DOSE-RESPONSE RELATIONSHIP FOR INHALED NITRIC OXIDE IN EXPERIMENTAL PULMONARY HYPERTENSION IN SHEEP

O. DYAR, J. D. YOUNG, L. XIONG, S. HOWELL AND E. JOHNS

SUMMARY

We have examined the effect of inhaled nitric oxide 4-512 p.p.m. in six sheep with pulmonary hypertension induced first with hypoxia and then with 6 μg kg⁻¹ of E. coli endotoxin. A similar dose-dependent reduction in pulmonary artery pressure occurred in pulmonary hypertension induced by hypoxia or endotoxin, with a maximum effect of 25-30% decrease with nitric oxide 64 p.p.m. Increasing the dose to 512 p.p.m. had no further effect. The ED₅₀ for inhaled nitric oxide was 39 p.p.m. for pulmonary hypertension induced by hypoxia and 48 p.p.m. for endotoxin-induced pulmonary hypertension. A dose-dependent increase in arterial oxygenation, which reached a maximum with nitric oxide 64 p.p.m., was seen with inhaled nitric oxide after endotoxin infusion. If the toxicity of inhaled nitric oxide can be determined, it may prove useful in the treatment of pulmonary hypertension secondary to the adult respiratory distress syndrome. (Br. J. Anaesth. 1993; 71: 702-708)

KEY WORDS


Pulmonary hypertension in the adult respiratory distress syndrome (ARDS) causes right ventricular failure and reduced left ventricular performance [1]. I.v. vasodilators such as nitroprusside, nitrates or prostacyclin reduce pulmonary artery pressures, but are not selective for the pulmonary circulation and so cause unacceptable systemic vasodilatation and hypotension [2, 3]. As i.v. vasodilators act throughout the pulmonary vasculature, they may increase intrapulmonary shunt by overcoming the effect of local hypoxic vasoconstriction, and so worsen the alveolar–arterial oxygen partial pressure difference and reduce arterial oxygenation [2].

Endothelium derived relaxing factor (EDRF) is an endogenous vasodilator which is either nitric oxide or a nitrosoylated compound with similar properties [4-6]. Inhaled exogenous nitric oxide causes selective pulmonary vasodilatation, without affecting systemic vascular resistance [7-11]. As nitric oxide is applied only to ventilated alveoli, an increase in shunt should not occur and oxygenation should not be altered.

Previous work using only one or two different doses has shown that nitric oxide is an effective pulmonary vasodilator, but dose–response studies have been performed only in association with pulmonary hypertension induced by a thromboxane analogue [9]. The effect of inhaled nitric oxide on oxygenation in ARDS has been examined at two doses in patients [7], but an animal model of ARDS has not been examined formally. This study was undertaken for three purposes. First, we have determined the dose–response curve for inhaled nitric oxide in the hypoxic sheep model—in particular, to determine if there is maximum effective dose. Second, we have determined the effects of nitric oxide on pulmonary pressures, vascular resistance and oxygenation in an animal model of ARDS. Third, we have compared the dose–response curves of nitric oxide on pulmonary hypertension caused by these different mechanisms.

MATERIALS AND METHODS

The experiments were conducted in accordance with local and governmental regulations concerning animal experimentation. Six Suffolk sheep (mean weight 39 kg, range 36-42 kg) were anaesthetized with pentobarbitone 18 mg kg⁻¹. Anaesthesia was maintained with infusions of pentobarbitone 5-15 mg kg⁻¹ h⁻¹ and fentanyl 5-25 μg kg⁻¹ h⁻¹. Fluid losses were replaced with Hartmann's solution or normal saline. Tracheotomy was performed using an 8-mm i.d. tube, and the lungs were ventilated to an arterial PcO₂ of 4.5-6.0 kPa using a volume preset ventilator (Oxford ventilator, Penlon, Abingdon, U.K.). Oxygen, nitric oxide–nitrogen mixtures and nitrogen were added as required to a reservoir bag and tube attached to the air entrainment port of the ventilator. Inspired oxygen concentrations were monitored using an 8-mm i.d. tube, and the lungs were ventilated to an arterial Pco₂ of 4.5-6.0 kPa using a volume preset ventilator (Oxford ventilator, Penlon, Abingdon, U.K.). Oxygen, nitric oxide–nitrogen mixtures and nitrogen were added as required to a reservoir bag and tube attached to the air entrainment port of the ventilator. Inspired oxygen concentrations were monitored using a Servomex paramagnetic oxygen analyser (Taylor Analytics, Crowborough, U.K.). Exhaled gas was passed through a vane respirometer for measurement of tidal volume and then to a 50-litre mixing chamber. Mixed expired and end-tidal carbon dioxide concentrations were measured using an infra-red absorption analyser (Normocap, Datex, Helsinki, Finland).

