Effects of inhaled nitric oxide on methacholine-induced bronchoconstriction: a concentration response study in rabbits

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ABSTRACT: Inhaled nitric oxide (NO), at a concentration of 80 ppm, counters the increase in respiratory resistance (Rrs) induced by methacholine, but fails to prevent a reduction in lung compliance (Crs) in a rabbit model. This study reports the effects of 3, 30 and 300 ppm of inhaled NO.

New Zealand White rabbits were intubated and mechanically ventilated with 30% oxygen during neurolept anaesthesia. Methacholine (3 mg·ml^{-1}) was nebulized, with or without NO inhalation.

Inhalation of 3 and 30 ppm NO had no effect on the induced bronchoconstriction, whereas 300 ppm fully blocked the increase in Rrs. The decrease in Crs due to methacholine was not countered by 3, 30 or 300 ppm NO. On the contrary, inhalation of 300 ppm NO in itself decreased Crs from 5.0±0.1 to 4.3±0.1 ml·cmH$_2$O^{-1}. Also, mean arterial pressure (60±7 to 54±5 mmHg), alveolar-arterial oxygen tension gradient (0.8±0.8 to 2.3±1.8 kPa) and methaemoglobin (0.5±0.2 to 1.5±0.5%) changed significantly on inhalation of NO 300 ppm prior to methacholine challenge.

We conclude that 3 and 30 ppm NO inhalation does not alter methacholine-induced bronchoconstriction. Inhalation of 300 ppm NO blocks an increase in resistance but fails to counter the reduction in compliance due to methacholine. This suggests that the bronchodilating effects of NO in rabbits *in vitro* are confined to the large airways.
Eur Respir J., 1994, 7, 698–702.

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The administration of inhaled nitric oxide (NO) as a selective pulmonary vasodilator [1, 2] is currently being investigated in several forms of severe respiratory failure [3–5]. It has also been shown that inhaled NO can act as a bronchodilator in guinea-pigs [6], and rabbits [7]. Inhaled NO, 80 ppm, has a bronchodilatory effect on patients with bronchial asthma, but compared to an inhaled β$_2$-agonist the effect is modest [8]. DUPUY *et al.* [6] showed that low concentrations (5 ppm) of NO could reduce respiratory resistance and that high concentrations (100 ppm) improved compliance, when an intravenous infusion of methacholine (MCh) was given to guinea-pigs. Inhalation of 80 ppm NO prevented the increase in respiratory resistance (Rrs) induced by methacholine nebulization in a rabbit model, but failed to improve lung compliance (Crs) [7]. These differences in response to MCh may be explained by a difference in species used and administration of MCh, and prompted this concentration-response study in the rabbit.

We investigated the effects of a low NO concentration (3 ppm), a concentration similar to that presently advocated for the treatment of pulmonary hypertension (30 ppm), and a high NO concentration (300 ppm), in our rabbit model, in which bronchoconstriction was induced by MCh nebulization.

Materials and methods

Animal preparation

We studied 18 New Zealand White rabbits of both sexes, with a body weight of 3.2–3.6 kg. They were vaccinated against *Pasteurella* and *Bordetella* and maintained on *ad libitum* water and 75 g of high protein pellets a day. Premedication of 0.5 ml Hypnorm (Janssen, Belgium) [9] was given *i.m.* The marginal ear vein was used for *i.v.* injections, whilst the ear artery was used for blood sampling and pressure monitoring. Before intubation, 1 ml Hypnorm *i.m.* and 5 mg diazepam *i.v.* were given. Intubation was performed with a cuffed tube 3.0 (Sheridan, USA). The rabbit was placed in the prone position on a heating pad to maintain normal body temperature. Artificial ventilation was given with a Siemens 900C ventilator (Siemens-Elema, Sweden), with an inspired

oxygen fraction (F_{IO_2}) of 0.3, an inspiratory to expiratory ratio of 1:2, tidal volume (V_T) 38 ml and a ventilatory frequency of about 30 breaths·min⁻¹. The ventilatory frequency was adjusted to keep the end-tidal carbon dioxide tension (P_{ETCO_2}) (Eliza duo, Gambro-Engström AB, Sweden) around 5 kPa, verified with blood gas analysis for determination of arterial CO_2 and O_2 tensions (Pa_{CO_2} and Pa_{O_2}) (ABL, 300, Radiometer, Denmark). The alveolar-arterial oxygen tension gradient (P_{A-aO_2}) was calculated, using the alveolar oxygen tension derived from the alveolar gas equation [10].

