

Selective protective effects of nitric oxide inhalation on allergen-induced acute airway reactions in the pig

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Summary

Background Nitric oxide (NO) is thought to be an important mediator of inflammatory processes during allergic reactions in the respiratory tract.

Objective This study was undertaken to investigate the effects of inhalation of NO on the allergen-induced acute airway reactions in the pig.

Methods Specific pathogen-free pigs were sensitized with *Ascaris suum* antigen and challenged with an allergen aerosol during mechanical ventilation and anaesthesia. One group ($n = 8$) was treated with inhaled NO (20 ppm) which was given from 30 min before allergen challenge until the experiments were completed at 120 min after challenge. A control group ($n = 8$) did not receive NO (< 0.001 ppm).

Results Inhalation of 20 ppm NO prevented the fall in arterial pO_2/FiO_2 levels that was observed in the control group (areas under the curve between 0 and 120 min were 3.7 ± 1.4 kPa/min in NO-treated pigs vs. 15.9 ± 3.4 in controls, $P < 0.01$, Mann–Whitney U -test) and it decreased baseline pulmonary arterial pressure (change from time-point $-30-0$ was $3.1 \pm 5.3\%$ in the control and $-19.9 \pm 3.5\%$ in the NO group, $P < 0.01$), which in turn resulted in a lower pulmonary arterial pressure during allergen challenge. NO also caused vasodilatation in the bronchial circulation, resulting in increased bronchial vascular conductance throughout the experiment. NO inhalation caused a small, but non-significant, reduction in the allergen-induced bronchoconstrictor response, whereas histamine release, as detected in urine, was not changed. Total protein levels in bronchoalveolar lavage (BAL) fluid were significantly decreased in the NO group at 120 min after challenge compared with 45 min (373 ± 101 μ g/mL vs. 631 ± 184 , respectively, $P < 0.05$, Wilcoxon matched pairs test), whereas levels in the control group did not change between these two time-points (513 ± 282 vs. 599 ± 354 , not significant).

Conclusion These findings indicate that NO inhalation improves ventilation/perfusion matching and causes some bronchodilatation during the allergen-induced acute airway reaction, whereas histamine release is not affected. Moreover, NO inhalation enhanced the clearance of extravasated protein in the airways, possibly through increased bronchial blood flow. Even though some protective effects were seen, this study does not support a therapeutic role for exogenous NO in acute allergic reactions.

Keywords: swine, acute allergic reaction, bronchial circulation, nitric oxide, *ascaris suum*, histamine, mast cell, neutrophil, arterial oxygenation

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Introduction

Nitric oxide (NO) is thought to be an important mediator of inflammatory processes in the airways. It is produced

endogenously in several cell types by a family of NO synthases (NOS) [1]. NO seems to have a dual role: when produced in large amounts it is thought to be deleterious, whereas production in low amounts seems to have a protective effect on bronchial hyper-responsiveness [2]. The harmful effects of NO are thought to be related to the simultaneous production of reactive oxygen species [1]. Evidence for a protective effect of NO during the acute allergic reaction comes from several animal studies. Guinea-pig studies have indicated that inhibition of endogenous NO production increases airway responsiveness to allergen and histamine challenges and it was suggested that this was due to direct bronchodilatory actions of endogenous NO [3,4].

A recent investigation, published by our group, showed that in pigs that were sensitized to *Ascaris suum* allergen, inhibition of endogenous NO increased the acute allergic response to challenge with allergen, but not with histamine. Moreover, histamine levels in bronchoalveolar lavage (BAL) fluid were significantly elevated after inhibition of endogenous NO production [5]. Based on these findings, it was proposed that a possible mechanism for the protective effect of NO could be an inhibitory effect on the mast cell. This study was therefore designed to clarify this possible direct inhibiting effect of NO on mast cell activation, by studying the opposite of our previously published study [5], namely inhalation of NO, rather than inhibition of endogenous NO production, during the acute allergic reaction. To our knowledge, inhalation of NO has not been previously studied in allergen-induced acute airway reactions.

Inhalation of NO is currently used in the clinic to treat pulmonary hypertension [6,7] and its positive effects are mainly due to pulmonary vasodilation and improved ventilation/perfusion matching [6]. In addition to being a vasodilator, NO has also been shown to have bronchodilating actions. Inhaled NO had positive effects during methacholine-induced bronchoconstriction in guinea-pigs [8] and rabbits [9]. In addition, it was found that inhalation of 80 ppm NO had a weak bronchodilatory effect in bronchial asthma patients [10]. Despite its bronchodilating actions, inhaled NO is not routinely used as a therapy for asthma [11]. In this study we made use of a previously described pig model [5,12] to study the effects of inhalation of NO during the acute allergic reaction.