A 6-French gauge arterial catheter was placed in the carotid artery and a 7-French gauge balloon-tipped pulmonary artery catheter was passed via the left internal jugular vein. Arterial, pulmonary artery, central venous and pulmonary artery occlusion pressures were measured using pressure transducers calibrated against mercury columns. A COM-2 cardiac output computer (Baxter Healthcare, Santa Ana, California, U.S.A.) was used to determine cardiac output by thermocathlab. An ABL330 blood gas analyser and OSM3 co-oximeter (both Radiometer, Copenhagen, Denmark) were used to measure blood-gas tensions and methaemoglobin concentrations.

After initial preparation, the lungs were ventilated using 60% inspired oxygen and the sheep were allowed to stabilize for 30 min. Intravascular pressures, arterial blood-gas tensions, methaemoglobin concentrations and ventilatory variables were recorded. The inspired oxygen concentration was then reduced to 12% by adding nitrogen to the air entrained by the ventilator, which caused an immediate increase in pulmonary artery pressure. The recordings were repeated and then nitric oxide was added to the inspired gas in concentrations of 4, 8, 16, 32, 64, 128, 256 and 512 parts per million by volume (p.p.m.), the doses being given in random order. Nitric oxide was stored as 2000 p.p.m. in nitrogen (to prevent oxidation to nitrogen dioxide), the flow of the mixture required (8 ml min$^{-1}$ to 1.6 litre min$^{-1}$) was calculated from the required final concentration and the total ventilation. In greater doses, the amount of nitrogen in the nitric oxide mix caused further reduction in inspired oxygen concentration, which was countered by decreasing the nitrogen flow. Nitric oxide–nitrogen flows were measured and controlled using needle valves and precision flowmeters calibrated for nitrogen (Meterate, Glass Precision Engineering, Hemel Hempstead, U.K. and 1100 series flowometers, KDG Ltd, Crawley, U.K.). At each dose of nitric oxide, the pulmonary artery pressure stabilized within 5–10 min and recordings were made. The nitric oxide was then stopped and the pulmonary artery pressure allowed to stabilize again. A new set of baseline recordings of intravascular pressures were made before each new dose of nitric oxide.

When all the doses of nitric oxide had been tested, the nitrogen was stopped and oxygen added to the inspired gas to an inspired concentration of 60–80%, to avoid any hypoxic pulmonary vasoconstriction. Endotoxin 6 µg kg$^{-1}$ (from E. coli serotype 0127:B8, Sigma, Poole, U.K.) was infused over 30 min. This resulted in a marked increase in pulmonary artery pressure, which remained increased after a small initial decrease when the infusion stopped. The dose–response study with inhaled nitric oxide was repeated, using the same range of doses in a different random order.

The haemodynamic variables (pulmonary vascular resistance, systemic vascular resistance) were derived using standard formulae. The deadspace: tidal volume ratio ($V_d:V_t$) was calculated from the Bohr equation using arterial instead of alveolar $P_{CO_2}$, after correction of the mixed expired carbon dioxide concentration for changes in water vapour content and temperature. Statistical comparisons were performed using analysis of variance and Duncan's multiple range test with "SPSS for Windows" release 5.0 (SPSS Inc., Chicago, U.S.A.) running under Windows version 3.1 on an 80486-based personal computer. $P \leq 0.05$ was taken as statistically significant.