Mean arterial pressure (MAP) was continuously monitored (Tram 7010, Marquette Electronics Inc., WI, USA). The anaesthesia was maintained with *i.v.* infusion of Hypnorm 0.1–0.3 mg·kg⁻¹·h and 2.5 mg diazepam *i.v.* as necessary. A muscle relaxant, 0.2 mg pancuronium bromide, was given *i.v.* At the end of the experiment, the muscle paralysis was antagonized with 0.15 mg neostigmine and 0.03 mg glycopyrronium. In addition, naloxone was given *i.v.* as needed to counteract hypoventilation due to residual Hypnorm effects.

Experimental procedure

The animals were divided into three groups of six rabbits each. The three groups inhaled 3, 30 or 300 ppm NO, respectively. The rabbits served as their own controls [11] and were, therefore, anaesthetized twice. Two weeks were allowed between the first and the second MCh provocation, with and without NO inhalation, in a randomized fashion. After a 30 min stabilization period, a double V_T was delivered to reopen any collapsed lung tissue. An inflation pressure (Pmax) limit was set at 25 cmH_2O.

NO was inhaled for 10 min prior to, as well as during, the nebulization of MCh with a concentration of 3 mg·ml⁻¹. Methacholine chloride was dissolved in distilled water with pH corrected to 7.01±0.12 (mean±95% confidence interval). Respiratory mechanics were measured and blood gases were obtained at baseline, at NO inhalation and within 2 min after MCh challenge. Respiratory mechanics were also measured 30 min after MCh challenge.

Determination of respiratory mechanics

Measurements of Crs and Rrs were obtained using the technique of rapid airway occlusion during constant-flow inflation [12, 13]. Rrs is the difference between Pmax and the pressure 2 s after the end-inspiratory pause, divided by the flow [14], with the endotracheal tube resistance of 28 $cmH_2O·l^{-1}·s$ subtracted. Crs was calculated as V_T divided by the end-inspiratory pressure minus the end-expiratory pressure. Pressure and flow were measured in the ventilator on the inspiratory side, and fed into a computer for on-line signal processing (MacII Fx computer with LabView 2 software, USA). For gas compression in the tubing, corrections were made for volume and flow values. A mean value of two "inspiratory

hold" manoeuvres was used for each point. The sampling of data was performed within 30 s of completion of the MCh challenge.

Statistical analysis

Statistical analysis was performed using Student's two-tailed test for paired data. The responses to MCh, with or without NO inhalation, were tested with analysis of variance (ANOVA) (SigmaStat, Jandel Scientific, Erkrath, Germany). Results are given as mean values ±95% confidence interval in the text and ±standard error of mean (SEM) in the figures. A value of $p < 0.05$ was considered statistically significant.

Results

Body weight and temperature, as well as pre-inhalation values of MAP, Pa_{O_2}, P_{ETCO_2}, Pmax, Rrs and Crs showed no change between the first and the second anaesthesia two weeks later (data not shown).

Effect of NO inhalation

Inhalation of NO 3 and 30 ppm did not significantly alter baseline data, whilst NO at a concentration of 300 ppm significantly altered MAP, P_{A-aO_2}, Crs and methaemoglobin (metHb) (table 1).

Table 1. – Effect of NO inhalation, 300 ppm

	Control	NO 300 ppm
Pmax cmH_2O	12.0±0.8	12.5±0.8
MAP mmHg	60±7	54±5*
HR bpm	234±11	235±10
P_{A-aO_2} kPa	0.8±0.8	2.3±1.8*
P_{ETCO_2} kPa	5.4±0.2	5.4±0.3
Rrs $cmH_2O·l^{-1}·s$	56±4	56±13
Crs $ml·cmH_2O^{-1}$	5.0±0.1	4.3±0.1*
metHb %	0.5±0.2	1.5±0.5*

Data are presented as mean value±95% confidence interval. Pmax: maximum insufflation pressure; MAP: mean arterial pressure; HR: heart rate; P_{A-aO_2}: alveolar-arterial oxygen tension gradient; P_{ETCO_2}: end-tidal carbon dioxide tension; Rrs: respiratory resistance; Crs: lung compliance; metHb: methaemoglobin. *: $p < 0.05$.