Materials and methods

The experiments carried out in this study were approved by the local ethics committee for animal research and were performed according to regulations issued by the Swedish National Board for Laboratory Animals.

Animals and sensitization

A total of 16 specific pathogen-free pigs of either sex (Seropig; Vallrums Lantbruks AB, Ransta, Sweden) was used in this study, with a mean body weight of 18.7 ± 0.7 kg. Male and female pigs have previously been shown not to react differently in allergen-induced acute and late-phase airway reactions [5,13,14]. The pigs were sensitized with subcutaneous injections of *Ascaris suum* antigen (for more details see [12]) in a suspension of $\text{Al}(\text{OH})_3$ at the ages of 3 and 8 weeks. Before the start of the experiments the pigs were classified according to their responsiveness to 50 μL of injected allergen into the skin. Pigs showing a classification of 2 (meaning that a dilution of 10^{-2} of the antigen gave a clear response in the skin) or more (response to a dilution of 10^{-3} or higher) were included in this study. They were then divided into a control group ($n = 8$) and a group that was treated with 20 ppm of inhaled NO ($n = 8$). These groups did not differ in their responsiveness to the skin tests. Median skin responsiveness was class 2 in both groups.

Anaesthesia and surgical preparation

One to two weeks after the second antigen injection the pigs were pre-medicated with ketamine hydrochloride (20 mg/kg i.m., Parke-Davis, Barcelona, Spain) and atropine (0.5 mg, NM Pharma, Stockholm, Sweden), and then anaesthetized with sodium pentobarbitone (15 mg/kg i.v., Apoteksbolaget, Umeå, Sweden) through an ear vein. Anaesthetic level was evaluated by applying pain stimuli to the digital skin with a forceps. After tracheotomy the pigs were mechanically ventilated with a Servo 900 ventilator (Siemens-Elema, Solna, Sweden). The pigs received a mixture of air and oxygen and inspired oxygen fraction (FiO_2) ranged from 23% to 32%, in order to get good arterial pO_2 values in each pig at baseline. Pancuronium bromide (0.2 mg/kg i.v., Pavulon; Organon, Boxtel, the Netherlands) was administered for muscle relaxation.

During the surgical preparation (which lasted approximately 90 min) the animals received a continuous infusion of pentobarbitone (10 mg/kg/h i.v.) and pancuronium bromide (0.5 mg/kg/h i.v.) through a cannula in a femoral vein. Femoral arteries were cannulated for blood sampling and measurements of mean arterial pressure (MAP) and heart rate (HR). The other femoral vein was cannulated and an Edwards Swan-Ganz balloon-tipped paediatric thermodilution catheter (Baxter, Irvine, CA, USA) was inserted into the pulmonary artery for continuous registration of pulmonary arterial pressure (PAP). The right ureter was cannulated for urine sampling. After a right side thoracotomy an ultrasonic flow probe (Transonic System,

Ithaca, NY, USA) was placed around the bronchial artery for measurement of absolute bronchial blood flow (BA).

Respiratory pressure was measured through a connection of the tracheal tube to a pressure transducer (Kent Scientific, Litchfield, CT, USA). A heated paediatric pneumotachometer (3500 series, Hans Rudolph, Kansas City, MO, USA) was used to measure air flow. Respiratory pressure and air flow signals were fed into a pulmonary computer (AP 200, ConMeTech AB, Uppsala, Sweden) for calculations of airway resistance (R_{aw}) and dynamic lung compliance (C_{dyn}). R_{aw} was calculated with the following formula: (peak pressure–pause pressure)/end inspiratory flow. The resistance of the tracheal tube was not subtracted for the calculation of R_{aw} . C_{dyn} calculations were made as follows: expiratory tidal volume/(pause pressure–end expiratory pressure). All measurements were recorded on a Grass polygraph (Grass Instrument, Quincy, MA, USA).

Ringer solution, containing 0.5% glucose, was given throughout the experiment at a rate of 20 mL/kg/h. Towards the end of the surgical preparation, the anaesthesia was changed to a combination of fentanyl (induction dose of 3 µg/kg during 5 min and thereafter 12 µg/kg/h, i.v.) and midazolam (induction dose of 0.04 mg/kg during 5 min and thereafter 0.16 mg/kg/h, i.v.), and pancuronium infusion was continued at the same rate as before. MAP and HR were continuously recorded and assessed for anaesthetic level. Heparin to a total of 1000 i.u. was given through the cannulas before the start of the experimental procedure. Arterial blood gas partial pressures and pH were regularly monitored using an automatic blood gas analyser (ILS 1610, Instrumentation Laboratory, Lexington, MA, USA) and were set to physiological levels at baseline. The settings were not changed thereafter. Body temperature was measured and maintained at 38–39 °C. After the experiments were completed the pigs were sacrificed with an overdose of sodium pentobarbitone.

