British Journal of Pharmacology (2001) 133, 615–624 © 2001 Nature Publishing Group All rights reserved 0007–1188/01 $15.00
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Nitric oxide- and nitric oxide donors-induced relaxation and its modulation by oxidative stress in piglet pulmonary arteries

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1 Inhaled nitric oxide (iNO) is widely used in the treatment of pulmonary hypertension while inhaled NO donors have been suggested as an alternative therapy. The differential susceptibility to inactivation by oxidative stress and oxyhaemoglobin of NO and two NO donors, sodium nitroprusside (SNP) and S-nitroso-N-acetyl-penicillamine (SNAP) were analysed in isolated endothelium-denuded pulmonary arteries from 2-week-old piglets stimulated with U46619.

2 NO, SNAP and SNP relaxed the arteries ($pIC_{30} = 7.73 \pm 0.12$, 7.26 ± 0.17 and 6.43 ± 0.13, respectively) but NO was not detected electrochemically in the bath after the addition of SNP and only at concentrations at which SNAP produced more than 50% relaxation.

3 The sGC inhibitor ODQ (10^{-6} M) or the sarcoplasmic Ca^{2+}-ATPase thapsigargin (2×10^{-6} M) markedly inhibited the relaxation induced by NO, SNAP and SNP.

4 Addition of oxyhaemoglobin (3×10^{-7} M) or diethyldithiocarbamate (1 mM) markedly inhibited NO- ($pIC_{30} = 6.88 \pm 0.07$ and 6.92 ± 0.18, respectively), weakly inhibited SNAP- and had no effect on SNP-induced relaxation. Xanthine oxidase (5 mu ml^{-1}) plus hypoxanthine (10^{-4} M) markedly inhibited NO- ($pIC_{30} = 6.96 \pm 0.12$) but not SNAP- or SNP-induced relaxation.

5 Superoxide dismutase (SOD), $MnCl_2$, diphenileneiodonium and exposing the luminal surface of the rings outwards (inversion) potentiated the relaxant responses of NO ($pIC_{30} = 8.52 \pm 0.16$, 8.23 ± 0.11, 8.01 ± 0.11 and 8.20 ± 0.10, respectively). However, SOD did not modify the NO detected by the electrode and had no effect on SNAP- or SNP-induced relaxation.

6 Therefore, the kinetics and local distribution of NO release of NO donors influence the susceptibility to the scavenging effects of oxyhaemoglobin and superoxide.

British Journal of Pharmacology (2001) **133,** 615–624

Keywords: Sodium nitroprusside; NO; SNAP; superoxide; oxidative stress; pulmonary artery; piglet

Abbreviations: DETCA, diethyldithiocarbamate; DPI, diphenileneiodonium; HX, hipoxanthine; iNO, inhaled nitric oxide; L-NAME, N^{ω}-nitro-L-arginine methylester, NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PPHN, persistent pulmonary hypertension of the newborn; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; sGC, soluble guanylate cyclase; SOD, superoxide dismutase; SNAP, S-nitroso-N-acetyl-penicillamine; SNP, sodium nitroprusside; U46619, 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; XO, xanthine oxidase

Introduction

Nitric oxide (NO) is a key mediator in the regulation of pulmonary vascular tone in the adult life and during the transition from the foetal to the postnatal pulmonary circulation (Abman *et al*., 1990; Barnes & Liu, 1995; Kinsella & Abman, 1995). Failure of the pulmonary circulation to undergo a normal transition results in persistent pulmonary hypertension of the newborn (PPHN), characterized by sustained elevation of pulmonary vascular resistance after birth, leading to right-to-left shunting of blood across the ductus arteriosus or foramen ovale and severe hypoxaemia (Levin *et al*., 1976; Kinsella & Abman, 1995; Hampl & Herget, 2000). An effective strategy for the treatment of PPHN must include vasodilators that decrease pulmonary vascular resistance without decreasing the systemic arterial pressure. Inhaled NO (iNO) is currently employed in the treatment of PPHN (Kinsella & Abman, 1995; Neonatal Inhaled Nitric Oxide Study Group, 1997; Roberts *et al*., 1997). iNO is advantageous over intravenous vasodilators because it causes selective pulmonary vasodilation due to its rapid inactivation by oxyhaemoglobin in the circulation. In addition, iNO produces greater arterial relaxation in the best ventilated lung areas, thus matching ventilation with perfusion. However, NO reacts with superoxide to form peroxynitrite at nearly diffusion-limited rate decreasing its biological activity (Beckman & Koppenol, 1996). Under physiologic conditions, this reaction is limited by the intracellular superoxide dismutase (SOD) that scavenges endogenous superoxide. However, when the concentration of NO is increased (e.g. by the use of iNO), or in the presence of increased superoxide concentrations significant concentrations of peroxynitrite may be produced (Beckman & Koppenol, 1996). Peroxynitrite directly causes oxidation, peroxidation, and nitration of biologically important molecules such as lipids, proteins, or DNA (Szabo, 1996a, b). Therefore, alterations of the interaction of NO with super-

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oxide are now being considered to play a major role in endothelial dysfunction associated to the pathogenesis of many vascular diseases including pulmonary hypertension (Cai & Harrison, 2000; Hampl & Herget, 2000).

Intravenous administration of NO donors produces nonselective pulmonary vasodilation and systemic hypotension (Kieler-Jensen *et al*., 1995). However, recent evidence suggests that inhalation of NO donors such as sodium nitroprusside (SNP) increases arterial oxygenation without altering systemic haemodynamics in infants with PPHN (Palhares *et al*., 1998). In addition, inhaled SNP produced sustained vasodilation and improved gas exchange in several models of experimental pulmonary hypertension (Yu *et al*., 1999; Yu & Saugstad, 1999; Adrie *et al*., 1998; Schutte *et al*., 1997). Therefore, aerosolization of NO donors may offer an alternative therapeutic approach to iNO in the treatment of PPHN. However, it is unclear how oxidative stress can modulate the vasodilator response to NO donors in pulmonary arteries.

Therefore, in the present study we have compared the mechanism of action, the susceptibility to oxidative stress and the inactivation by oxyhaemoglobin of NO and two NO donors, SNP and S-nitroso-N-acetyl-penicillamine (SNAP), in isolated pulmonary arteries from 2-week-old piglets.