**RESULTS**

Mean pulmonary artery pressure before hypoxia was 22 mm Hg, increasing to 37 mm Hg after the inspired oxygen was reduced to 12%. Mean pulmonary artery pressure before endotoxin was 18 mm Hg, increasing to 39 mm Hg after endotoxin infusion. Mean pulmonary artery pressure decreased with increasing doses of nitric oxide, but most of the effect occurred between 4 and 64 p.p.m. (fig. 1). There was little additional effect. The sd for the
Fig. 2. Effect of inhaled nitric oxide on pulmonary vascular resistance (PVR) during hypoxia (■) and endotoxemia (□). Error bars = 1 sd. Pre = Before hypoxia or endotoxin. * Significant difference from baseline (0 p.p.m.) 

$P < 0.05$.

Table 1. Effect of nitric oxide on cardiac output (CO), deadspace: tidal volume ratio ($V_{D}:V_{T}$), methaemoglobin concentrations (MeHb) and systemic vascular resistance (SVR) (mean (SD))

<table>
<thead>
<tr>
<th>Nitric oxide (p.p.m.)</th>
<th>CO (litre min$^{-1}$)</th>
<th>$V_{D}:V_{T}$</th>
<th>MeHb (%)</th>
<th>SVR (dyn s cm$^{-1}$)</th>
<th>CO (litre min$^{-1}$)</th>
<th>$V_{D}:V_{T}$</th>
<th>MeHb (%)</th>
<th>SVR (dyn s cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before hypoxia or endotoxin</td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>4.6 (0.6)</td>
<td>0.44 (0.1)</td>
<td>0.6 (0.1)</td>
<td>2017 (20)</td>
<td>6.4 (0.6)</td>
<td>0.25 (0.19)</td>
<td>0.7 (0.6)</td>
<td>1001 (232)</td>
</tr>
<tr>
<td>4</td>
<td>7.5 (1.0)</td>
<td>0.43 (0.2)</td>
<td>0.5 (0.2)</td>
<td>1235 (210)</td>
<td>7.6 (1.1)</td>
<td>0.54 (0.2)</td>
<td>0.8 (0.2)</td>
<td>1179 (310)</td>
</tr>
<tr>
<td>8</td>
<td>6.8 (1.4)</td>
<td>0.39 (0.2)</td>
<td>0.9 (0.2)</td>
<td>1184 (197)</td>
<td>6.8 (1.5)</td>
<td>0.55 (0.1)</td>
<td>1.5 (0.2)</td>
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<td>16</td>
<td>7.7 (1.4)</td>
<td>0.36 (0.2)</td>
<td>0.8 (0.2)</td>
<td>986 (237)</td>
<td>7.7 (1.5)</td>
<td>0.51 (0.1)</td>
<td>1.8 (0.9)</td>
<td>1366 (692)</td>
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<td>32</td>
<td>7.2 (1.2)</td>
<td>0.36 (0.2)</td>
<td>1.2 (0.2)</td>
<td>1153 (237)</td>
<td>7.2 (1.5)</td>
<td>0.49 (0.1)</td>
<td>1.5 (0.1)</td>
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<td>64</td>
<td>7.4 (0.8)</td>
<td>0.26 (0.4)</td>
<td>1.1 (0.4)</td>
<td>1218 (176)</td>
<td>7.4 (1.9)</td>
<td>0.46 (0.1)</td>
<td>1.8 (1.0)</td>
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<tr>
<td>128</td>
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<td>0.9 (0.5)</td>
<td>1152 (512)</td>
<td>6.4 (1.2)</td>
<td>0.48 (0.5)</td>
<td>2.3 (1.0)</td>
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<td>256</td>
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<tr>
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<td>0.3 (0.5)</td>
<td>1.7 (0.5)</td>
<td>1079 (424)</td>
<td>7.2 (1.4)</td>
<td>0.46 (0.1)</td>
<td>2.2 (1.1)</td>
<td>1133 (419)</td>
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</table>

mean pulmonary artery pressure after endotoxin were much smaller than those during hypoxia (coefficients of variation for endotoxin 9–13%, for hypoxia 15–34%) because endotoxin produced a more stable increase in pulmonary artery pressure than hypoxia, with less within- and between-subject variability. The increase in pulmonary artery pressure caused by hypoxia was reversed almost totally by nitric oxide, but the increase caused by endotoxin could not be reversed fully. The ED$_{50}$ (dose of nitric oxide required to produce 50% of maximal percentage reduction in pulmonary artery pressure) calculated from the pooled data was 39 p.p.m. for hypoxia and 48 p.p.m. for endotoxaemia.