NO delivery system

NO was delivered to the pigs via the inhalation tubing connected between the ventilator and the tracheal tube. The concentration in the delivering gas cylinder was 201 ppm NO in N₂ (AGA AB, Stockholm, Sweden). It was delivered to the inhaled air of the pig by means of an electrically regulated valve (Bürkert, Ingelfingen, Germany) that opened with each inhalation upon a signal from the ventilator. Flow was adjusted so that the final peak concentration of NO delivered to the pig was 20 ppm. The final peak concentrations of NO in the inhaled air were measured on-line via an outlet just before the tracheal tube with a fast-response chemiluminescence analyser (77 AM; EcoPhysics, Dürnten, Switzerland). After connecting the NO delivery system, total ventilatory minute volume was

adjusted to the same level as before. FiO_2 fell by 4.2% in the NO group after start of inhalation with NO. FiO_2 was not adjusted, so as to give the same baseline pO_2 in both groups at $t = 0$ h. Inhaled NO₂ peak levels were measured (700 AL; EcoPhysics) and were found to be < 0.1 ppm at inhalation of 20 ppm NO. Control animals received medical breathing air (AGA AB, concentration of NO < 1 ppb) to the same volume and flow as the NO-treated animals.

Experimental protocol and sample collection

Inhalation of 20 ppm NO or medical breathing air was started 30 min before challenge with *Ascaris* allergen and was continued until the end of the experiments. The concentration of 20 ppm NO was chosen because it had been shown to have protective effects in a pig model of acute lung injury (unpublished observations). *Ascaris* extract was diluted in a standardized way in saline to a volume of 2 mL and this solution was put into an ultrasonic nebulizer (NB 108, Engström Medical, Stockholm, Sweden). The nebulizer produced an aerosol which was delivered to the pig between the ventilator and the endotracheal tube. A maximum of 2 mL of aerosolized diluted antigen was given, but if an increase of 3 cmH₂O in peak tracheal pressure was observed, the aerosol was stopped before all antigen was given. Therefore, the period during which antigen was delivered varied between 5 and 10 min. The amount of antigen given was 1.49 ± 0.15 mL in the control group and 1.66 ± 0.13 mL in the NO group (non-significant, Mann–Whitney *U*-test). Bronchoalveolar lavages (BAL) were performed 45 and 120 min after *Ascaris* challenge by inserting a fibre-optic bronchoscope for paediatric use (Olympus, Tokyo, Japan) into the right middle lobe of the lungs. A volume of 20 mL saline (37 °C) was used at both time-points. MAP, HR, PAP, C_{dyn} , R_{aw} and BA were continuously recorded on a Grass Polygraph (Grass Instrument, Quincy, MA, USA). Blood and urine samples were taken at regular time intervals. The experiments were finished at 120 min after *Ascaris* challenge.

After collection, the urine and BAL samples were kept on ice until they were centrifuged for 20 min at 170 g at +4 °C. The supernatants were aliquoted and stored at –80 °C until further analysis.

Sample analyses

Differential counts of BAL cytopins were performed after fixation with 100% methanol for 5 min and staining with May–Grünwald (Sigma, St Louis, MO, USA) for 5 min and Giemsa (Merck) for 15 min. Three hundred cells per cytopsin slide were counted.

Total protein content in the BAL fluids was measured with the Pierce BCA protein assay (Pierce Chemical Company, Rockford, IL, USA), which uses enhanced colourimetric detection of Cu^+ produced in the reaction of protein with alkaline Cu^{2+} . Urine creatinine concentrations were determined with a colourimetric assay (Sigma). BAL and urine histamine levels were measured with a radioimmunoassay kit (Immunotech, Marseille, France). Histamine levels in the urine are expressed per mol creatinine.

Calculations and statistics

Data are presented as mean $\pm$ SEM. Bronchial vascular tone is presented as bronchial vascular conductance (BVC), calculated from blood flow divided by MAP. Statistical analyses were performed with the Mann–Whitney *U*-test to detect significant differences between the treatments at certain time-points. Area under the curve (AUC) was calculated from 0 to 45 min to avoid disturbances from the BAL at 45 min (except for arterial blood gas values where 0–120 min was

used) and the values at time-point 0 min were taken as baseline. Data were considered significant at $P < 0.05$. The Wilcoxon matched pairs test was performed to detect differences between two time-points within one group. Friedman and Dunn's multiple comparison tests were used to detect differences over time. Analyses were performed with a commercial software package (Prism; Graphpad, San Diego, CA, USA) for the Macintosh computer.

