumped through the artery at a constant flow rate (2 ml min^{-1}) by means of a Gilson Minipuls peristaltic pump (P_1). It reached the vessel either directly (route A) or after first having passed through a curved glass tube (*E*; route B). Light from a tunable argon ion laser (*L*; Spectra Physics, type 160-8) could be directed into the tube through a quartz end window (*Q*) to irradiate solutions *en route* to the preparation. The tube was tapered and its outer surface coated with silver, to optimize the dispersion of light within its lumen. Six polypropylene outlet tubes (*O*; 1-6) of different lengths were available to vary the time delay (from 0.72–3.12 min) between formation of photolytic products in tube *E* and their arrival at the artery.

Laser light could also be made to illuminate the vessel directly, by reflecting the expanded beam off a front silvered mirror (*M*).

A transducer (*T* Sensym type SCX 150NC, Farnell Electronic Components, Leeds) located upstream from the artery (*V*) was used to monitor the perfusion pressure. Its output was displayed on a chart recorder. Temperature was maintained at 32°C (±2°C) by adjusting the flow rate (via pump P_2) through the external perfusion circuit. A Spectra Physics type 404 Power Meter was used to measure the laser beam output power.

Experimental protocols

All experiments were performed in a darkroom, with a red safelight (Kodak; 50W) as the sole means of illumination. Two protocols were used:

Potentiation of photorelaxation during the plateau phase of S-type responses Arteries were precontracted with Krebs solution containing phenylephrine (K+PE solution; via route A, Figure 1) and directly exposed to light by reflecting the laser beam off the front-silvered mirror (*M*, Figure 1; beam diameter at preparation: 2 cm). The illumination conditions (beam power = 0.2 or 2 mW; λ=514.5 nm; 1 min exposures) were selected to produce small-amplitude (<10% agonist-induced pressure) photorelaxant responses (Furchgott *et al.*, 1961). A single bolus injection (10 μl) of cluster *A/B* was then introduced into the internal perfusate, through a resealable rubber membrane (*R*, Figure 1). The concentrations used were sufficient to induce moderate *S*-type responses (Flitney *et al.*, 1992b). Arteries were again exposed to laser light (as above) at regular intervals (12 min), for periods up to 5 h. The photosensitivity of arteries was increased by this treatment, producing enhanced photorelaxant responses, or EPRs. The amplitude of EPRs is given throughout as the drop of pressure recorded at the end of the period of irradiation expressed as a percentage of the perfusion pressure existing immediately prior to switching on the laser.

Perturbation of steady-state vasodilator responses to A/B by laser irradiation of solutions en route to the artery Vessels were perfused with K+PE solution via route B (Figure 1). Pressure recordings were made during continuous infusions of cluster *A/B* (0.3 μM or 1.0 μM), initially in the dark, and then during intermittent laser irradiation of the incoming solution (in tube *E*, Figure 1). Exposure of solutions to light (λ=457.9 or 514.5 nm; 5 min) produced an additional vasodilatation during the period of illumination. This is termed the light-induced vasodilator response, or LIVR.

LIVRs were recorded under different illumination conditions (beam power, wavelength, delay time) and in the presence of either oxyhaemoglobin (Hb; 5 μM), superoxide dismutase (SOD; 150 units ml^{-1}) or the NOS inhibitor, N^G-monomethyl-L-arginine (L-NMMA). The decrease of pressure, measured at the end of the illumination period, is expressed throughout as a percentage of that recorded immediately prior to switching on the laser.

Photodecomposition of cluster B

Spectrophotometric measurements Photolysis of compound *B* was studied under conditions which simulated those used for

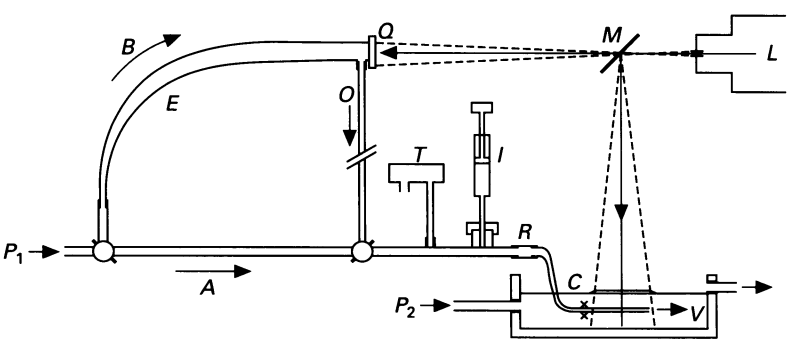

Figure 1 Apparatus for perfusing segments of rat tail artery. P_1 and P_2, peristaltic pumps; *V*, vessel; *C*, plastic cannula; *R*, resealable rubber diaphragm; *I*, microinjector; *T*, pressure transducer; *O*, outlet tube (6 available, of different lengths); *E*, exposure tube; *Q*, quartz end-window; *M*, front-silvered mirror; *L*, argon-ion laser. Vessels perfused directly (route A) or via exposure tube (route B).

recording LIVRs. Solutions (0.3 or 0.9 μM) were exposed to laser light in the photolysis tube (flow rate: 2 ml min^{-1}; $\lambda = 457.9$ nm and 514.5 nm) and collected in a spectrophotometer cuvette (10 cm path length). Absorption spectra were recorded (Cecil double-beam UV-VIS spectrophotometer; type CE 594) and the decrease in absorbance at 360 nm ($\varepsilon = 14{,}940$ M^{-1} cm^{-1}) was measured to determine the quantity of *B* photolysed.