The effect of inhaled nitric oxide on pulmonary vascular resistance (PVR) is shown in figure 2. The values for pulmonary vascular resistance before hypoxia and endotoxin were 236 and 119 dyn s cm$^{-1}$, respectively. The increase in pulmonary vascular resistance caused by endotoxin was not reversed fully by nitric oxide, but even the smallest dose (nitric oxide 4 p.p.m.) was able to reverse the pulmonary vascular resistance increase caused by hypoxia. The decrease in pulmonary vascular resistance to less than the pre-hypoxic baseline was probably caused by the marked increase in cardiac output that occurred with hypoxia (table 1).

Cardiac output was unchanged by inhaled nitric oxide both during hypoxia and after infusion of endotoxin (table 1). This implies that dose-dependent changes in pulmonary artery pressure and pulmonary vascular resistance are an effect of nitric
NITRIC OXIDE DOSE-RESPONSE

Fig. 3. Effect of inhaled nitric oxide on arterial oxygen partial pressure ($P_{O_2}$) during hypoxia (■) and endotoxaemia (□). Error bars = 1 SD (so for data during hypoxia are in general smaller than the plotting symbols). Pre = Before hypoxia or endotoxin. * Significant difference from baseline (0 p.p.m.) ($P < 0.05$).

Nitric oxide concentration (p.p.m.)

Fig. 4. Effect of inhaled nitric oxide on alveolar-arterial oxygen partial pressure difference ($P_{O_2} - P_{A_2}$) during hypoxia (■) and endotoxaemia (□). Error bars = 1 SD (so for data during hypoxia are smaller than the plotting symbols). * Significant difference from baseline (0 p.p.m.) ($P < 0.05$).

Nitric oxide concentration (p.p.m.)

oxide on pulmonary vasculature and not secondary to altered cardiac output. $V_{D}:V_{T}$ and systemic vascular resistance were unchanged by nitric oxide.

After infusion of endotoxin there was a dose-dependent increase in $P_{O_2}$ up to 64 p.p.m.; after this there was no further increase (fig. 3). Nitric oxide had no effect on the hypoxaemia caused by 12% oxygen. The inspired oxygen partial pressure during the endotoxin studies varied slightly between sheep (60–80%). Figure 4 shows the alveolar-arterial oxygen partial pressure difference which corrects for this; there is a dose-dependent decrease up to 64–128 p.p.m.

The maximum methaemoglobin concentration observed was 3.7%, and the mean values increased with increasing dose of nitric oxide (table I). It was apparent that the increase in methaemoglobin concentration was both time- and dose-dependent. In one animal, nitric oxide 512 p.p.m. was given for 20 min at the end of the experiment and the methaemoglobin concentration increased linearly with time to 11%. The onset of pulmonary vasodilatation was rapid—within one breath with the greater doses. Reversal of this effect after the nitric oxide was discontinued occurred at 5–10 min.

**DISCUSSION**

Nitric oxide is a colourless gas formed by combustion in a reducing atmosphere. It is present in concentrations of up to 9 p.p.m. in atmospheric air [12] and in concentrations of up to 1000 p.p.m. in cigarette smoke [13]. Endogenous nitric oxide is synthesized from L-arginine by nitric oxide synthases in mammalian endothelial cells [14], macrophages [15] and nerve tissue [16]. Synthesis may be blocked by Nω-
monomethyl arginine or other false substrates and cells deficient in L-arginine release reduced amounts of nitric oxide [17]. It is highly lipid soluble and diffuses rapidly from the endothelium into vascular smooth muscle. Nitric oxide activates intracellular soluble guanylate cyclase [18], increasing intracellular cyclic guanosine monophosphate which, in turn, inhibits calcium release from the sarcoplasmic reticulum and inhibits calcium entry through receptor-operated channels. This causes relaxation of smooth muscle [18].