Results

Pulmonary and cardiovascular effects

R_{aw} values at 0 min were 6.23 ± 1.57 cmH₂O/L/s in the control group and 6.68 ± 0.70 cmH₂O/L/s in the NO group (non-significant, Mann–Whitney *U*-test). Challenge with allergen resulted in rapid increases of R_{aw} in both the control and the NO group (Fig. 1a). Inhalation of NO did result in a reduced increase of R_{aw} , but this was not significant (see also Table 1 for the AUC values). C_{dyn}

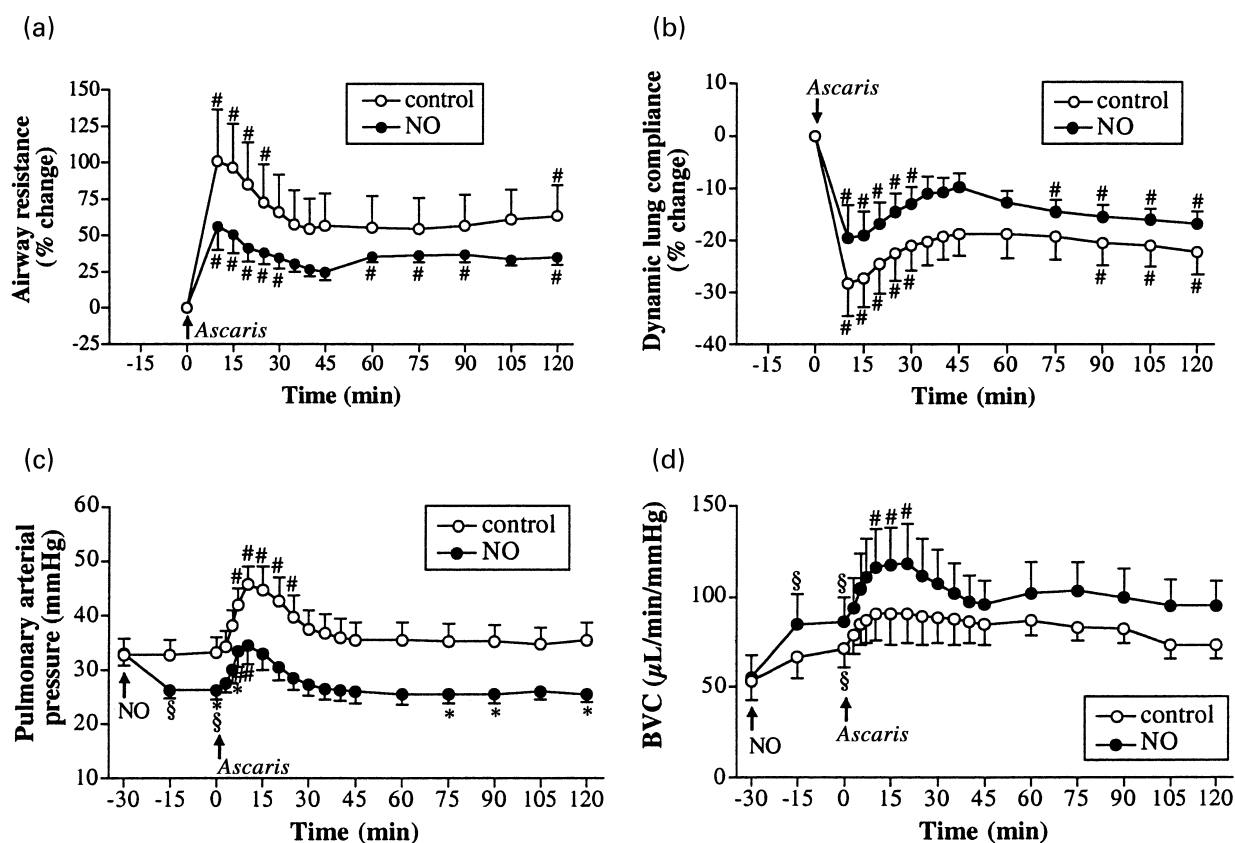


Fig. 1. Effects of NO inhalation on allergen-induced changes in: (a) airway resistance (R_{aw}); (b) dynamic lung compliance (C_{dyn}); (c) pulmonary arterial pressure (PAP); and (d) bronchial vascular conductance (BVC). Allergen challenge was given as an aerosol during 5–10 min, starting at $t = 0$ min. Inhalation of 20 ppm NO in the NO group started at $t = -30$ min and was continued until $t = 120$ min. Data are expressed as mean $\pm$ SEM. * $P < 0.05$ vs. the control group; # $P < 0.05$ vs. $t = 0$ min; § $P < 0.05$ vs. $t = -30$ min.