Effect of NO inhalation on methacholine-induced bronchoconstriction

Inhalation of 3 ppm NO. MCh nebulization resulted in a rapid increase in Rrs in the control situation, from a baseline of 47±8 to 77±20 $cmH_2O·l^{-1}·s$ ($p < 0.01$). The Rrs tended to increase from 55±8 to 86±41 $cmH_2O·l^{-1}·s$ (NS) during NO inhalation when MCh was nebulized (fig. 1). Crs fell due to MCh nebulization in the control situation, from a baseline of 4.6±0.5 to 3.4±0.9 $ml·cmH_2O^{-1}$ ($p < 0.01$). With NO inhalation, Crs decreased from 4.3±0.3
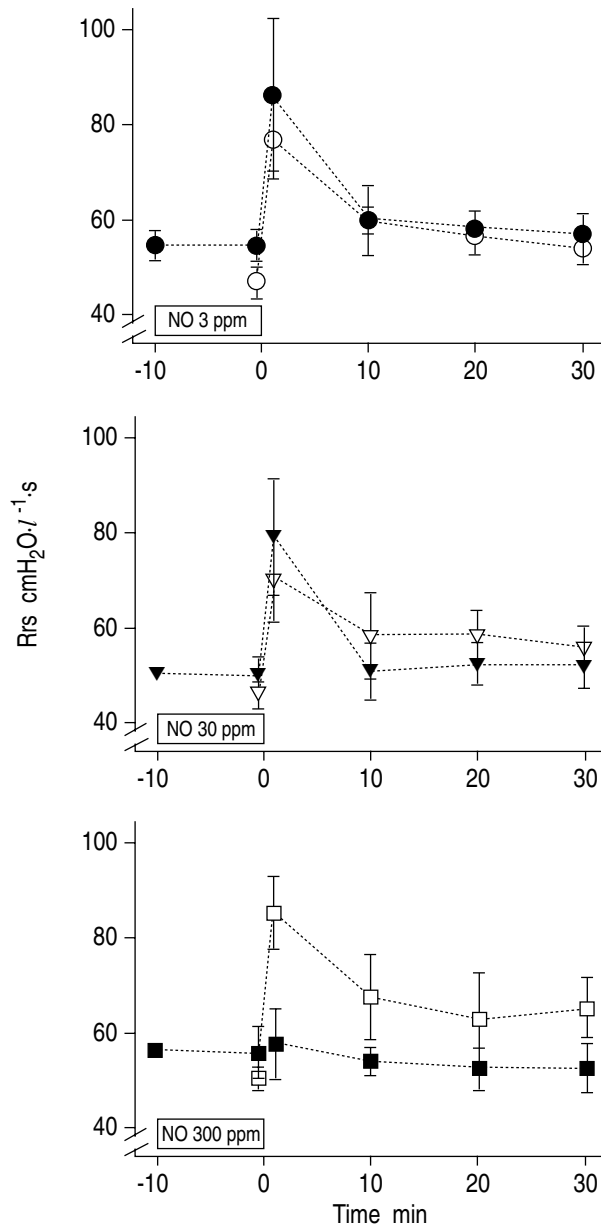
Fig. 1. – Change in respiratory resistance (Rrs) to methacholine (MCh) with or without nitric oxide (NO) inhalation. Rrs is shown prior to MCh nebulization, within one minute after the nebulization and during a follow-up period of 30 min. Hollow symbols: control (without NO); filled symbols: NO was inhaled 10 min before as well as during MCh nebulization. Inhalation of 3 ppm (●) and 30 ppm NO (▼) did not block the increase in Rrs due to MCh. An absence of response to MCh was seen when 300 ppm NO (■) was inhaled. $p<0.01$, analysis of variance (ANOVA).
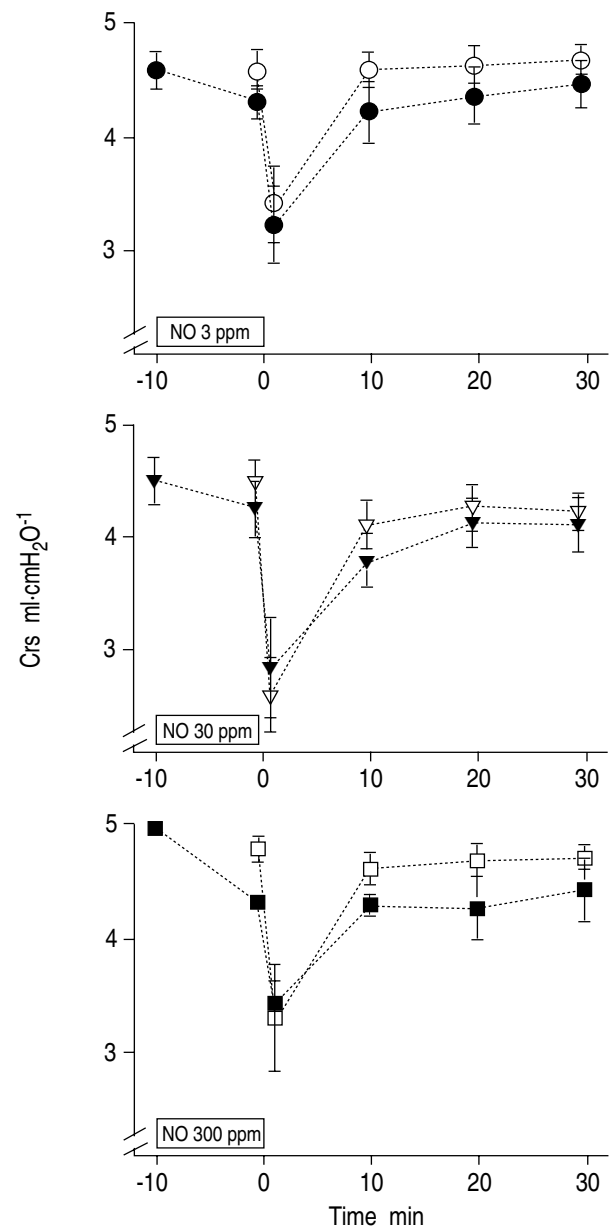
Fig. 2. – Change in lung compliance (Crs) to methacholine (MCh) with and without nitric oxide (NO). Crs is shown prior to MCh nebulization, within one minute after the nebulization and during a follow-up period of 30 min. Hollow symbols: control (without NO); filled symbols: NO was inhaled 10 min before as well as during MCh nebulization. Inhalation of 3 ppm (●) and 30 ppm NO (▼) did not block the decrease in Crs due to MCh. Inhalations of 300 ppm (NO) (■) decreased Crs ($p<0.05$, analysis of variance (ANOVA) but the response to MCh was not altered from the control situations.