Methods

Tissue preparation

Two-week-old male piglets (3–4 kg) were used in this study. Piglets were killed in the local abattoir and the lungs were rapidly immersed in cold (4°C) Krebs solution (composition in mM: NaCl 118, KCl 4.75, $NaHCO_3$ 25, $MgSO_4$ 1.2, $CaCl_2$ 2.0, KH_2PO_4 1.2 and glucose 11) and transported to the laboratory. The pulmonary arteries (third branch with an internal diameter of about 0.5–1.5 mm) were carefully dissected free of surrounding tissue and cut into rings of 2–3 mm length (Pérez-Vizcaíno *et al*., 1997; 1998). To avoid interferences with endogenous NO, the endothelium was removed in all preparations by gently rubbing the intimal surface of the rings with a metal rod. The endothelium removal procedure was verified by the inability of acetylcholine (10^{-6} M) to relax arteries precontracted with noradrenaline (10^{-6} M). In order to determine the location of the superoxide source, in some experiments the pulmonary artery rings were inverted so that the adventitia was facing inwards and the intima outwards. A set of rings inverted twice (so that they showed the original inward/outward orientation) were used as controls for the inversion procedure.

Endothelium-denuded pulmonary rings were mounted between two hooks under 0.5 g of tension in a 5 ml organ bath filled with Krebs solution at 37°C gassed with a 95% O_2-5% CO_2 gas mixture as previously described (Pérez-Vizcaíno *et al*., 1997; 1998). The contraction was measured by an isometric force transducer (model PRE 206-4, Cibertec, Madrid, Spain). Contractile tension was recorded by a REGXPC computer program (Cibertec, Madrid, Spain). NO concentrations were measured using an amperometric electrode (ISO-NO, WPI Inc., FL, U.S.A.) and recorded in a computer together with the force data by using data acquisition hardware and data recording software (Powerlab and Chart v3.4, respectively, AD Instruments Pty Ltd., Castle Hill, Australia). The electrode is selective for NO because it is covered by a membrane which only allows gases to permeate through. The electrode was calibrated according to the manufacturer by the conversion of known concentrations of NO_2Na into NO in the presence of H_2SO_4 and KI. This reaction also allowed us to measure nitrites contaminating the NO saturated solution. The electrode was placed in the organ bath, external to the vessel, at a distance of 5–7 mm from the tissue and drugs were applied equidistant (5–10 mm) from both.

After equilibration the rings were pre-contracted with the thromboxane A_2 mimetic 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619, 10^{-7} M). In previous experiments, we demonstrated that this concentration produces approximately 70–80% of the maximal U46619-induced contraction in piglet pulmonary vessels (Pérez-Vizcaíno *et al*., 1996). Thereafter, rings were treated with vehicle (Krebs solution or dimethylsulphoxide) or one of the following drugs: the soluble guanylate cyclase (sGC) inhibitor ODQ (10^{-6} M), the inhibitor of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) thapsigargin (2×10^{-6} M), oxyhaemoglobin (3×10^{-7} M), superoxide dismutase (SOD, 100 u ml^{-1}), the SOD mimetic $MnCl_2$ (10^{-4} M), xanthine oxidase (XO, 5 mu ml^{-1}) plus hypoxanthine (HX, 10^{-4} M), the SOD inhibitor diethyldithiocarbamate (DETCA, 10^{-3} M), the inhibitor of the mitochondrial electron transport chain rotenone (5×10^{-5} M), the NADPH oxidase inhibitor diphenileneiodonium (DPI, 10^{-5} M), the NO synthase inhibitor L-NAME (10^{-4} M), the cyclo-oxygenase inhibitor indomethacin (10^{-5} M), the cytochrome P450 inhibitor SKF 525A (10^{-5} M), the lipo-oxygenase inhibitor AA861 (10^{-5} M) or the xanthine oxidase inhibitor oxypurinol (10^{-4} M). Dimethylsulphoxide had no effect on U46619-induced tone or NO-induced vasodilation and therefore, the control results shown indicate experiments in which Krebs solution was used as vehicle. Rings were exposed to these drugs for 15 min except to hypoxanthine which was added just before initiating the concentration-response curves to NO, SNAP or SNP. These treatments produced no changes (1 ± 0.6% for XO plus HX, 4 ± 2% for indomethacin, -2 ± 1% for oxypurinol, 6 ± 4% for rotenone, -0.5 ± 2% for DPI, -5 ± 5% for SKF525A and -10 ± 5% for AA861), weak significant increases (8 ± 4% for ODQ, 12 ± 1% for oxyhaemoglobin, 13 ± 6% for L-NAME) or weak significant decreases (-16 ± 4% for thapsigargin, -18 ± 4% for DETCA) in the tone induced by U46619. Since these changes were always less than 20%, the concentration of U46619 was kept constant at 10^{-7} M. The curves to NO were performed by non cumulative addition of increasing volumes of Krebs solution saturated with NO (Villamor *et al*., 2000). Each concentration of NO was added once the effect of the previous concentration disappeared (e.g. Figure 1). Addition of similar volumes of Krebs saturated with N_2 had no effect. To prepare the NO saturated solutions, a vial containing 20 ml of Krebs solution was initially bubbled with N_2 for 15 min and then continuously bubbled with NO (450 p.p.m.). The first concentration of NO was added to the organ bath 5 min after initiating the bubbling (steady-state NO values were reached within 2–3 min). The concentration of NO in the saturated solution as measured by the electrode was $8.9\pm0.5\times10^{-7}$ M ($n=4$) which is in good agreement with the value (8.6×10^{-7} M)