Table 1. Area under curve values

	Time period (min)	Control	NO
R _{aw} (% change/min)	0–45	3117 ± 1058	1585 ± 374
C _{dyn} (% change/min)	0–45	932 ± 203	672 ± 154
PAP (mmHg/min)	0–45	351 ± 77	167 ± 31
BVC (% change/min)	0–45	738 ± 274	1151 ± 343
pO ₂ /FiO ₂ (kPa/min)	0–120	15.9 ± 3.4	3.7 ± 1.4**
pCO ₂ (kPa/min)	0–120	668 ± 38	637 ± 26
pH (per min)	0–120	5.1 ± 1.4	1.8 ± 1.0*
metHb (% of total Hb/min)	0–120	191 ± 23	286 ± 23*

Allergen challenge was given as an aerosol during 5–10 min, starting at $t = 0$ min. Inhalation of 20 ppm NO in the NO group started at $t = -30$ min and was continued until $t = 120$ min. AUC was calculated from 0 to 45 min or from 0 to 120 min, as indicated in the table, and the values at $t = 0$ min were taken as baseline. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$ vs. the control group.

starting values at $t = 0$ min were 18.01 ± 1.53 mL/cmH₂O in the control group and 18.25 ± 1.17 mL/cmH₂O in the NO group. C_{dyn} decreased upon challenge with allergen and this decrease was not significantly affected by inhalation of NO (Fig. 1b and Table 1).

MAP did not change significantly during these experiments, nor were differences between groups observed (data not shown). PAP decreased significantly after NO inhalation was started and remained lower than in the control group until the experiments were completed (Fig. 1c). In both groups allergen challenge resulted in an increase in PAP which lasted approximately 45 min. Apart from 5 min after the start of challenge, the values did not differ between the two groups during the 45 min peak. AUC calculations did not show significant differences (Table 1). BVC increased significantly as a result of NO inhalation, both at $t = -15$ and 0 min, but also in the control group the BVC values were significantly increased at $t = 0$ compared with -30 min (Fig. 1d). After allergen challenge BVC increased significantly in the NO group, but BVC values did not differ significantly between groups at any timepoint during the experiment, nor did AUC calculations show any differences (Table 1).

Effects on arterial blood gas values

Baseline ($t = 0$ min) pO₂ values were 14.8 ± 1.3 kPa in the control group and 13.9 ± 0.7 kPa in the NO group. Arterial pO₂/FiO₂ values in the control group decreased significantly as a result of allergen challenge and remained lower than starting values until the end of the experiments (Fig. 2). On the other hand, pO₂/FiO₂ values in the NO group did not change after allergen challenge and were significantly higher than in the control group (see Table 1

for AUC values). Arterial pCO₂ levels did not change significantly over time and no differences between the two groups were observed (Table 2), nor were the calculated AUC values between 0 and 120 min different (Table 1). Arterial pH values in the control group decreased after allergen challenge, but no differences were found with the NO group. On the other hand, AUC values for arterial pH between 0 and 120 min were significantly lower in the NO group compared with the control group (Table 1). Methaemoglobin (metHb) increased as a result of inhalation of NO and remained higher than in the control group until the experiments were completed (Table 2). AUC values for metHb from 0 to 120 min were significantly higher in the NO than in the control group (Table 1).

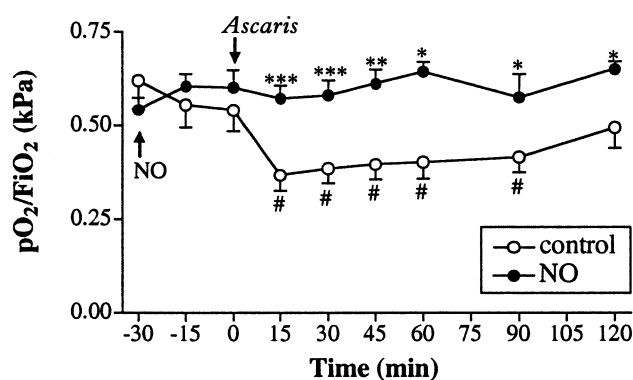


Fig. 2. Effects of NO inhalation on allergen-induced changes in arterial pO₂/FiO₂ values. Allergen challenge was given as an aerosol during 5–10 min, starting at $t = 0$ min. Inhalation of 20 ppm NO in the NO group started at $t = -30$ min and was continued until $t = 120$ min. Data are expressed as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the control group; # $P < 0.05$ vs. $t = 0$ min.