Electrochemical measurements of NO released photochemically The concentration of NO in the tube effluent was measured with an electrochemical detector (Iso-NO, World Precision Instruments, Sarasota, FA., USA). The tip of the probe was inserted horizontally into the outlet tube (*O*), so that the polymer membrane covering the working electrode was flush with the inner wall. The probe was calibrated with known [NO]s generated by reduction of nitrite with excess ascorbic acid. The reaction produces molar equivalents of NO from NO_2^-. The electrode tip was immersed in ascorbic acid dissolved in oxygen-free, nitrogen-saturated water in a closed vessel (60 ml; 0.1 M). The solution was stirred vigorously and different volumes of oxygen-free sodium nitrite (2.5 mM) were injected into the vessel to generate known [NO]s. The sensitivity of the electrode was temperature-dependent and the calibration was carried out at 32°C. The current generated by oxidation of NO at the working electrode increased linearly with [NO_2^-] in the reaction mixture over the range 0–5 μM ($r^2 = 0.999$), with a slope (electrode sensitivity) of 1.49 pA nM^{-1} NO generated.

The probe was used to make measurements of [NO] produced photochemically by irradiating solutions of cluster *B* (1 μM) with different beam intensities under conditions which exactly simulated those used for recording LIVRs. NO reacts readily with oxygen-derived species and becomes physiologically inactive. The rate of inactivation was determined measuring the probe current as a function of delay time, by passing irradiated solutions through outlet tubes of different lengths.

Drugs and reagents used

Nitrosylated iron-sulphur clusters were synthesized in the laboratory, as described previously (Flitney *et al.*, 1992b). (−)-Phenylephrine hydrochloride, superoxide dismutase (lot number: 110H93211), haemoglobin and ascorbic acid were obtained from Sigma Ltd. L-NMMA was a gift from Dr Harold Hodson of the Wellcome Research Laboratories, Beckenham, Kent. All other reagents were 'Analar' grade obtained from BDH Ltd.

Results

Enhanced photorelaxation produced by irradiating arteries during S-type responses

Furchgott *et al.* (1961) showed that exposure to light causes pre-contracted vascular smooth muscle to relax. Here we show that vessels previously injected with solutions of cluster *A*/*B* exhibit greatly enhanced photorelaxant responses (EPRs) when irradiated during the plateau phase of an *S*-type response.

Typical recordings of EPRs are shown in Figure 2a-c. Control PRs were elicited first (see Methods). Arteries were then given a single bolus injection (10 μl) of either 250 μM *A* or 500 μM *B* and afterwards subjected to repeated periods of laser irradiation. This treatment produced a marked and long-lasting (up to 5 h) potentiation of the photorelaxant response (Figure 2a,b). The addition of SOD (150 units ml^{-1}) to the internal perfusate further enhanced the sensitivity of vessels to light (Figure 2c).

The characteristics of EPRs are compared to control photorelaxations in Figure 3. The results show, first, that the time interval between switching on the laser and the earliest detectable drop of pressure (*L*) decreased, while the maximum rate of drop of pressure (*dP*/*dt*) and amplitude of the photorelaxant response (*P*) both increased. There was no significant effect on the half-time for recovery ($t_{1/2}$) in the dark. Second, enhancement of the photorelaxant response was greater for cluster *B* than for *A* under comparable illumination conditions. And third, increasing the beam power (from 0.2 to 2.0 mW) increased the potentiating effect of both compounds.

Perturbation of steady-state vasodilator responses to A/B by laser irradiation of solutions en route to the artery

Characteristics of laser-induced vasodilator responses (LIVRs) The main features of the LIVR are illustrated in

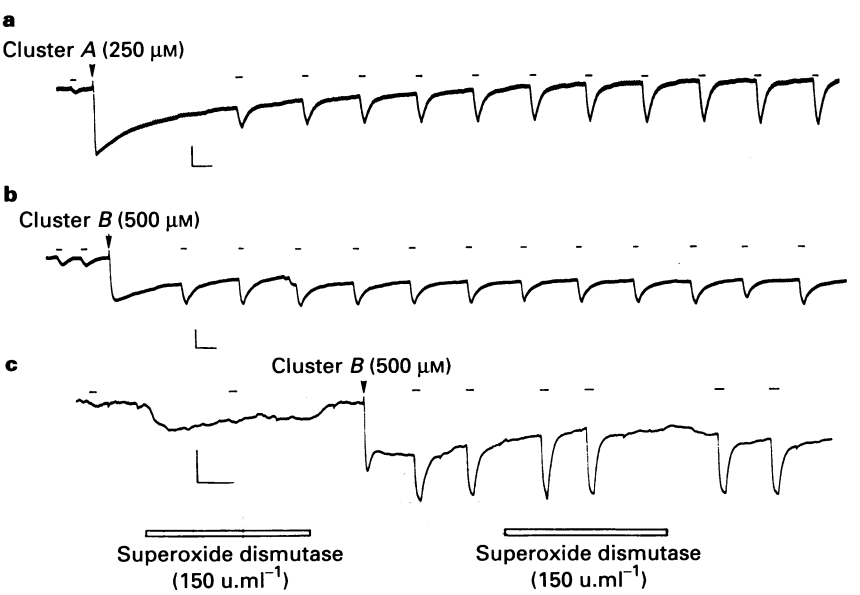

Figure 2 (a,b) Show enhanced photorelaxations (EPRs) recorded during the plateau phase of S-type responses produced by bolus injections of cluster *A* and *B* respectively. Control photorelaxations are shown to the left of the vertical arrows. Periods of irradiation (1 min duration; $\lambda = 514.5$ nm; beam power = 0.2 mW) are indicated by the horizontal lines. (c) Potentiating effect of SOD on EPRs. Amplitude of EPRs given as maximum drop in pressure expressed as a percentage of pressure immediately before irradiating the vessel. Scale bar (all traces) vertical (pressure) = 20%; horizontal = 5 min).