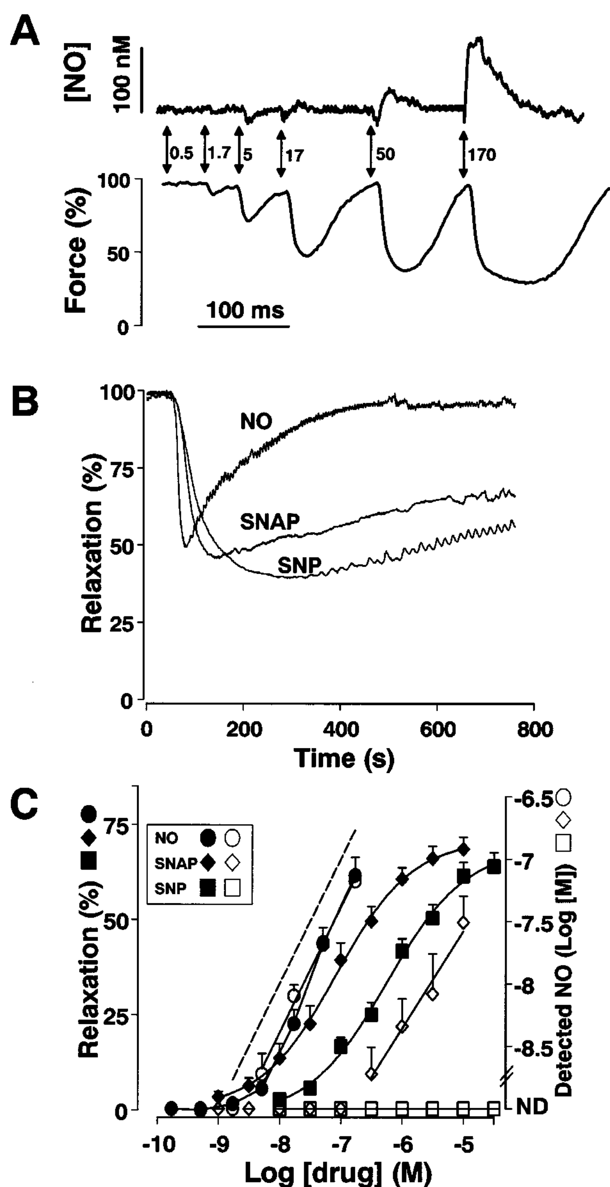

Figure 1 Relaxant responses to NO, SNAP and SNP in endothelium-denuded piglet pulmonary arteries and electrochemically detected NO in the organ bath. (A) Original simultaneous recordings of the relaxant effects of NO and the NO detected in the organ bath. NO was added as indicated by the arrows (concentrations are expressed in nM). A small artefact (downward deflection) can be observed upon addition of the NO solution mostly due to the extremely high sensitivity of the electrode to changes in temperature. (B) Representative traces of the time-course of the effects of a single concentration of NO (5×10^{-8} M), SNAP (3×10^{-7} M) and SNP (3×10^{-6} M). (C) The averaged results of relaxation (solid symbols) induced by NO (n=9, circles), SNAP (n=4, diamonds) and SNP (n=3, squares) and the NO detected electrochemically (open symbols). Results (mean ± s.e.mean) of relaxation are expressed as a percentage of control tone induced by 10^{-7} M U46619. The dashed line indicates the theoretical NO added (note that detected NO is lower than NO added indicating NO loss during pipetting and diffusion in the bath). ND not detectable.

calculated from the solubility of NO at 25°C (1.931×10^{-3} M) considering the dilution factor (450 p.p.m.). However, as described in the Results section, there was still certain loss of NO during the pippeting and diffusion in the organ bath before NO was detected by the electrode. The concentration of nitrite in the saturated NO solution after 10 min of NO bubbling was $4.1\pm0.4\times10^{-7}$ M (n=6) and the threshold for nitrite-induced relaxation in pulmonary arteries was 0.3 mM.

Drugs

U46619 and SNAP were from Alexis Biochemicals (Läufelfingen, Switzerland), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) from Tocris Cookson Ltd (Bristol, U.K.), SKF525A from RBI (Smith Kline Beecham, U.K.), AA861 from Takeda (Japan) and all other drugs from Sigma Chemical Co. (Alcobendas, Spain). Drugs were dissolved initially in distilled deionized water (except for thapsigargin, AA861 and ODQ which were dissolved in dimethyl sulphoxide and hypoxanthine which was dissolved in NaOH 0.1%) to prepare a 10^{-2} M or 10^{-3} M stock solution and further dilutions were made in Krebs solution. Oxyhaemoglobin was prepared by reduction of human haemoglobin as described (Angulo *et al.*, 1996).

Statistical analysis

Results are expressed as means ± s.e.mean and n reflects the number of animals from which the arterial rings were obtained. Individual cumulative concentration-response curves were fitted to a logistic equation. The drug concentration exhibiting 50% of the maximal effect (E_{max}) was calculated from the fitted concentration-response curves for each ring and expressed as negative log molar (pD_2). Statistically significant differences between groups were calculated by an ANOVA followed by a Newman Keuls test. $P<0.05$ was considered statistically significant.

Results

NO, SNAP and SNP-induced relaxation and NO detected electrochemically

Stimulation of endothelium-denuded PA with U46619 (10^{-7} M) induced a sustained contractile response of 958 ± 51 mg (n=30). The addition of NO resulted in a transient signal in the NO sensitive electrode accompanied by a relaxant response (Figure 1A). The time courses of the relaxant responses to a single concentration of the three drugs (Figure 1B) indicate that the relaxant effect of NO developed faster (the maximal relaxant effect was achieved at 26 ± 2 s, n=4) than SNAP (86 ± 8 s, n=4), being SNP the slowest of the three drugs (250 ± 12 s, n=4). However, while NO-induced relaxation was very transient (full recovery was achieved within 6–8 min), the relaxant responses to SNAP and SNP lasted longer than 20 min. The addition of NO, SNAP and SNP induced a concentration-dependent relaxation (Figure 1C), the order of potency being NO > SNAP > SNP. The maximal relaxant responses (E_{max}) of SNAP and SNP were 69 ± 3 and 64 ± 4% of the precontraction value, respectively, while the E_{max} of NO was not achieved at the maximal concentration tested. NO-induced relaxations were transient and the peak concentrations of NO as measured by the NO-sensitive electrode in the organ bath were lower than the added concentrations (dashed line in

Figure 1C) because of its short half life (NO decayed in the organ bath monoexponentially with a half life of approximately 15 s). Addition of SNAP produced an increase in detected NO but, in contrast to added NO, the electrode only detected NO at concentrations at which SNAP produced more than 50% relaxation while addition of SNP up to 3×10^{-5} M resulted in no detectable NO in the organ bath (Figure 1C).

Mechanism of NO- and NO donor-induced relaxation

The treatment with the sGC inhibitor ODQ (10^{-6} M) after U46619 exposure markedly inhibited the relaxation induced by NO, SNAP and SNP (Figure 2). Addition of the inhibitor of the sarcoplasmic Ca^{2+}-ATPase (SERCA) thapsigargin (2×10^{-6} M) also significantly shifted the concentration-response curves to NO, SNAP and SNP (Figure 2, Table 1).

Scavenging effects of oxyhaemoglobin

Addition of oxyhaemoglobin (3×10^{-7} M) markedly inhibited the relaxation induced by NO (Figure 3A). In the presence of oxyhaemoglobin, only the highest concentration of NO tested (2×10^{-7} M, i.e. near to the concentration of oxyhaemoglobin used) produced a significant relaxant effect. SNAP-induced relaxation was only partially inhibited by oxyhaemoglobin which produced a rightward shift of the concentration-response curve while SNP-induced relaxation was unaffected (Figure 3B, C, Table 1).