Table 2. Effects of NO inhalation on allergen-induced changes in arterial blood values

	t = – 30 min	t = 0 min	t = 15 min	t = 60 min
pCO ₂ (kPa)				
Control	5.08 ± 0.41	5.12 ± 0.30	5.79 ± 0.31	5.49 ± 0.34
NO	5.40 ± 0.30	5.30 ± 0.22	5.53 ± 0.26	5.33 ± 0.24
pH				
Control	7.42 ± 0.02	7.40 ± 0.02	7.35 ± 0.02#	7.36 ± 0.02
NO	7.39 ± 0.02	7.39 ± 0.01	7.38 ± 0.02	7.39 ± 0.02
MetHb (% of total Hb)				
Control	1.83 ± 0.22	1.80 ± 0.15	1.50 ± 0.23	1.55 ± 0.22
NO	1.79 ± 0.17	2.24 ± 0.18§	2.38 ± 0.20*	2.36 ± 0.18*

Allergen challenge was given as an aerosol during 5–10 min, starting at t = 0 min. Inhalation of 20 ppm NO in the NO group started at t = – 30 min and was continued until t = 120 min. Data are expressed as mean ± SEM. **P* < 0.05 vs. the control group; #*P* < 0.05 vs. t = 0 min; §*P* < 0.05 vs. t = – 30 min.

Findings in BAL fluid and urine

Total protein levels were measured in BAL fluid samples taken at both 45 and 120 min after allergen challenge. In the NO group, levels at 120 min were significantly lower than levels at 45 min. This was not observed in the control group (Fig. 3a). Histamine levels in BAL fluid were found to be below the detection limit of the assay in both groups (data not shown). Neutrophil counts in BAL fluid were found to be elevated in the NO group compared with the control group, and this was significant at 120 min (Fig. 3b). BAL lymphocyte and macrophage numbers were not affected by NO inhalation. Small numbers of eosinophils

were found in one control pig and in one pig treated with NO. No eosinophils were found in BAL fluid of the other pigs at either time-point. BAL recovery at 45 min in the control group was 11.8 ± 1.4 mL, and in the NO group 12.9 ± 0.6 mL. At 120 min BAL recovery was 13.0 ± 1.3 mL in the control group and 13.0 ± 0.5 in the NO group. These values were not significantly different (Mann–Whitney *U*-test).

Histamine concentrations in urine increased after allergen challenge, with a significant increase in the NO group at t = 30 min (Fig. 4). No differences were observed between the two groups, nor between calculated AUC between 0 and 90 min (data not shown).

Discussion

This study was carried out in order to examine the possible protective effects of inhalation of NO during the acute allergic reaction. Inhalation of 20 ppm NO completely prevented the fall in arterial oxygenation that occurred in the control group after airway challenge with allergen. NO was also clearly shown to have protective effects on the increase observed in PAP after allergen challenge. This was mainly caused by the NO-induced decrease in baseline, whereas the allergen-induced reactions were not affected by NO. These effects of NO inhalation are well established and it is on these effects that the use of NO therapy in the clinic is based [6].

Besides effects on pulmonary vascular tone, inhalation of NO has also been shown to have bronchodilatory actions. In a guinea-pig model it was demonstrated that inhaled NO had a bronchodilatory effect on methacholine-induced bronchoconstriction [8] and in rabbits it was shown that NO inhalation prevented the increase in airway resistance