Figure 4. The horizontal lines associated with each pressure recording indicate the periods (5 min) of laser irradiation and the beam powers (mW) used. It is important to emphasise that arteries were carefully shielded from stray light throughout these experiments, to avoid evoking photorelaxant responses.

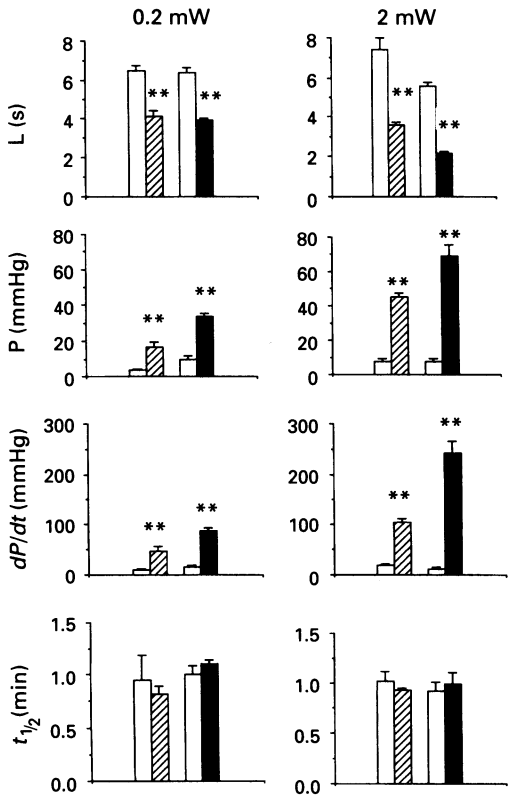

Figure 3 Effect of bolus injections of cluster *A* (250 μM; hatched columns) and *B* (500 μM; solid columns) on various parameters of photorelaxant responses: L = interval between switching on laser and first detectable drop of pressure; P = maximum drop in pressure; *dP/dT* = rate of pressure change; $t_{1/2}$ = half-time for pressure recovery after switching off laser. Open columns = parameters from control photorelaxations recorded prior to injection of clusters *A*/*B*. Beam powers (λ = 514.5 nm) indicated at top of figure. **Indicates statistically different from control photorelaxations at $P = 0.01$ level (Student's *t* test).

LIVRs produced by irradiating solutions of cluster *B* (0.3 mM) under different illumination conditions (beam power, wavelength) are shown in Figure 4a (λ = 457.9 nm) and b (λ = 514.5 nm). The LIVR typically comprised an initial, rapid drop of pressure which commenced after a short delay (here ~45 s; using outlet tube No.1) and was complete within ca. 1 min. Thereafter, pressure either stayed constant throughout the remainder of the illumination period, or it continued to fall but at a reduced rate. After illumination ceased, the vessel reconstricted: pressure increased rapidly at first, and then more slowly, terminating at (or near) the pre-illumination (control) level.

The results from experiments of this type for both clusters are summarised in Figure 5a (cluster *A*) and 5b (cluster *B*). Here the amplitude of the LIVR is plotted as a function of $\log_{10}$ beam power. The resulting curves show (a) the LIVR increased in size with increasing beam power; (b) irradiating solutions with light at 457.9 nm was more effective at producing LIVRs than at 514.5 nm; (c) cluster *B* was more light-sensitive than cluster *A*; and (d) increasing the concentration (of cluster *B*) increased the amplitude of LIVRs produced under standard illumination conditions.

LIVRs are mediated by NO released photochemically Figure 6a-d shows that LIVRs are mediated by *exogenous* (cluster-derived) NO. First, LIVRs were suppressed by adding oxyhaemoglobin (Hb; 5 μM) to the internal perfusate; the effect was fully reversible (Figure 6a). In contrast, the addition of 100 μM L-NMMA, sufficient to abolish endothelium-dependent relaxations to carbachol, failed to inhibit the LIVR (Figure 6b). In some cases a small reduction was observed, but more often the responses were slightly potentiated by L-NMMA.

Second, LIVRs were progressively reduced in amplitude when the interval between solution leaving the photolysis tube and entering the artery (= delay time) was extended by increasing the length of the outlet tube (Figure 6c). Figure 7 summarises results from a series of experiments in which different tube lengths (Nos 1–6) were selected and LIVRs were recorded in the absence and presence of SOD. In the absence of SOD, the responses decayed exponentially with increased delay times, falling to 50% of their maximum value after 58.9 s (λ = 514.5 nm; open circles) and 57.9 s (λ-457.9 nm; filled circles). Addition of 150 u ml^{-1} SOD to the internal perfusate prevented attenuation of the LIVRs (Figure 6d and Figure 7; filled triangles). SOD was also found to potentiate sub-maximal LIVRs (not shown).