Nitric oxide is inactivated by combination with haemoglobin with the formation of methaemoglobin. Nitric oxide binds to haemoglobin with an affinity 1500 times that of carbon monoxide [19]; thus nitric oxide which enters the vascular lumen is inactivated rapidly. This accounts for the selective pulmonary vasodilatation seen with inhaled nitric oxide, which presumably acts on the luminal surface of pulmonary vessels. Any nitric oxide entering the blood is inactivated and so does not cause systemic effects. Cigarette smoke, which contains nitric oxide up to 1000 p.p.m., is known to cause pulmonary vasodilatation [20]. These properties of inhaled nitric oxide were first demonstrated by Higenbottam and colleagues [21] in patients with primary pulmonary hypertension. Inhaled nitric oxide has been shown also to cause pulmonary vasodilatation in humans with pulmonary hypertension resulting from mitral valve disease [22], ARDS [7], hypoxia [23] and in neonates with primary pulmonary hypertension of the newborn [24, 25].

In animal models, inhaled nitric oxide has reduced or reversed pulmonary vasoconstriction caused by hypoxia [9], heparin–prothrombin reaction [10], a thromboxane analogue [9], and infusion of endotoxin [11]. Dose–response data for the reduction of pulmonary artery pressure have been presented for inhaled nitric oxide in lambs using thromboxane analogue-induced pulmonary vasoconstriction [9, 10]. These data suggest that a maximum effect would be reached at about 80 p.p.m., but greater doses were not used. Frostell and colleagues showed also that inhalation of nitric oxide 80 p.p.m. for 6 min had no effect on pulmonary or systemic haemodynamics of animals with normal lungs breathing 60–70% oxygen [9]. Archer and co-workers, using an isolated perfused rat lung model, showed that nitric oxide injected in solution into the pulmonary artery produced a transient, dose-dependent reduction in hypoxic pulmonary vasoconstriction [26].

We are unaware of any formal dose–response studies of inhaled nitric oxide in the hypoxic sheep or in an endotoxin-induced ARDS model in sheep. We therefore sought to confirm existing evidence that nitric oxide reduces hypoxic pulmonary vasoconstriction in a dose-dependent fashion and that there is a greater useful dose beyond which further vasodilatation does not occur, and to see if the same effect occurred in the endotoxic sheep model of ARDS.

Our methods were similar to those of previous studies. The hypoxia induced in our sheep ($F_{1\text{O}_2}$ 0.12) was not as severe as that used by Frostell and colleagues [9] ($F_{1\text{O}_2}$ 0.06–0.08), yet it caused a marked increase in both pulmonary artery pressure and cardiac output. We tried initially to reduce $F_{1\text{O}_2}$ to 0.1, but two sheep showed signs of myocardial ischaemia on ECGs, therefore a greater $F_{1\text{O}_2}$, was used. Endotoxaemia produces a biphasic response in the sheep pulmonary circulation [27]: a brief initial phase of marked pulmonary hypertension is followed by a phase of less severe hypertension and abnormal microvascular permeability. A dose of endotoxin 6 μg kg$^{-1}$ was chosen on the basis of previous (unpublished) work which showed sustained and reasonably constant pulmonary hypertension over many hours after administration. Endotoxin produces an acute model of ARDS with an increased alveolar–arterial oxygen partial pressure difference, microvascular permeability and lung water, but cannot mimic long-term changes such as fibrosis.

Nitric oxide caused a dose-dependent reduction in the mean pulmonary artery pressure that was similar during hypoxia and after endotoxin, with a maximum effect produced with 128 p.p.m. and no significant change thereafter. The $ED_{50}$ values were similar. There would thus be no reason to use doses greater than 128 p.p.m., as maximum pulmonary vasodilatation would have been achieved, while toxicity, as determined by methaemoglobin concentrations, would continue to increase with greater doses. The decrease in pulmonary vascular resistance that occurred with increasing doses of nitric oxide showed a similar pattern.