Effects of superoxide generating systems

Two means of increasing superoxide were used in our experiments: XO which generates superoxide as a byproduct of the conversion of HX into uric acid (i.e. an exogenous source of superoxide) and DETCA which inhibits superoxide dismutase (SOD) (i.e. the main endogenous scavenger of superoxide). The addition of XO (5 mu ml^{-1}) plus HX (10^{-4} M) and DETCA (10^{-3} M) markedly inhibited NO-induced relaxation (Figure 4). However, XO plus HX significantly reduced the NO detected by the electrode while DETCA had no effect (Figure 6). In contrast, SNAP-induced relaxation was unaffected by XO plus HX but partially inhibited by DETCA which produced a parallel rightward shift of the curve. Moreover, SNP-induced relaxation was unaffected by either XO plus HX or DETCA (Figure 4, Table 1).

Effects of superoxide scavengers

SOD and $MnCl_2$ (a SOD mimetic; Hussain & Ali, 1999; Mackenzie *et al*., 1999) were used as scavengers of superoxide in order to analyse the possible role of superoxide on NO, SNAP and SNP-induced relaxation. Both treatments potentiated the relaxant responses of NO (Figure 5, Table 1). However, SOD did not modify the NO detected by the electrode (Figure 6). In contrast, SOD and $MnCl_2$ had no effect on SNAP- or SNP-induced relaxation.

Source of superoxide

To inhibit the activity of the superoxide generating systems (Cai & Harrison, 2000) which could be responsible for the superoxide-driven NO destruction we used DPI (inhibitor of membrane NAD(P)H oxidase), L-NAME (inhibitor of NO synthase), indomethacin (inhibitor of cyclo-oxygenase), AA861 (inhibitor of lipo-oxygenase), SKF525A (inhibitor of Cit P450 oxidase), oxypurinol (inhibitor of xanthine oxidase) and rotenone (inhibitor of mitochondrial electron transport chain). DPI significantly potentiated the relaxant effect of NO while other inhibitors had no effect on this response (Figure 7A).

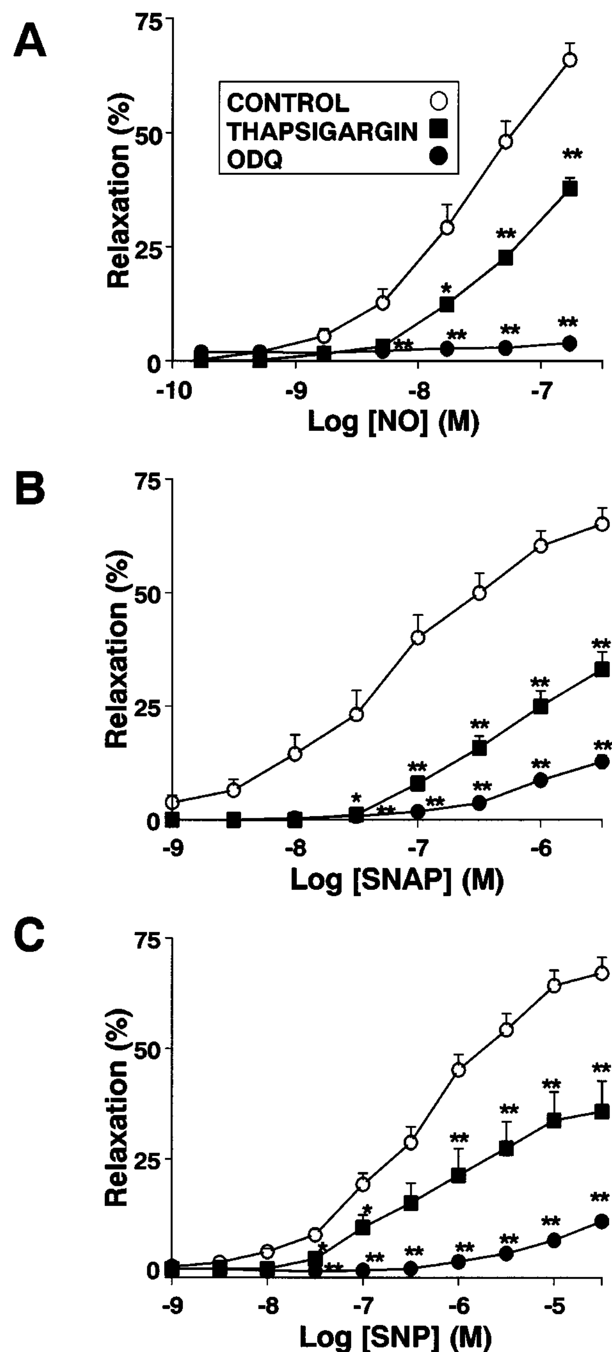

Figure 2 Effects of the sGC inhibitor ODQ (10^{-6} M) and the SERCA inhibitor thapsigargin (2×10^{-6} M) on (A) NO-, (B) SNAP- and (C) SNP-induced relaxation in endothelium denuded pulmonary arteries stimulated with 10^{-7} M U46619. Results are expressed mean $\pm$ s.e.mean ($n=5-7$) and ** denote $P<0.05$ and $P<0.01$ vs control.

Table 1 pIC_{30} values for the relaxant effects of NO, SNAP and SNP in endothelium-denuded pulmonary arteries in the absence or presence of oxyhaemoglobin or agents modulating superoxide concentrations

	NO	*SNAP*	*SNP*
Control	7.73±0.12 (10)	7.26±0.17 (9)	6.43±0.13 (9)
Oxyhaemoglobin	6.88±0.07 (4)**	6.37±0.11 (4)**	6.31±0.22 (4)
XO+HX	6.96±0.12 (5)**	7.09±0.13 (7)	6.55±0.30 (4)
DETCA	6.92±0.18 (6)**	6.69±0.11 (6)*	6.41±0.11 (5)
SOD	8.52±0.16 (7)*	7.40±0.16 (6)	6.17±0.27 (5)
$MnCl_2$	8.23±0.11 (6)	7.24±0.27 (6)	6.18±0.17 (5)

Values are mean±s.e.mean (n). * and ** $P<0.05$ and $P<0.01$ vs control, respectively.

