Modulation of Airway Responsiveness to Acetylcholine by Nitric Oxide in a Rabbit Model

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Abstract. Nitric oxide (NO) is an important mediator in the regulation of bronchial muscle tone and airway responsiveness. We investigated the influence of exogenous NO on airway responsiveness to acetylcholine aerosols (ACH) in normal and in hyperresponsive rabbits. White New Zealand rabbits were anesthetized, intubated, and breathed room air spontaneously. Responses of respiratory parameters in ACH challenge tests were measured. In group A the influence of NO on ACH infusion-induced airway constriction was measured. Airway responses to aerosols from 0.25 to 8.0% ACH solutions in saline were measured with 150 and 300 ppm NO inhalation (groups B and C) and compared with the same animals' responses without NO. Moreover, we examined the influence of NO synthase inhibition on airway responsiveness (group D) and the modulatory effect of NO in hyperresponsive animals (group E). 300 ppm NO inhalation significantly decreased the bronchoconstrictor response to intravenously administered ACH (group A). However, the baseline value of dynamic elastance (E_{dyn}) was only marginally lower under the influence of 300 ppm NO. During inhalation of 150 or 300 ppm NO, responses to nebulized 2.0% and less ACH solutions remained nearly unaltered. Responses to aerosols of 4.0 and 8.0% diminished significantly (groups B and C). Following 40 min of aerosolized *N*-nitro-*L*-arginine-methyl ester (*L*-NAME) solution (a NO synthase inhibitor, 1.2 mM) inhalation, the response of E_{dyn} to ACH increased significantly in group D. In group E, animals inhaled 500 mg/m³ ammonium persulfate (APS), an oxidant with various industrial applications, after the first ACH challenge test (0.2, 1.0, and 2.0% ACH). After 2 h of APS exposure, the ACH-induced

Abbreviations: ACH, acetylcholine hydrochloride; AHR, airway hyperresponsiveness; APS, ammonium persulfate; AR, airway responsiveness; ARDS, adult respiratory distress syndrome; ΔP_{es} , respiratory changes in esophageal pressure; E_{dyn} , dynamic elastance; *L*-NAME, *N*-nitro-*L*-arginine-methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; N.S., not significant; S.E.M., standard error of the mean; $\bar{V}$, minute volume; V' , respiratory airflow; V_T , tidal volume; $\bar{x}$, mean

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broncho constriction was increased significantly in the challenge test. After another 2 h of APS inhalation, the airway responsiveness to ACH was tested under the influence of 300 ppm NO. NO significantly decreased the response to ACH to almost the same level as before APS exposure. The results indicate that responses to high ACH concentrations as well as an APS-induced increase in ACH responsiveness were effectively reduced by high concentrations of inhaled NO.

Key words: Acetylcholine—Airway responsiveness—Ammonium persulfate—L-NAME—Nitric oxide—Occupational lung diseases—Rabbits.

Introduction

The modulating effects of nitric oxide (NO) on neurotransmission [4, 19], airway muscle tone, and airway responsiveness (AR) have been recognized for several years [1, 17, 26]. NO has many clinical applications including the normalization of pulmonary vascular pressure in newborns with severe persistent hypertension [10, 20] and in the treatment of adult respiratory distress syndrome (ARDS) [6, 18, 22, 23]. In addition, it serves as vasodilator in chronic obstructive pulmonary disease [21]. Current data suggest that inhalation of nitric oxide by patients with mild asthma during methacholine-induced bronchospasm results in a minor but significant relaxation of airway tone [9]. NO is produced in different cell types during the NO synthase-catalyzed conversion of L-arginine to L-citrulline. NO stimulates soluble guanylate cyclase, and the production of cyclic guanosine monophosphate causes a relaxation of smooth muscles. Applying an inhibitor of NO synthase, *N*-nitro-L-arginine-methyl ester (L-NAME), blocks NO production [1].

In the rabbit model of Högman et al. [8], inhalation of NO effectively reduced the bronchoconstrictor responses to methacholine. Folkerts et al. [7] demonstrated the contribution of a NO deficiency in the respiratory epithelium in the development of virus-induced AHR.