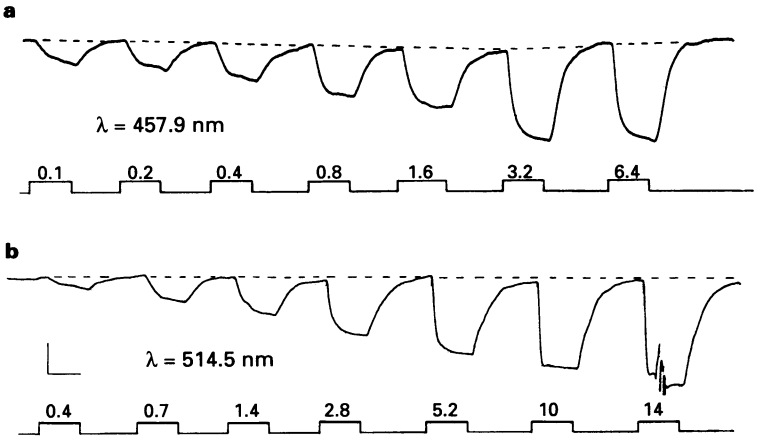

Figure 4 Light-induced vasodilator responses (LIVRs) produced by irradiating solutions of cluster *B* (0.3 μM) *en route* to the preparation. See Figure 1. Lower line in each trace shows periods of irradiation (5 min) and laser beam powers used. Wavelengths indicated near each recording. Qualitatively similar responses were obtained with cluster *A*. Note greater sensitivity to shorter wavelength light. Scale bars: vertical = 20% drop in perfusion pressure; horizontal = 4 min.

Electrochemical measurements of NO released during photolysis of cluster B

Photolysis of cluster B *is oxygen dependent* Preliminary experiments showed that the photodecomposition of cluster *B* displays a requirement for dissolved molecular oxygen. Solutions (100 μM) were made up in either oxygen-saturated water or in water previously bubbled with oxygen-free nitrogen. Scintillation vials were completely filled with solution (*ca.* 10 ml), tightly stoppered with screw-cap lids and sealed with parafilm to prevent entry of air. Half of the vials were then placed on a viewing box and exposed to light (up to 5 h) and the other half remained in the dark.

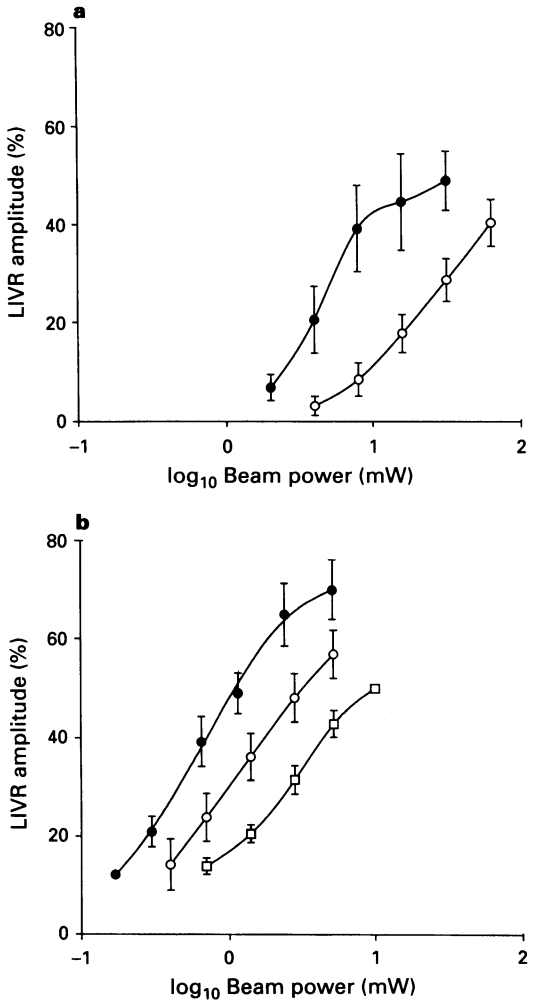

Figure 5 LIVR amplitudes plotted as a function of $\log_{10}$ beam power (mW). (a) Experiments using cluster *A* (1.6 μM); (●), λ=457.9 nm; (○), λ=514.5 nm. (b) Experiments using cluster *B*; (●), λ=457.9 nm; ○, □, λ=514.5 nm. (○,●), 1 μM; (□), 0.3 μM. Sensitivity of both clusters to light is greater at the shorter wavelength. All data obtained with the shortest outlet tube (No.1; delay time = 0.73 min).

Table 1 shows that the absorbance (measured at 360 nm; see below) of oxygen-containing solutions decreased to 26.3±1.9% of the initial value after 5 h irradiation. For oxygen or nitrogen saturated solutions kept in the dark the absorbance decreased to 93.5±0.4% and 99.2±0.01%, respectively. When *B* was dissolved in water saturated with oxygen-free nitrogen and exposed to light, the absorbance decreased to only 87.7±0.4%, which is similar to the spontaneous decomposition rate for solutions which were kept in the dark.

This result was confirmed subsequently in experiments with the NO-sensitive probe, which consistently failed to detect any increase of NO when *B* was irradiated in solutions saturated with oxygen-free nitrogen. For this reason, electrochemical measurements of [NO] were made in Krebs solution bubbled with 95%O_2/5%CO_2 maintained at a temperature of 32°C, to simulate as closely as possible the conditions of our experiments with isolated arteries.