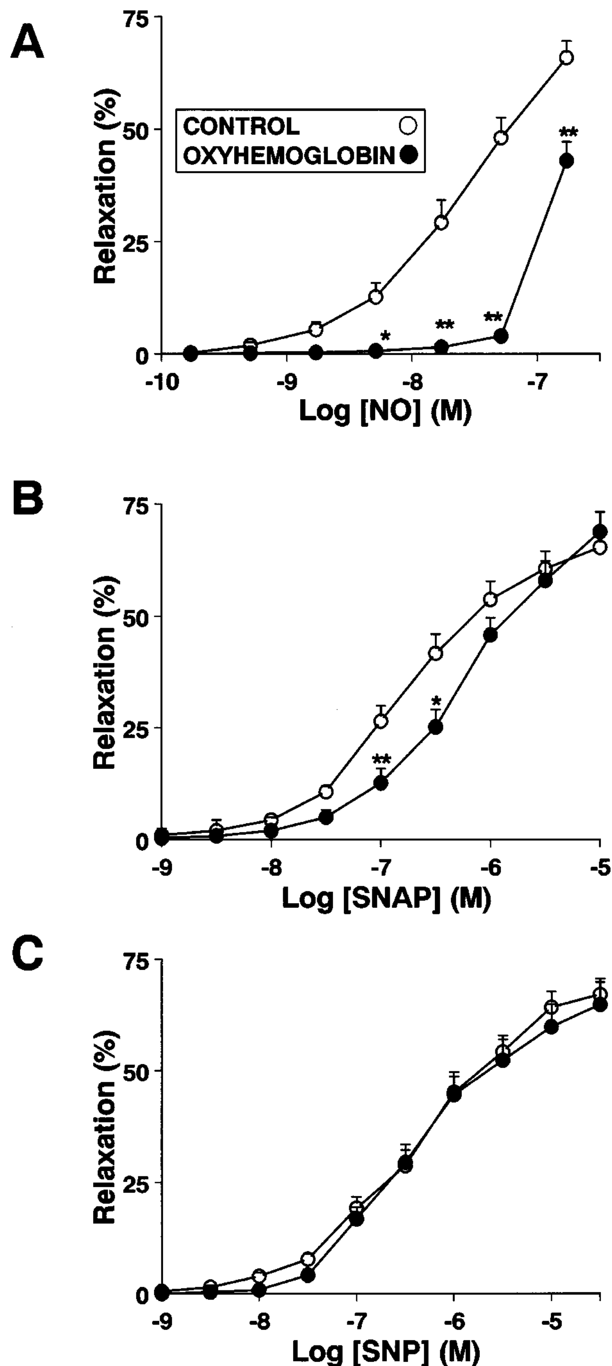

Figure 3 Effects of the oxyhaemoglobin (3×10^{-7} M) on (A) NO-, (B) SNAP- and (C) SNP-induced relaxation in endothelium denuded pulmonary arteries stimulated with 10^{-7} M U46619. Results are expressed mean±s.e.mean ($n=5-6$). * and ** denote $P<0.05$ and $P<0.01$ vs control.

In order to determine the localization of the superoxide source, relaxation was measured in rings which were oriented so that the adventitial or the luminal surface were preferentially exposed to NO. The rings were mounted: (a) with the adventitia facing outward (as all previous experiments); (b) inverted (adventitia facing inward); or (c) inverted twice (adventitia facing outward, control for the inverting procedure). These procedures did not change the contractile response to U46619 (790±129 mg, $n=8$, 890±130 mg, $n=7$ and 915±177 mg, $n=6$, respectively). The relaxation of NO was higher in rings with the adventitia facing inward (inverted) as compared to that in arteries in which adventitia was facing outward (control, non inverted) (Figure 7B). In contrast, in arteries which were inverted twice, NO-induced relaxations were similar to those in control, non inverted, arteries. Thus, orientation, but not the inversion procedure, seems to be responsible for the higher relaxant response to NO. This potentiating effect of exposing the adventitial surface inwards on NO-induced relaxation was similar to that of effect of DPI in non inverted arteries. Furthermore, in inverted rings, DPI was without effect (Figure 7B).

Discussion

The observations made in the present study indicate that NO, and the NO donors SNAP and SNP share a common mechanism of action for pulmonary arterial relaxation but, due to the different NO-releasing profiles, they show different kinetics and a different susceptibility to the scavenging effects of oxyhaemoglobin and superoxide. A model showing our interpretation of the results of the present paper is shown in Figure 8.

Electrochemical detection of NO, scavenging effects of oxyhaemoglobin and inhibitory effects of ODQ and thapsigargin

The electrochemical NO-sensitive electrode placed in the organ bath is unable to detect NO generated by the endothelium unless it is located very close to the artery (e.g. in the lumen, Simonsen *et al.*, 1999; authors unpublished observations). Similarly, the electrode will detect NO from added NO gas and from spontaneous NO releasers but it is not expected to detect NO from NO donors which only generate NO within the tissue and will not detect changes due to altered tisular metabolism of NO. NO in the bath was not detected by the electrode upon addition of SNP and was only observed after the addition of higher concentrations of