Using our rabbit model for occupational lung diseases [11, 12], we investigated the influence of exogenous NO on bronchoconstrictor responses to acetylcholine (ACH). We then examined the influence of NO synthase inhibition (L-NAME) on AR to ACH. Finally, the influence of NO inhalation on the constrictor response to ACH in hyper-responsive animals was tested. AHR was induced by exposure to ammonium persulfate (APS), a potent oxidant used in numerous industrial bleaching processes and in the hair cosmetic industry as a component of hair bleach powders. The substance is suspected to cause lung diseases after exposure in the workplace [2, 5]. Indeed, APS in a concentration of 50 mg/m³ in the air caused AHR after an exposure of 4 h in our rabbit model for occupational lung diseases [13–15].

Materials and Methods

Experimental Setup

White New Zealand rabbits derived from the same breed and of similar age and body weight (3.5–4.0 kg) were premedicated with ketamine hydrochloride (25 mg/kg, Parke-Davis, Berlin, Germany, Ketanest®) and

xylazin (5 mg/kg, Bayer, Leverkusen, Germany, Rompun®) and placed in a supine position. Anesthesia was kept constant by continuous infusion of 0.2 mg/kg/h of thiopentobarbital sodium (Byk Gulden, Konstanz, Germany, Trapanal®) via a catheter inserted into the femoral vein. The body temperature was maintained at $39 \pm 0.5^\circ\text{C}$ by means of a thermocontroller connected to a heating pad. The animals were intubated (Mallinckrodt, Athlone, Ireland, 3.0-mm inner diameter) and breathed room air spontaneously. All animals were in healthy condition, free from signs of acute airway infections, and had not suffered from any previous infections. (For a more detailed description of the rabbit model and the statistical data analysis see Refs. 11 and 12.)

Recording of Respiratory and Cardiovascular Parameters

The respiratory airflow (V') was recorded by a Fleisch's head (00) attached to the tracheal tube. Tidal volume (V_T) was obtained by electrical integration of the inspiratory flow signal. Dynamic elastance (E_{dyn}) representing airway resistance was calculated from the differences in the esophageal pressure (ΔP_{es}) and V_T . Catheterization of the femoral artery was used to measure cardiovascular parameters and to collect small blood samples (about 0.4 ml) to examine blood gases and correlated acid-base parameters. Data were recorded on a polygraph and, after A/D conversion of the measured signals, on a personal computer [11, 12].

Airway Challenges with ACH Aerosols

Changes in airway responses to aerosols from 0.2% and 2% ACH solutions (acetylcholine hydrochloride, Sigma Chemie, Deisenhofen, Germany) in saline generated by a commercial nebulizer (Pari, Clinic II) were investigated when respiratory and cardiovascular parameters were constant after induction of anesthesia. 0.13 ml of solution was nebulized in 5.7 liters of room air per minute and stored in a reservoir bag. The particle size ranged from 0.5 to 5.5 μm . During the challenge tests, animals inhaled about 1.1 liter of ACH aerosols from ACH solutions in saline corresponding to 0.48 mg of ACH (2.0% solution). These doses were equivalent to those commonly used in human airway challenge tests for the detection of AHR. No animal effectively responded to 0.2% ACH aerosol, and responses to 2% ACH were $50\% \leq \Delta E_{\text{dyn}} \leq 150\%$ of the baseline value. Doubling the baseline values is the common criterion for a significant obstructive response in humans [25]. Therefore, we focused on the responses to 2.0% ACH, which could be quantitatively and reproducibly detected with our recording device and be augmented after exposure to toxic agents [11, 12]. To evaluate the AR in the different groups, ACH concentrations of 0.2, 1.0, 2.0, 4.0, and 8.0% ACH were used in the experiments.

Generation of NO Atmospheres

During NO atmosphere inhalation, animals breathed spontaneously from an airstream of 100 ml/s which had no influence on the baseline respiratory and cardiovascular parameters. NO (1% NO 2.8 in N₂ 5.0, Messer-Griesheim, Oberhausen, Germany) was added into the airstream near the respiratory valve to reach the final NO concentration. NO₂ was removed by a gas filter (NOXON®, Messer Griesheim, Oberhausen, Germany, $c(\text{NO}_2) < 50$ ppb). NO atmospheres of the inhaled gases were measured continuously with an infrared photometer (UNOR 610, MAIHAK, Wuppertal, Germany). The photometer detected NO concentrations >80 ppm, and the maximum display delay was 20 s. Two-point calibration was performed with air (zero) and NO in N₂ (300 ppm) before the experiments.