Spectrophotometric measurements of the tube effluent under different illumination conditions revealed a proportional decrease in absorbance ('bleaching') across the range of wavelengths extending from 250–600 nm. A plot of the change in absorbance ($\Delta A/A$, measured at λ=360 nm) versus beam power showed that the photodecomposition of *B* (1 μM solution) increased linearly with increasing powers, up to *ca* 80 mW at 514.5 nm and *ca* 30 mW at 457.9 nm, ranges which far exceeded those used when recording LIVRs (see Figures 4, 5 and 6). The fractional decomposition of *B* per mW of light ($\times 10^3$) was found to be 14.06±0.43 at 457.9 nm and 3.64±0.23 at 514.5 nm (mean±s.e.mean; n=9). These values were independent of concentration over the range 0.3–10 μM.

In principle, these measurements allow the overall efficiency (E_λ) of the photolytic process, expressed in terms of the steady-state [NO] formed (nM mW^{-1}), to be estimated from $E_\lambda = fcn$, where f = the fractional breakdown of *B* (mW^{-1}) at wavelength λ, c = the concentration of *B* (nM) used, and n = the number of mol of ligated nitrosyl groups released as free NO mol^{-1} cluster photolysed. However, the value of n (=1–7) has not so far been determined (but see appendix) and this expression gives the [NO] formed at *source*; it does not allow for losses incurred (inactivation) *en route* to the artery.

The rate of inactivation was therefore estimated by irradiating solutions of *B* (10 μM) under standard illumination conditions (10 mW at 457.9 nm; 40 mW at 514.5 nm) and recording the decrease in probe current for increasing lengths of outlet tube. The current declined exponentially with delay time, decreasing by 50% ($t_{1/2}$) in *ca* 21 s. Linear regression

Table 1 Change in absorbance (fraction of starting value) measured at 360 nm with time for solutions of cluster *B* (100 μM) kept in the dark (columns 1 and 2) or exposed to light (columns 3 and 4)

	Dark		*Light*	
Time (h)	O_2	N_2	O_2	N_2
0	1.00	1.00	1.00	1.00
1	0.987±0.005	0.998±0.001	0.511±0.021	1.00±0.007
2	0.973±0.008	0.995±0.004	0.378±0.012	0.970±0.002
3	0.963±0.012	0.993±0.002	0.298±0.013	0.945±0.009
4	0.950±0.004	0.991±0.001	0.255±0.015	0.900±0.011
5	0.935±0.004	0.992±0.001	0.263±0.019	0.877±0.004

Values are mean±s.e.mean; n=6.
Decrease in absorbance for solutions which were saturated with oxygen-free nitrogen and exposed to light (column 4) was approximately the same as for solutions kept in the dark. Solutions containing oxygen and exposed to light (column 3) showed a large decrease in absorbance (ca. 75%) over a 5 h period.