The pulmonary vasodilatory effect of inhaled nitric oxide was seen with concentrations of nitric oxide 4 p.p.m., confirming its potency in small doses [8–10, 24]. A dose of 4 p.p.m. represents an inhaled dose of about 0.8 μg kg$^{-1}$, and, if equilibrated with extracellular fluid, would be a 12–13 nmol litre$^{-1}$ solution. Ambient concentrations of nitric oxide in air may exceed this value: up to 9 p.p.m. has been detected as an air pollutant [12]. Nitric oxide was also very specific in effect: no changes in systemic vascular resistance were detected, even with the greatest dose.

In ARDS, systemically administered vasodilators cause an increase in alveolar–arterial oxygen partial pressure difference [2, 3], presumably by vasodilating vessels that are constricted by local hypoxia and so perfusing underventilated areas. It was expected that inhaled nitric oxide would have no effect on arterial oxygenation after endotoxin administration—the nitric oxide simply would not reach underventilated areas. Instead, a dose-dependent improvement in alveolar–arterial oxygen partial pressure difference and arterial oxygenation was noted, with a plateau at 128 p.p.m. and greater and the decrease in pulmonary artery pressure was correlated with the decrease in alveolar–arterial difference ($r = 0.54$, $P < 0.0005$; linear regression). Mean $P_{a\text{O}_2}$ increased by a factor of almost three. This must represent an improvement in the ventilation–perfusion relationships within the lung, presumably as a result of redistribution of blood flow. A study in patients with ARDS using the multiple inert gas elimination technique has also suggested that this occurs [7]. The nitric oxide had no measurable effect on arterial oxygenation during the administration of
hypoxic gas mixtures, but the alveolar–arterial oxygen partial pressure difference was so small that there was no room for improvement.

The toxicity of nitric oxide has been reviewed extensively elsewhere [10]. Nitric oxide binds to haemoglobin, forming methaemoglobin and a small amount of nitrosoyl haemoglobin [28]. Methaemoglobin is reduced back to haemoglobin and nitrates mostly by NADH–methaemoglobin reductase and the reduction of normal methaemoglobin production uses only a fraction of the capacity of this pathway [29]. When methaemoglobin production is increased by inhaling nitric oxide, this extra capacity is used. Although the maximum methaemoglobin concentration in these experiments did not exceed 3.7%, the exposure at each dose was only about 15 min; longer exposure of one animal showed a linear increase in methaemoglobin concentration with time. More work is required to determine the dose–time–methaemoglobin concentration relationship. For long term studies, methaemoglobin may not be a suitable marker of nitric oxide toxicity. Mice exposed to nitric oxide 10 p.p.m. for 2 weeks had normal methaemoglobin concentrations, but had signs of increased red cell turnover, with splenomegaly and increased bilirubin concentrations [30].

Gaseous nitric oxide is oxidized to nitrogen dioxide in the presence of oxygen at a rate determined by the oxygen concentration and the square of the nitric oxide concentration [31]. With the greatest concentrations of nitric oxide and oxygen used in this study, 25% oxidation would be expected to occur in about 1 min; with nitric oxide 4 p.p.m. during hypoxia this would take 12 h. The nitric oxide–nitrogen mix was added to the inspired gas at the point closest to the animal in the ventilation system that would ensure even mixing. The dwell time for the mixture in the ventilation system was short (10 s), so it would seem unlikely that there was significant oxidation of the nitric oxide.

In summary, we have presented data showing that inhaled nitric oxide is a potent and selective pulmonary vasodilator, with a similar dose–response relationship for pulmonary hypertension caused by hypoxia or endotoxaemia. The maximum effect occurs between 64 and 128 p.p.m.; no benefit was found with greater doses. A previously unreported dose-dependent decrease in alveolar–arterial oxygen partial pressure difference was found during nitric oxide inhalation in endotoxaemia. Provided inhaled nitric oxide can be shown to be free of serious toxicity, it may prove useful in the treatment of pulmonary hypertension complicating ARDS.

REFERENCES


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