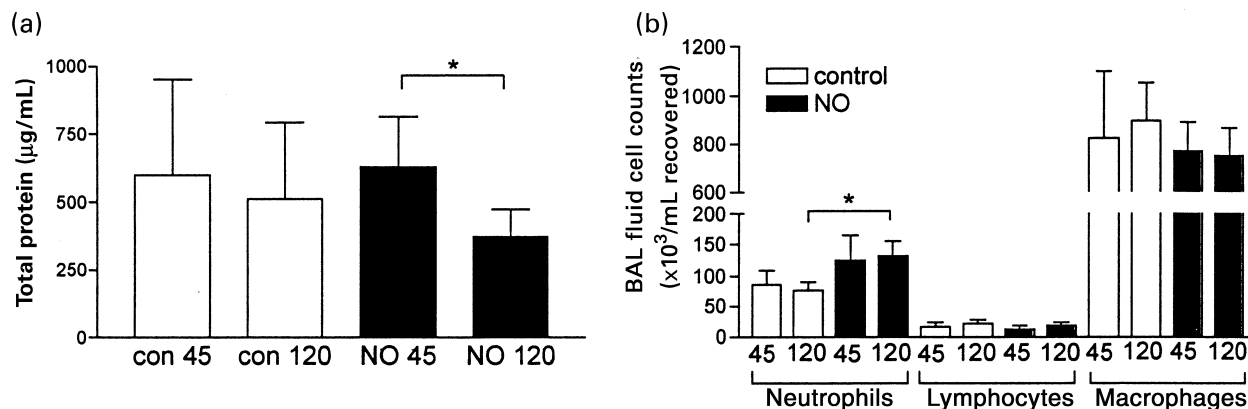


Fig. 3. Effects of NO inhalation on allergen-induced changes in (a) total protein concentrations and (b) leucocyte counts in BAL fluid. Allergen challenge was given as an aerosol during 5–10 min, starting at t = 0 min. Inhalation of 20 ppm NO in the NO group started at t = – 30 min and was continued until t = 120 min. BAL was performed at timepoints 45 and 120 min after challenge. Data are expressed as mean ± SEM. **P* < 0.05.

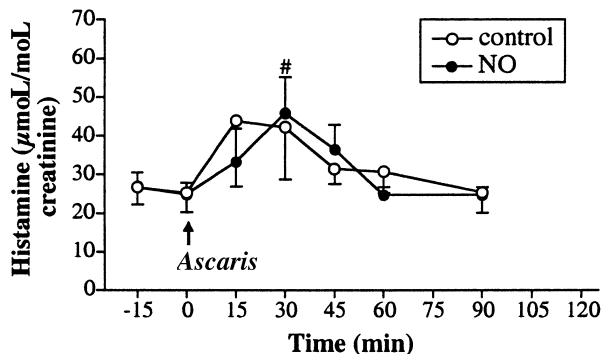


Fig. 4. Effects of NO inhalation on allergen-induced changes in histamine concentrations in urine. Allergen challenge was given as an aerosol during 5–10 min, starting at $t = 0$ min. Inhalation of 20 ppm NO in the NO group started at $t = -30$ min and was continued until $t = 120$ min. Data are expressed as mean $\pm$ SEM. # $P < 0.05$ vs. $t = 0$ min.

in response to nebulized methacholine [9]. This bronchodilatory action is thought to occur via the interaction of NO with guanylate cyclase, that in turn elevates 3,5 guanosine monophosphate (cGMP) levels in bronchial smooth muscle cells [2]. In human bronchial asthma, the bronchodilatory effects of NO inhalation have been found to be weaker [10,15] than in guinea-pig and rabbit studies.

In the current study on pigs, we have not been able to demonstrate a significant bronchodilatory effect of inhaled NO, even though we found indications that there was such an effect, as suggested by the lower R_{aw} and higher C_{dyn} values after NO inhalation. However, a large individual variation was observed, both in the control group and in the NO-treated group, which made it more difficult to observe clear effects. The variation in the control group seemed to be higher than in the NO group, which could be another indication for protective effects of inhalation of NO. A possible explanation for the lack of bronchodilatory effects of inhalation of NO in certain individuals could be different degrees of oedema formation in the airway mucosa in asthmatics [16]. Evidence for the role of the formation of oedema was given in a study on rabbits that demonstrated that the inhibitory effect of inhalation of NO on bronchoconstriction could be blocked by nebulization of hypertonic saline or by hyperventilation with dry gas [17], possibly due to the development of oedema which acted as a diffusion barrier.

Another explanation could be that oedema formation in the airway wall caused by hyperosmolarity, induces release of substances that inactivate or bind the NO molecule, or act on the smooth muscle directly, making the cell resistant to the relaxing stimuli from NO [18]. Evidence that oedema formation in the airway wall indeed took place in the current study comes from the total protein measurements in

BAL fluid, which were higher than in a study which examined the effects of LPS-induced acute lung injury on BAL fluid total protein levels [19]. However, not all of the NO that was inhaled in the current study could have been trapped or inactivated, as the bronchial circulation was still reactive to inhalation of NO also after challenge with allergen and subsequent oedema formation. On the other hand, the increased bronchial blood flow could lead to increased trapping of NO by binding to haemoglobin, thus preventing NO from reaching the bronchial smooth muscle. In addition, it must be mentioned that a higher dose of inhaled NO might have had a more profound bronchodilating effect, but the current dose of 20 ppm was chosen because it had been shown to have clear effects in pig models of acute lung injury, both in our hands (unpublished observations) and in others [20].