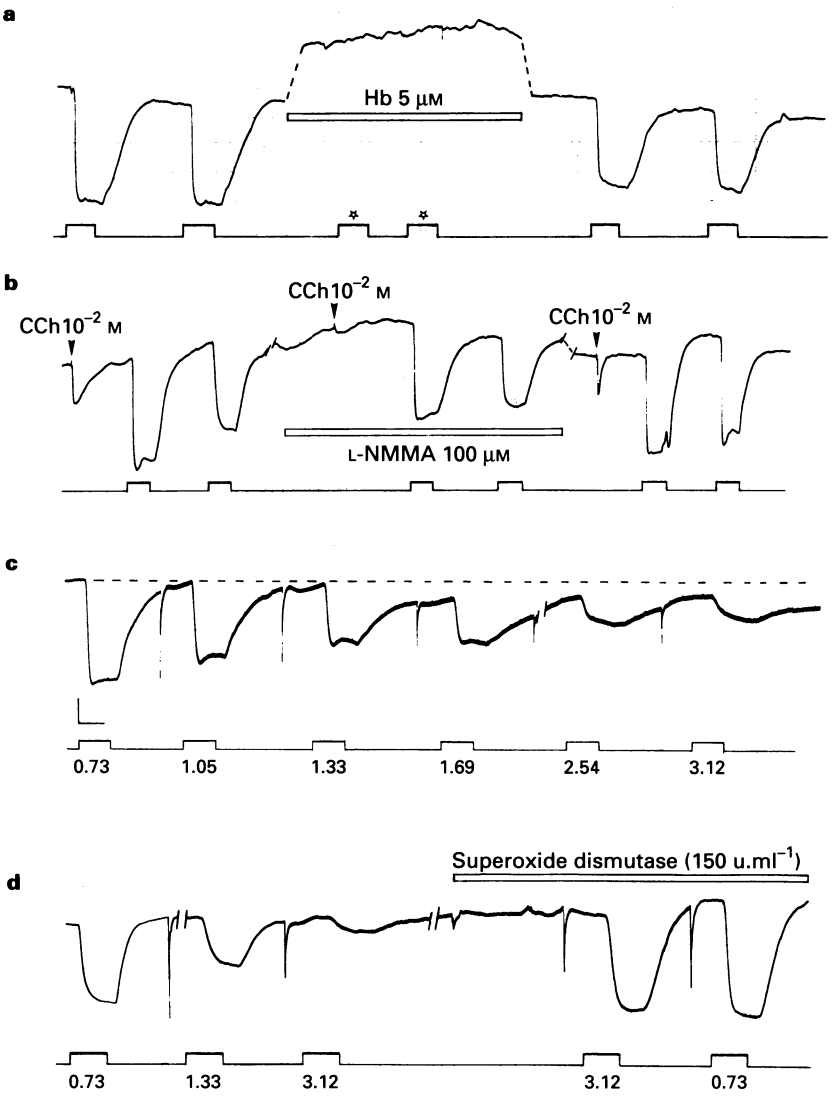

Figure 6 Experiments designed to show that LIVRs are due to NO released photochemically. (**a**) Blockade of paired LIVRs ($\lambda = 514.5$ nm; 10 mW and 457.9 nm; 3.3 mW for first and second responses, respectively) by addition of 5 μM oxyhaemoglobin (Hb) to the internal perfusate during interval shown by horizontal box. Hb increased perfusion pressure by around 50% (dashed lines indicate broken trace) and entirely eliminated LIVRs (asterisks). Note that suppression of LIVRs by Hb is fully reversible on wash-out (**b**). LIVRs were not affected by the NOS inhibitor L-NMMA when used at a concentration which blocked endothelium-dependent responses to carbachol (CCh; arrows). Paired responses produced by light at 514.5 nm and 457.9 nm as for Figure 6a. (**c**) Increasing the length of the outlet tubing (= time delay (min), indicated on lower trace) caused a progressive reduction in size of LIVRs produced under standardized illumination conditions (here, 10 mW at 514.5 nm; cluster *B* 0.3 μM). (**d**) Addition of SOD to the internal perfusate prevented attenuation of the LIVR with increasing delay times. Responses recorded at delay times indicated on lower trace. Note that LIVR at 3.12 min is fully restored by SOD. Rapid deflections of pressure trace in (c) and (d) indicate switching of 6 way tap to select different outlet tubes. Scale bars for all traces (shown in c): vertical = 20% drop in perfusion pressure; horizontal = 4 min.

analyses of semi-logarithmic plots of [NO] against delay time (min) gave the following equations: for 514.5 nm, $y = -0.946x + 2.598$ ($r^2 = 0.989$) and for 457.9, $y = -0.810x + 2.678$ ($r^2 = 0.993$).

Log dose-response curves relating LIVR amplitudes to [NO] generated photochemically The [NO] *reaching the preparation* (delay time = 45 s, for tube No.1) produced by irradiating a 1 μM solution of *B* at each wavelength is plotted as a function of beam power in Figure 8a. The [NO] increased asymptotically towards a maximum value. These measurements were used to construct a log dose-response curve (Figure 8b) of LIVR amplitudes as a function of [NO], using (a) the data presented in Figure 5, all from experiments with outlet tube No.1; and (b) the results presented in Figure 7, where LIVRs were recorded using different lengths of outlet tubing (tubes No.1–6; square symbols), after making the appropriate correction for each delay time. The data lie on a curve extending over three decades, with a threshold [NO] of <0.1 nM and an estimated ED_{50} value of 3.73 nM.

Discussion

Light potentiation of vasodilator responses to iron-sulphur clusters is mediated by photochemical release of NO

Vasodilator responses produced by nitrosylated iron-sulphur clusters are enhanced by light: this applies to 'steady state' responses, generated by continuous infusions of *A*/*B* (Figure 4), and to *S*-type responses, resulting from single bolus injections (Figure 2). The potentiating effects of light are greater at the shorter wavelength and at higher beam powers (Figures 3,

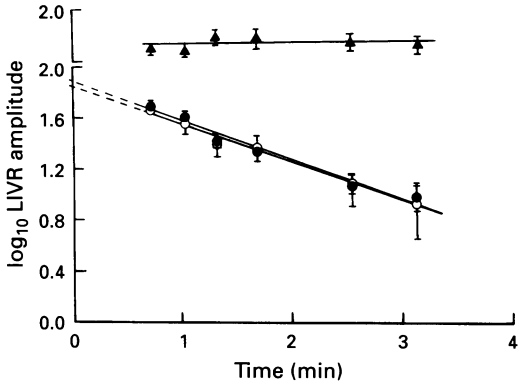

Figure 7 Semi-logarithmic plot of the decrease in LIVR amplitude (mean ± s.e.mean; $n = 5$) with increasing delay times. LIVRs decayed exponentially with $t_{1/2}$ values of 58.9 s (514.5 nm; 10 mW; ○) and 57.9 s (457.9 nm; 3.3 mW; ●). LIVRs produced by irradiating cluster *B* (0.3 μM). Addition of SOD to the internal perfusate prevented loss of the LIVR (▲; mean ± s.e.mean; $n = 3$). Note break in ordinate axis for clarity.