to 3.2 ± 0.9 ml·cmH$_2$O^{-1} ($p<0.05$) (fig. 2). The P$_A$-ao$_2$ increased from 1.5 ± 0.4 to 4.9 ± 2.6 kPa ($p<0.05$) in the control situation when MCh was nebulized. During NO inhalation the P$_A$-ao$_2$ changed from 1.7 ± 1.7 to 6.1 ± 5.9 kPa (NS). In conclusion, there was no significant difference in response to MCh, whether NO had been inhaled or not.

Inhalation of 30 ppm NO. In the control situation, when MCh was nebulized Rrs increased from 46 ± 8 to 70 ± 21 cmH$_2$O·l^{-1}·s ($p<0.05$). With NO inhalation, Rrs increased

from 50 ± 8 to 79 ± 31 cmH$_2$O·l^{-1}·s ($p<0.05$) (fig. 1). Crs decreased due to MCh in the control situation to 2.6 ± 0.8 ml·cmH$_2$O^{-1}, from a baseline value of 4.5 ± 0.5 ml·cmH$_2$O^{-1} ($p<0.001$). With NO inhalation, Crs decreased from 4.2 ± 0.6 to 2.8 ± 1.1 ml·cmH$_2$O^{-1} ($p<0.05$) (fig. 2). P$_A$-ao$_2$ increased from 0.9 ± 0.5 to 5.7 ± 3.9 kPa ($p<0.05$) in the control situation when MCh was nebulized. During NO inhalation, P$_A$-ao$_2$ changed from 1.1 ± 0.4 to 6.4 ± 4.5 kPa ($p<0.05$). There was, thus, no significant difference in response to MCh, whether NO had been inhaled or not.

Inhalation of 300 ppm NO. The Rrs increased from 50±5 to 85±20 $cmH_2O \cdot l^{-1} \cdot s$ (p<0.01) in the control situation. With NO inhalation, the Rrs did not change. The baseline value was 45±13 $cmH_2O \cdot l^{-1} \cdot s$, and with NO inhalation during MCh nebulization 46±18 $cmH_2O \cdot l^{-1} \cdot s$, significantly different compared to the response to MCh without NO (p<0.01, ANOVA) (fig. 1). In the control situation, Crs decreased from 4.8±0.3 to 3.3±1.2 $ml \cdot cmH_2O^{-1}$ (p<0.05), and with NO inhalation the Crs decreased from 4.3±0.1 to 3.4±0.4 $ml \cdot cmH_2O^{-1}$ (p<0.01) (fig. 2). $P_A\text{-}a_{O_2}$ in the control situation was 1.3±0.7 and increased with MCh nebulization to 6.2±4.2 kPa (p<0.05). When NO was added, the $P_A\text{-}a_{O_2}$ increased from 2.25±1.8 to 4.6±2.9 kPa (p<0.01) due to MCh nebulization.