The modest effects of inhalation of NO in this study contrast with the distinct effects of inhibition of endogenous NO production by N^G -nitro-L-arginine (L-NNA) in the same model making use of *Ascaris suum*-sensitized pigs [5]. In the current study no evidence could be found for a modulating role of inhaled NO on histamine release by mast cells. On the other hand, inhibition of endogenous synthesis of NO during the acute allergic reaction causes a clear-cut increase of histamine concentrations in BAL fluid [5]. These findings, combined with the data obtained in the current study, indicate that endogenously produced NO is responsible for the possible inhibiting effect on mast cells and that high doses of exogenous NO do not have any additive effect. Evidence for an involvement of NO in the activity of mast cells has been shown in several other studies [21,22]. However, as almost all histamine values measured in BAL fluid were below the detection limit of the assay, it is possible that we missed the peak in histamine release by mast cells by performing the BAL at 45 min after challenge rather than, for example, after 30 min. Moreover, the increase in BVC as a result of NO inhalation caused an increased bronchial blood flow that might possibly have removed the released histamine even faster than in the control group. The opposite effect was noted during inhibition of endogenous NO synthesis, when a decreased BVC may have been one of the explanations for the enhanced histamine levels found in BAL fluid [5].

Total protein analysis in BAL fluid can be seen as a measure of vascular leakage, and inhibition of endogenous NO synthesis has been demonstrated to increase total protein levels 45 min after allergen challenge during the acute airway reaction in the pig [5]. Other studies have presented conflicting results with regard to the role of NO in vascular leakage, with data that suggest a protective role of NO [23] and data that show the opposite [24]. It may be that under basal conditions, when NO is produced in low amounts, endogenous NO suppresses vascular leakage,

whereas NO enhances vascular leakage when it is produced in large amounts during inflammation [25]. In the present study, however, total protein concentrations in BAL fluid of the NO group were significantly lower at 120 min after allergen challenge, compared with at 45 min, whereas total protein levels in the control group remained at an elevated level. This implies that inhalation of NO could be responsible for an increased clearance of excessive fluid and protein formed in the airway mucosa as a result of the acute allergic reaction. A possible explanation for this could be the increased bronchial blood flow induced by NO inhalation, as the bronchial circulation is of importance in reabsorbing fluid and inflammatory cellular fragments [26]. Decreased pulmonary vascular resistance as a result of NO inhalation could also have contributed to the lower total protein in BAL fluid at 120 min, through reduced capillary pressure [27].

Inhalation of NO caused an increase in the number of neutrophils in BAL fluid, at 45 and 120 min after allergen challenge, compared with control animals. This could possibly be explained by the increased formation of NO₂ and reactive oxygen species in the group that received inhaled NO. NO₂, which is formed when NO reacts with O₂, has been shown, at a concentration of > 10 ppm, to be able to induce intrapulmonary accumulation of neutrophils [28]. It has also been shown that four consecutive daily exposures to 2 ppm of NO₂ induced a neutrophilic airway inflammation in the bronchial wash in humans [29]. However, no short-term (1.5 h) effects of 2 ppm NO₂ inhalation were observed on neutrophil numbers in BAL or bronchial wash [30]. When measuring levels of inhaled NO₂ during these experiments we found that these were < 0.1 ppm, which seems to be too low to be able to be responsible for the significant increase in the numbers of neutrophils observed after 120 min.

The role of neutrophils in chronic asthma is uncertain. They have been found to appear during the transition phase, between the immediate bronchoconstrictive reaction and the late phase [31], and 120 min could therefore be a timepoint at which they could be found to be increased in BAL fluid. NO, thus, seems to initiate a change in the time pattern at which the neutrophils appear. In a pig model studying sepsis-induced acute lung injury an opposite finding was reported. In that study it was found that inhalation of 20 ppm NO resulted in a reduction of BAL neutrophils at 5 h after induction of acute lung injury and it was suggested that inhaled NO interferes with alveolar-capillary membrane permeability [20]. This indicates that in these two different types of airway inflammation models inhaled NO seems to have opposite effects. It must be added that in the current study it is not possible to compare the findings with baseline neutrophil values, as BAL was not performed at baseline in order to avoid disturbances.

In conclusion, this study showed that inhalation of 20 ppm NO during the allergen-induced acute airway reaction has protective effects on arterial oxygenation and pulmonary arterial pressure. In contrast, only a small non-significant bronchodilatory effect of NO could be observed and, taken together, this indicates that the fall in arterial oxygenation during the acute airway reaction is caused by a ventilation/perfusion mismatch at the pulmonary circulation level and not at the bronchiolar level. Histamine release from mast cells in the airways could not be shown to be changed, in contrast to a previous study that demonstrated that inhibition of endogenous NO synthesis markedly increased histamine release. This indicates that endogenously produced NO is responsible for a possible modulating role of mast cells, whereas exogenous NO in high amounts does not add to this effect. However, NO inhalation was responsible for an enhanced clearance of total protein that had formed in the airway mucosa as a result of the acute allergic reaction, possibly through increased bronchial blood flow.

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