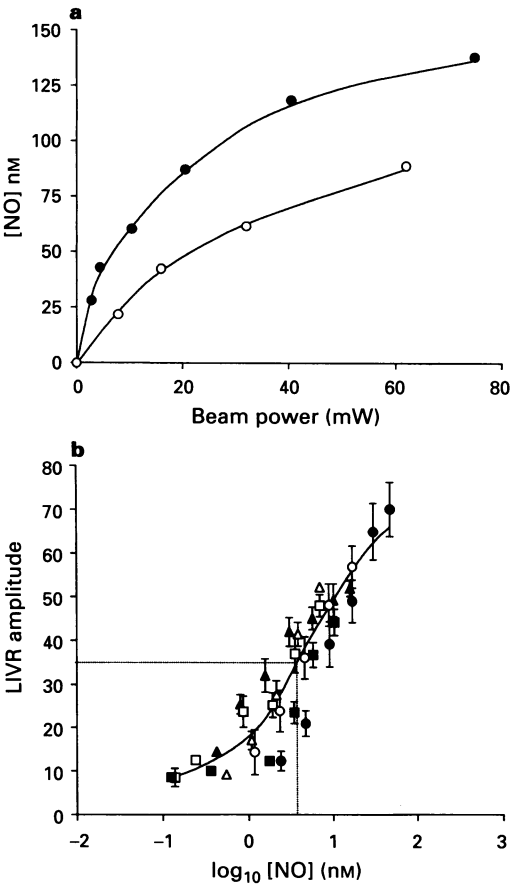

Figure 8 (a) Concentration of NO generated photochemically and recorded with the NO sensor (delay time = 0.73 min; tube No.1 as used for experimental results presented in Figures 4 and 5). Cluster *B* (10 μM) irradiated with light of wavelength 457.9 nm (●) and 514.5 nm (○) at beam energies (mW) indicated on abscissa scale. (**b**) LIVR amplitudes plotted as a function of $\log_{10}$ [NO]. Note that data for experiments in which different delay times were used (from Figure 7) is also included after making a correction to allow for the rate of inactivation of NO in our apparatus ($t_{1/2} = 21$ s). See text for details. The threshold [NO] required to elicit LIVRs is < 0.1 nM and the ED_{50} is estimated (vertical dashed line) to be around 3.7 nM.

4, 5). They are facilitated by exogenous SOD (Figure 2c and 6d), the enzyme which dismutes O_2^- (McCord & Fridovich, 1969), a known inactivator of NO (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986), and they are blocked by Hb (Figure 6a) a scavenger for NO (Antonini & Brunori, 1971; Martin *et al.*, 1985). The failure of L-NMMA, a stereospecific inhibitor of endothelial NOS (Rees *et al.*, 1990), to suppress LIVRs (Figure 6b) shows that this type of potentiation cannot be caused by stimulation of EDNO synthesis. Instead, the results demonstrate that both compounds are photodegradable and that the enhanced vasodilator effects caused by light are due to accelerated release of ligated nitrosyl groups as free NO. This conclusion was confirmed (in the case of cluster *B*) by electrochemical measurements using the NO sensor, which showed that NO release was greater at the shorter wavelength and that it increased with increasing beam powers (Figure 8a).

Similar results to ours were reported by Matthews *et al.* (1994), working with guinea-pig taenia caeci pre-loaded by exposure to 50 μM cluster *B* for 30 min. Exposure to visible light (400–800 nm) caused relaxation of carbachol or KCl pre-contracted strips which could be blocked (*inter alia*) by oxyhaemoglobin, but not by N^G-nitro-L-arginine, a NOS inhibitor similar to L-NMMA. The magnitude of the light-induced relaxation was related to wavelength, intensity and duration of illumination. They attributed the relaxant effect of light to the photochemical release of NO and consequent activation of the guanylate cyclase-cyclic GMP pathway.

The role of oxygen and superoxide in the photodecomposition of B *and in the 'inactivation' of NO released photochemically*

The results show that oxygen and/or oxygen-derived species are involved both in the photolysis of *B* and in the subsequent fate of the NO produced.

Table 1 shows that the decomposition of *B* is greatly reduced (*ca* 6.4×) in oxygen-free, nitrogen-saturated solutions exposed to light (< 11% over 5 h) as compared to oxygen saturated solutions (> 70% over 5 h). The chemical basis for this observation has not been investigated, but clearly it could have important implications for the generation of NO from *B in vivo*, where the oxygen tension can vary between different tissues, or even between different regions within the same tissue, for example in solid tumours which invariably contain both normoxic and hypoxic regions.

NO combines with O_2^- to form peroxynitrite in a rapid, near-diffusion-limited reaction (Saran *et al.*, 1990). SOD prevents this reaction sequence by catalysing the dismutation of O_2^- to hydrogen peroxide (McCord & Fridovich, 1969). The ability of SOD to prevent loss of the LIVR with increasing delay times (Figures 6d and 7) shows that inactivation of cluster-derived NO is due to it reacting with O_2^- anions generated during photolysis. McCord & Fridovich (1973) showed that irradiation of oxygenated water with u.v. light produces O_2^- and hydroxyl (OH) radicals in equimolar amounts. The less energetic wavelengths used here are unlikely to generate O_2^- by this means, though it is worth noting that only very small amounts (nM quantities) would be required to react with the NO produced. An alternative explanation, and one we favour, is that O_2^- is formed as a consequence of reactions between dissolved molecular oxygen and one or more of the photodecomposition products of *B*. Ferrous iron complexes, produced in the photolysis tube by fragmentation of the FeS framework, are possible candidates, since they are known to auto-oxidize and form O_2^- from oxygen.

Relative photosensitivities of A *and* B

Cluster *B* invariably produces larger LIVRs and EPRs than *A* under comparable illumination conditions (Figures 3 and 5). This implies that *B* is more photosensitive than *A*. Unfortunately, we were unable to study the photodecomposition of *A* for comparison with *B* because its absorption

spectrum showed marked, time-dependent changes, even when solutions were kept in the dark. Unlike cluster *B*, *A* is insoluble in water and so it was first dissolved in dimethyl sulphoxide (DMSO) prior to use. DMSO is a co-ordinating solvent which can cause the iron-sulphur framework to fragment and then spontaneously re-assemble as cluster *B*, the more stable of the two configurations. The reaction proceeds through the formation of mononuclear (dinitrosyl) solvo-complexes. The conversion of *A* to *B* by DMSO has been detected by infra-red spectroscopy (Glidewell; personal communication) under conditions which are comparable to those used here.