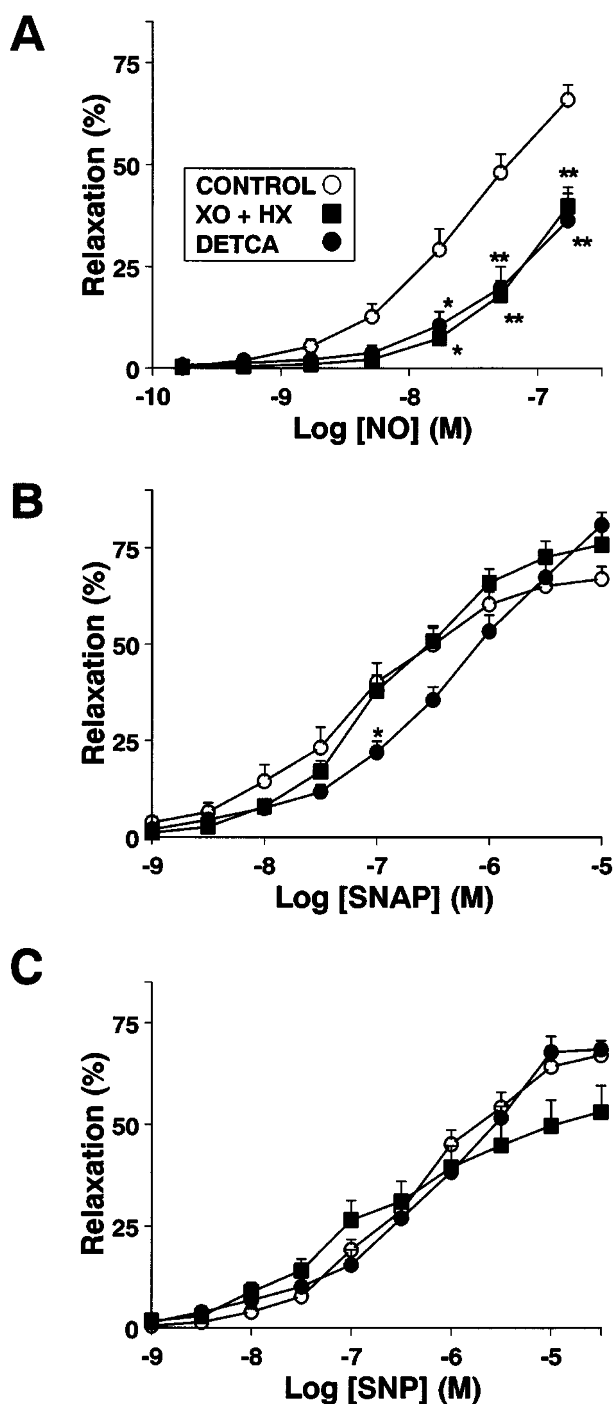

Figure 4 Effects of increased levels of superoxide on (A) NO-, (B) SNAP- and (C) SNP-induced relaxation in endothelium denuded pulmonary arteries stimulated with 10^{-7} M U46619. Superoxide was increased by adding XO (5 mu ml^{-1}) plus HX (10^{-4} M) or by inhibiting SOD with DETCA (1 mM). Results are expressed mean ± s.e.mean (n = 5–8). * and ** denote $P<0.05$ and $P<0.01$ vs control.

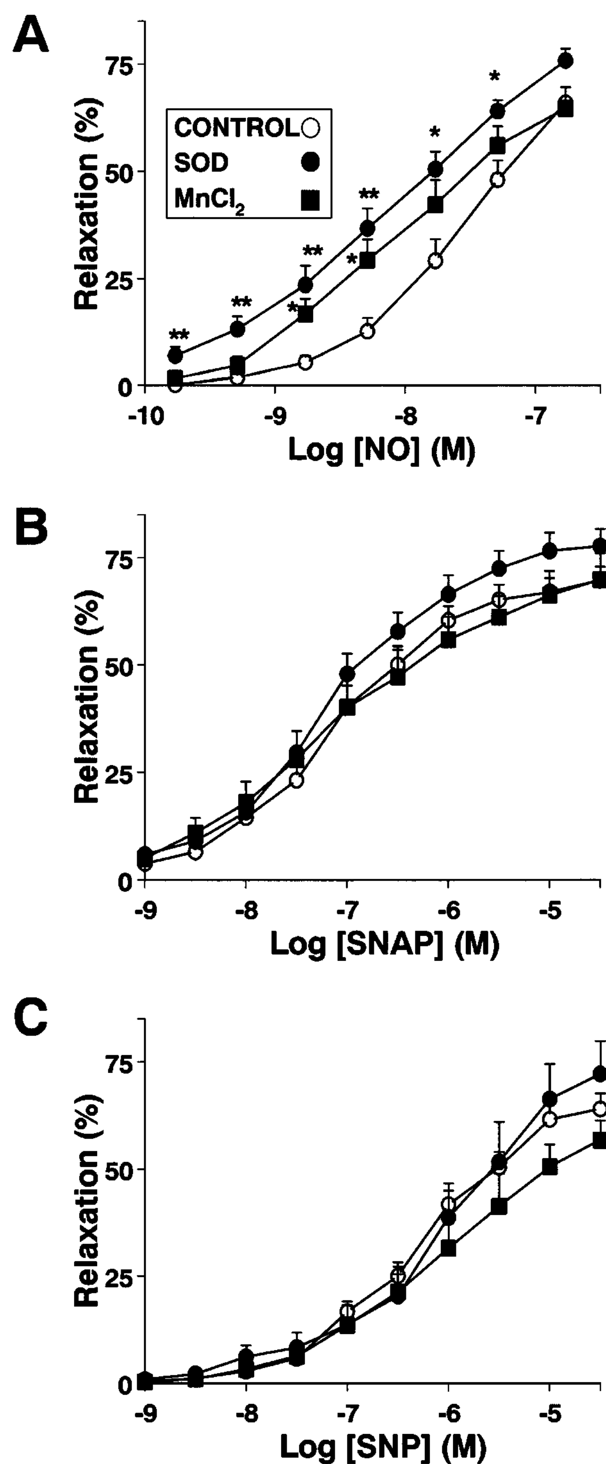

Figure 5 Effects of the superoxide scavengers superoxide dismutase (SOD, 100 u ml^{-1}) and $MnCl_2$ (10^{-4} M) on (A) NO-, (B) SNAP- and (C) SNP-induced relaxation in endothelium denuded pulmonary arteries stimulated with 10^{-7} M U46619. Results are expressed mean ± s.e.mean (n = 4–8). * and ** denote $P<0.05$ and $P<0.01$ vs control.

SNAP than those inducing relaxation. These results are consistent with those reported for SNAP in isolated platelets and measuring NO release by the rate of conversion of oxyhaemoglobin to methahaemoglobin espectrophotometrically (Salas *et al.*, 1994). Accordingly, oxyhaemoglobin, which is unable to penetrate into the cells and only scavenges extracellular NO, markedly inhibited NO-, weakly inhibited SNAP- and had no effect on SNP-induced relaxation. All these results indicate that extratisular release of NO is not

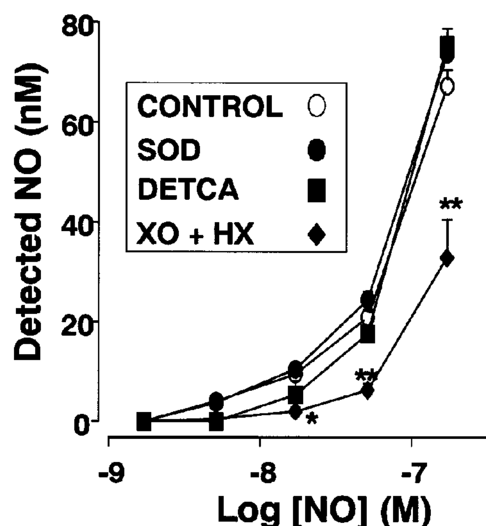

Figure 6 Effects of SOD (100 u ml^{-1}), XO (5 mu ml^{-1}) plus HX (10^{-4} M) and DETCA (1 mM) on the NO concentrations detected electrochemically in the organ bath after the addition of NO. Results are expressed mean ± s.e.mean (n=5–9). * and ** denote $P<0.05$ and $P<0.01$ vs control.