Discussion

This study in rabbits shows that low nitric oxide concentrations, *i.e.* 3 and 30 ppm, do not alter the increase in resistance or the reduction in compliance when bronchoconstriction is induced by methacholine nebulization. This study also shows that a high NO concentration, *i.e.* 300 ppm, completely blocks the increase in resistance, but fails to counter the reduction in compliance due to MCh nebulization. Interestingly, we found that 300 ppm NO inhalation in itself decreases Crs and mean arterial blood pressure, and increases $P_A\text{-}a_{O_2}$ and methaemoglobin concentration.

We found previously that 80 ppm NO blocked the increase in resistance to a subsequent MCh nebulization [7]. Our findings in the previous as well as the present study are at variance with those of DUPUY *et al*. [6], who found that even at 5 ppm NO significantly reduced the increase in resistance caused by intravenous MCh in the guinea-pig. Moreover, DUPUY *et al*. [6] showed a significant improvement in compliance with 100 and 300 ppm NO during MCh infusion, whereas we saw no protective effect on compliance with 300 ppm NO during MCh nebulization. These differences may be due to open *versus* closed chest preparations, the difference in species studied and/or systemic *versus* local administration of MCh. We interpret the different effects of NO on Rrs and Crs to dilation of mainly large airways, with less or only minor effects on small airways. It is worthy of note that isolated canine tracheal muscle strips tended to relax more than bronchial strips when NO was added to the tissue bath [15].

It also seems that higher inspired concentrations of NO are required to dilate airways in our rabbit model [7] (and present study) and in subjects with hyperreactive airways and asthmatics [16, 8], than are required to dilate pulmonary vessels [1–3]. This may be due to lower sensitivity to NO in the bronchial wall, or to a poor penetration from the airway lumen to the bronchial smooth muscle. A difference in the sensitivity to NO has been demonstrated in *in vitro* experiments [15]. Canine airway smooth muscle was 10 times less sensitive compared to vascular smooth muscle. It has also been shown that inhaled NO dilates bronchial vessels and increases bronchial blood flow [17], an effect that has been shown for MCh also [18]. A hyperaemic airway mucosa may interfere with the diffusion of inhaled NO to the airway smooth muscle, and there may be inactivation of NO by the haemoglobin. The pathogenesis of asthma has been shown to be closely linked to the presence of airway inflammation [19] with a hyperaemic and swollen airway lining.

In asthmatic patients, 80 ppm NO inhalation did not have a consistent bronchodilator effect [8], perhaps due to low receptor sensitivity or poor penetration. This has led to advocation for giving higher NO concentrations. However, a number of negative effects must then be considered, including NO_2 formation, and the decrease in Crs and increase in $P_A\text{-}a_{O_2}$ found in our present study.

Increased NO concentrations will facilitate the formation of NO_2, a bronchial irritant. NO_2 concentrations as low as 0.5 ppm increase airway responsiveness [20] and higher concentrations may cause pulmonary oedema [21, 22]. Thus, inhaled NO_2 levels must be kept very low and be closely monitored. The decrease in Crs may possibly be related to vascular dilation and airway oedema, with subsequent small airway narrowing and closure. The unexpected increase in $P_A\text{-}a_{O_2}$ may be due to diffusion of NO into the lung tissue and blood, reaching the dilating pulmonary vessels in poorly ventilated areas. More research is clearly needed before inhaled NO of higher concentrations can be advocated in the treatment of asthma.

In summary, high NO concentrations, *i.e.* ≥80 ppm, are needed to produce any effect on the increase in resistance when bronchoconstriction is induced by methacholine nebulization. However, lung compliance still remains low. This indicates that the bronchodilatory effects of NO are mainly confined to large airways, with less or only minor effects on small airways. The observation that 300 ppm NO in itself changes lung compliance, alveolar-arterial oxygen tension gradient and mean arterial pressure needs further investigation.

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