Sensitivity of arteries to NO released photochemically

The log dose-response curve of Figure 8b shows that the [NO] required to produce a detectable (threshold) LIVR is probably below 0.1 nM. The ED_{50} value of 3.73 nM is considerably less than that for endothelium-dependent relaxations reported by Jacobs *et al.* (1990; rabbit aorta; 63 nM) and by Sakuma *et al.* (1990; 20–250 nM, for a variety of arteries), but rather close to the value cited by Kelm & Schrader (1990; using rat aorta and guinea-pig coronary resistance vessels: 5–7 nM). The ED_{50} obtained here differs from that reported previously (*ca.* 16 nM; Flitney *et al.*, 1993b,c) because (a) we had earlier assumed that all 7 ligated nitrosyl groups would be released during photolysis, whereas our recent experiments, using the electrochemical probe, suggest that this is probably not so and the figure may be as few as 3 (see Appendix); and (b) we used the decline in amplitude of the LIVR with delay time to estimate the $t_{1/2}$ value for inactivation of NO which is actually greater (*ca* 2.8 ×) than the value obtained directly using the NO probe. The reason for the difference is because the relation between LIVR amplitude and [NO] is non-linear. Finally, it is worth emphasizing here that LIVRs are necessarily superimposed on a 'background' vasodilator response, due to the spontaneous release of NO in the dark (Flitney *et al.*, 1992b) and this may have affected the sensitivity of arteries to 'extra' NO produced during photolysis.

Therapeutic and investigative potential of iron-sulphur nitrosyl clusters

EDNO is a major determinant of peripheral resistance *in vivo* (Vallance *et al.*, 1989; Aisaka *et al.*, 1989; Rees *et al.*, 1989; Gardiner *et al.*, 1990; Chu *et al.*, 1991). Significantly, EDRs are attenuated in hypertension (Winquist *et al.*, 1984; Luscher & Vanhoutte, 1986; Otsuka *et al.*, 1988; Tesmafariam & Halpern, 1988; Sunano *et al.*, 1989) and in atherosclerosis (Harrison *et al.*, 1987; Chappell *et al.*, 1987; Henry *et al.*, 1987; Fostermann *et al.*, 1988; Guerra *et al.*, 1989). The hypotensive (or anti-hypertensive) actions of clinical 'nitrovasodilators' are generally short-lived and so continuous drug infusions may be required to produce sustained effects (Kreye, 1980). We showed earlier (Flitney *et al.*, 1992b; 1983a,c) that iron sulphur cluster nitrosyls enter endothelial cells remarkably quickly and accumulate therein, releasing NO slowly. The photosensitive nature of these compounds clearly adds an important new dimension to their therapeutic and investigative potential. Their capacity to accumulate within endothelial cells and then later release NO rapidly on exposure to light opens up the possibility of improving the perfusion of selected vascular beds and perhaps also of delivering potentially cytotoxic quantities of NO to specific target sites, for example to solid tumours. Likewise, their photosensitivity offers a simple and effective means for achieving controlled 'step' increments of NO in pre-loaded tissues (Matthews *et al.*, 1994; Boulton *et al.*, 1994), or even in liquid samples, which could be exploited for the purpose of investigating proposed biochemical or pharmacological roles for NO.

This work was supported by grants to F.W.F. from the British Heart Foundation and Cancer Research Campaign. We are indebted to Dr Harold Hodson of the Wellcome Research Laboratories for a generous gift of LNMMA. Our grateful thanks go to Mr Haitham Al-Sa'doni, who assisted with the electrochemical measurements, and to David Roche, Sean Earnshaw and Karen Johnston for their expert photographic assistance.

Appendix

The value of *n*, the number of ligated nitrosyl groups released as NO during the photochemical reaction, is obtained from electrochemical measurements of NO and spectroscopic measurements of the quantity of *B* photolysed under defined conditions of illumination. It will be recalled that [NO] fell exponentially with delay time and that linear regression analyses of semi-logarithmic plots of the data returned the following equations:

514.5 nm (40 mW) $y = -0.946x + 2.598$

457.9 nm (10 mW) $y = -0.810x + 2.678$

(where [NO] is in nM and time is in min).

The flow rate of solution through the system was held constant (2 ml min^{-1}) and the volume of the photolysis tube was 4.5 ml. Thus, on average, each molecule of NO generated photochemically spent 1.125 min *before* reaching the entrance to the outlet tube. Placing $x = -1.125$ min into the above equations returns the following values for the [NO] generated in the photolysis tube:

514.5 nm (40 mW) = 4594.6 nM or 114.9 nM mW^{-1}

457.9 nm (10 mW) = 3883.7 nM or 388.4 nM mW^{-1}

The fractional decomposition of *B* at each wavelength (mW^{-1}), measured spectroscopically, was found to be 0.0036 at 514.5 nm and 0.014 at 457.9 nm. The concentration of *B* was 10 μM. Thus, if all 7 ligated nitrosyls are released we should expect the [NO] formed in the photolysis tube to be $0.0036 \times 10 \times 7 = 0.252$ μM (514.5 nm) and $0.014 \times 10 \times 7 = 0.98$ μM (457.9 nm). The actual amounts were significantly less, by factors of $114.9/252 = 0.46$ (514.5 nm) and $388.4/980 = 0.40$ (457.9 nm). This means that between $0.4 \times 7 = 2.8$ and $0.46 \times 7 = 3.2$ NOs are released per molecule of *B* photolysed. The mean value for these experiments is 3.

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(Received August 4, 1995
Revised January 16, 1996
Accepted January 22, 1996)