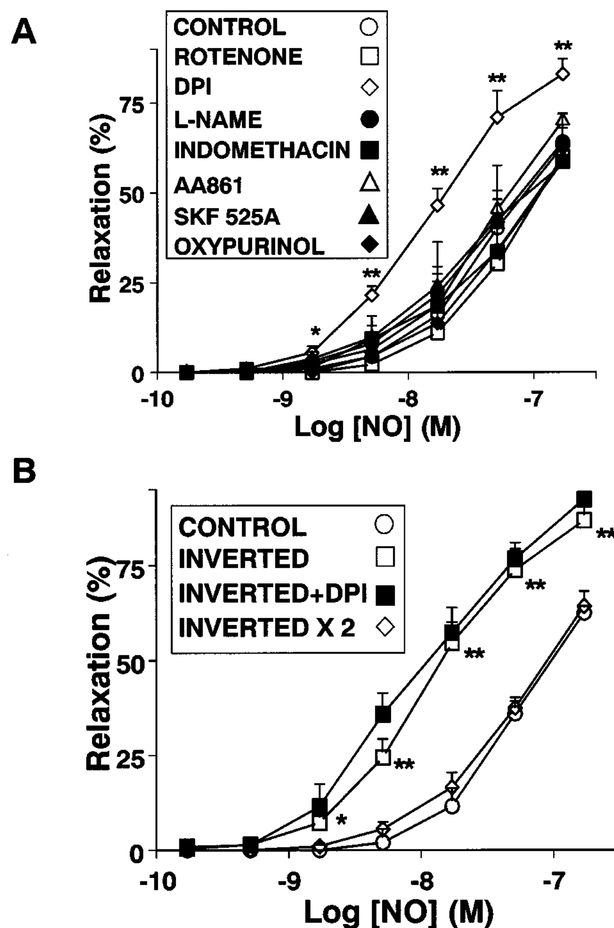

Figure 7 (A) Effects of inhibitors of the main enzymatic systems producing superoxide, rotenone (5×10^{-5} M), DPI (10^{-5} M), L-NAME (10^{-4} M), indomethacin (10^{-5} M), AA861 (10^{-5} M), SKF 525A (10^{-5} M) and oxypurinol (10^{-4} M) on NO-induced relaxation in endothelium denuded pulmonary arteries stimulated with 10^{-7} M U46619 (n=5–6). (B) Effects of the inversion procedure. Control are normal non inverted arteries, inverted indicates arteries in which the adventitia faces inward and inverted ×2 indicates arteries inverted twice in which (as control arteries) the adventitia faces outward. Results are expressed mean ± s.e.mean (n=4–6). * and ** denote $P<0.05$ and $P<0.01$ vs control.

the mechanism for the relaxant effect of SNP and only marginally of SNAP. SNP (and possibly SNAP) requires cellular metabolism to produce NO (Kowaluk *et al.*, 1992; Murphy, 1999). Even when it is widely assumed, we cannot definitely affirm that SNAP- and SNP-induced relaxation is due to tisular release of NO. In fact, a stereoselective S-nitrosothiol recognition site in the sGC has been reported (Davidson *et al.*, 1996). On the other hand, SNP releases cyanide in addition to NO which exerts multiple effects including the activation of sGC (Ignarro *et al.*, 1986). Nevertheless, the potent and similar inhibitory effects of the sGC inhibitor ODQ on NO- and SNAP- or SNP-induced relaxation indicate that NO-independent effects, if existing, are cyclic GMP-dependent. Furthermore, the inhibition of the SERCA, which is the main target of the NO/cyclic GMP pathway in these arteries (Pérez-Vizcaíno *et al.*, 1997; Cogolludo *et al.*, 2001) also produced a similar effect for the three drugs.

NO and superoxide

Increased oxidative stress was induced in our experiments using an exogenous source of superoxide (by means of the XO plus HX system) or preventing the degradation of endogenously generated superoxide, by means of the SOD inhibitor DETCA (Cocco *et al.*, 1981). XO plus HX reduced the peak NO concentrations detected by the electrode as well as the NO-induced relaxations indicating that NO reacts rapidly with the superoxide generated by the XO plus HX system during the diffusion process in the organ bath. However, the SOD inhibitor DETCA only reduced NO-induced relaxations without affecting the NO detected by the electrode, confirming that increased superoxide levels after the inhibition of SOD were restricted to the tissue and not extended to the organ bath. Conversely, exogenously added SOD or the membrane permeable SOD mimetic $MnCl_2$ (Mackenzie *et al.*, 1999; Hussain & Ali, 1999) increased NO-induced relaxations but again no changes in detected NO were observed. These results indicate that, under basal conditions, superoxide is produced within the tissue in concentrations sufficient to inactivate exogenously added NO and this effect is potentiated when superoxide metabolism is prevented by inhibiting the main superoxide scavenging mechanism SOD. Since SOD is not membrane permeable, the fact that exogenously added SOD potentiated NO-induced relaxation suggests that at least part of the superoxide which inactivates NO must be extracellular. However, the endogenous superoxide-dependent NO inactivation is restricted to the vessel area and, therefore, undetectable by the electrode in the bath. Although we have used a high concentration of oxygen (95%), this does not modify the relaxant response to NO as compared to lower concentrations such as 21 or 0% in piglet pulmonary arteries

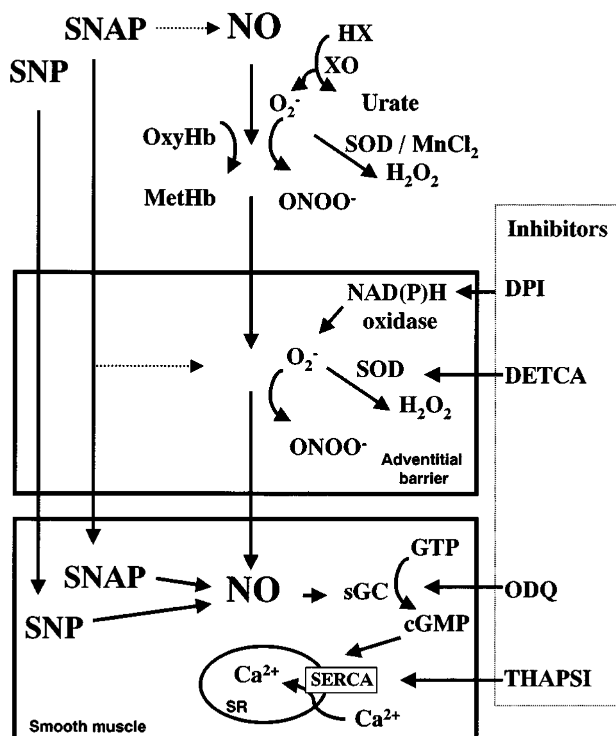

Figure 8 A model showing the hypothesized role of the adventitial oxidant barrier and the different susceptibility of NO, SNAP and SNP. See text for explanation.

(Villamor *et al.*, 2000) indicating that tissue superoxide is not the result of such high oxygen concentrations.

The source of superoxide in the pulmonary arteries was analysed using inhibitors of the main enzymatic superoxide generating systems, i.e. membrane NAD(P)H oxidase, NO synthase, cyclo-oxygenase, lipo-oxygenase, cytochrome P450 oxidase, xanthine oxidase and the mitochondrial electron transport chain (Thannickal & Fanburg, 2000). To inhibit the activity of these superoxide generating systems, we used DPI (Hancock & Jones, 1987), L-NAME, indomethacin, AA861, SKF 525A (Corriu *et al.*, 1996), oxypurinol and rotenone (Hatefi, 1976), respectively. Only DPI had a potentiating effect on NO-induced relaxation which suggests that, as described in other vessels (Di Wang *et al.*, 1998), the main source of superoxide in the piglet pulmonary artery is the membrane NAD(P)H oxidase. We analysed the localization of the superoxide source by measuring relaxation in rings which were oriented so that the adventitial or the luminal surface were preferentially exposed to NO (Steinhorn *et al.*, 1994; Di Wuang *et al.*, 1998). NO-induced relaxation was reduced in rings in which NO had to cross the adventitia before reaching the smooth muscle (non inverted or double inverted rings) as compared to that in arteries in which adventitia was facing inward (inverted rings). Furthermore, the NAD(P)H oxidase inhibitor DPI had no effect in inverted rings indicating that the main source of NAD(P)H oxidase is located in the adventitia. Therefore, the adventitial NAD(P)H oxidase acts as a chemical barrier for NO. Accordingly, Di Wuang *et al.* (1998) found that the inmunostaining for the NAD(P)H oxidase proteins gp19^{phox}, p22^{phox}, p47^{phox} and p67^{phox} was almost exclusively located in the adventitial layer in the rat aorta.

NO donors and superoxide

Unlike NO, the relaxant effects of SNAP were unaffected by exogenous SOD, $MnCl_2$ or by the XO plus HX system and only weakly inhibited by DETCA while none of these agents modified SNP-induced relaxation. Thus, the NO donor-induced relaxation is unaffected or hardly affected by endogenous or even exogenously added superoxide. These data, taken together with those obtained with the NO-sensitive electrode and those obtained with oxyhaemoglobin, indicate that NO is released from these NO donors in a different compartment from that of the endogenous source of superoxide (the adventitia). Thus, the NO releasing compartment must be located in or near the site of action of NO, i.e. the smooth muscle cell, not readily accessible to endogenous superoxide.

Implications

The adventitial barrier for NO is of unclear physiological significance because endogenous NO diffuses from the endothelium, its main physiological source, to the adjacent smooth muscle cells. However, iNO used therapeutically must cross the adventitial layer in its way from the alveoli to the pulmonary artery smooth muscle cells. Therefore, even when the adventitia is a thin layer in the small pulmonary arteries, the oxidant stress generated therein or in the surrounding parenchyma may inactivate iNO.

Under physiologic conditions, superoxide-driven NO inactivation and hence peroxynitrite production is limited by the intracellular superoxide dismutase SOD (Beckman & Koppenol, 1996). However, when the concentration of NO is increased (e.g. by the use of iNO), or under conditions of increased oxidant stress such as those requiring iNO (e.g. pulmonary hypertension associated to sepsis, respiratory distress syndrome, or other forms of pulmonary insult), it is likely that significant concentrations of peroxynitrite are produced (Beckman & Koppenol, 1996). Thus, the beneficial effects of iNO might be partly counteracted by the peroxynitrite-induced lung damage (Hampl & Herget, 2000). In fact, preliminary results indicate that intravenous SOD may be beneficial as an adjunctive therapy for iNO (Albert *et al.*, 1999). A possible strategy to deliver NO to the smooth muscle cells avoiding an excessive formation of peroxynitrite could be to replace iNO with inhaled NO donors which are less susceptible to react with superoxide. This reduced inactivation is also associated with a decreased inactivation by oxyhaemoglobin which will result in a lower risk of clinically significant metahaemoglobinaemia (Kinsella & Abman, 1995). However, since NO inactivation by oxyhaemoglobin is the main determinant of pulmonary selectivity (due to the rapid removal of NO from the blood) this benefit may be at the cost of an increased systemic vasodilatation. At present, the limited experimental and clinical information about the use of inhaled NO donors, mostly with SNP, indicates that these drugs can produce a selective pulmonary effect (Palhares *et al.*, 1998; Yu *et al.*, 1999; Yu & Saugstad, 1999; Adrie *et al.*, 1998; Schutte *et al.*, 1997).

In conclusion, the present data indicate that the kinetics and local distribution of NO release from NO donors have a strong influence not only in the potency and duration of the relaxant effect but also on their susceptibility to the

scavenging effect produced by oxyhaemoglobin and superoxide. The reduced inactivation of NO donors by superoxide as compared to NO suggests that inhalation of these drugs may result in pulmonary vasodilation independent of the oxidant status and possibly a reduced peroxynitrite induced lung injury.

The authors are grateful to Dr C. Sánchez-Ferrer for his help with the preparation of oxyhaemoglobin and C. Rivas for excellent technical assistance. This work was supported by a CICYT (SAF 99/0069) and CAM (08.4/0019/1998) Grants. F. Pérez-Vizcaíno and A. Cogolludo are supported by a grant from Communidad Autónoma de Madrid.

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(Received January 5, 2001
Revised March 20, 2001
Accepted April 3, 2001)