Influence of NO on ACH Infusion-Induced Airway Constriction

In group A 0.1% ACH solution was infused (about 0.09 ml/min) into the femoral vein causing a constant level of bronchoconstriction. After obtaining steady state (about 5 min after infusion started), the animals

inhaled 300 ppm NO in air. Once the E_{dyn} had stabilized (5–10 min after NO inhalation started), the NO inhalation and then the ACH infusion were stopped in sequence. Respiratory and cardiovascular parameters were measured in each step.

Influence of NO on ACH Inhalation-Induced Airway Constriction

In groups B and C we tested the influence of 150 or 300 ppm NO on AR after inhalation of 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0% ACH. Initially, the basal response to ACH was tested. Each concentration was inhaled for 1 min at intervals of 15 min after the responses declined to baseline values. In a second test, the challenge was repeated under the influence of NO after 60 min of recovery. Prior to the second challenge test, the animals inhaled NO [150 (group B) or 300 ppm (group C)] for 30 min. NO was administered directly after each ACH inhalation.

Influence of NO Synthase (NOS) Inhibitor L-NAME on AR to ACH

In group D we examined the constrictor response to ACH (0.2, 1.0, and 2.0%) under the influence of the NOS inhibitor L-NAME (Sigma Chemie). Following the first ACH challenge test, the animals inhaled nebulized L-NAME solution at a concentration of 1.2 mM [23] for 40 min. The aerosols were generated by the nebulizer used for ACH aerosol generation. Thereafter, the response to ACH was tested. ACH challenge tests were repeated after 45 min.

Influence of NO on APS-Induced AHR to ACH (Group E)

In group E the animals were exposed to nebulized APS solution $[(\text{NH}_4)_2\text{S}_2\text{O}_8 \text{ p.A., Merck, Darmstadt, Germany}]$ to induce an AHR in the same way as reported in a previous paper [15]. After the first ACH challenge test with 0.2, 1, and 2% ACH solutions, the animals inhaled 500 mg/m³ APS in air for 2 h. The AR to ACH was tested afterward, and the animals inhaled APS for 2 more h. A third ACH provocation test was performed under the influence of 300 ppm NO inhalation.

Statistical Analysis

The data in text and figures represent the mean ($\bar{x}$) $\pm$ standard error of the mean (S.E.M.). Differences in the responses of airway mechanical parameters to ACH challenge were calculated. The significance of the values was tested by Student's *t*-test [24], and $p < 0.05$ was considered significant.

Results

Influence of NO and L-NAME on Baseline Values

Inhalation of 150 ppm NO did not change baseline values significantly (Fig. 1). However, E_{dyn} decreased slightly from 23.9 ± 1.4 to 22.3 ± 0.9 mmHg/dlV_T under the influence of 300 ppm NO (Fig. 1, $p < 0.05$). During L-NAME inhalation, E_{dyn} increased from 22.0 ± 1.1 to 25.0 ± 1.3 mmHg/dlV_T (Fig. 1, $p < 0.05$).

Fig. 1. Influence of NO inhalation (150 ppm, *left*; and 300 ppm, *middle*) and L-NAME inhalation (*right*) on the baseline value of E_{dyn} .

Influence of NO on the ACH Infusion-Induced Airway Constriction

NO effectively reduced ACH infusion-induced airway constriction (group A). ACH infusion caused an increase in E_{dyn} from 21.7 ± 0.6 to 35.7 ± 2.1 mmHg/dlV_T. Under the influence of 300 ppm NO in air, E_{dyn} decreased significantly to 29.6 ± 1.3 mmHg/dlV_T ($p < 0.005$). After NO inhalation ceased, E_{dyn} increased again significantly and dropped after terminating ACH infusion (Fig. 2).

Influence of NO on ACH Inhalation-Induced Bronchoconstriction (Groups B and C)

In these experiments we tested whether NO had any influence on the constrictor response to ACH in our rabbit model. No significant alterations of control values of E_{dyn} before ACH inhalation were measured without or after NO inhalation. NO inhalation did not effect constrictor responses in provocation tests with ACH solution concentrations of less than 2%. However, the response to aerosolized 4% ACH solution diminished from 76.9 ± 8.5 to 62.3 ± 7.8 mmHg/dlV_T under the influence of 150 ppm NO (Fig. 3). Similarly, 300 ppm NO did not influence the constrictor response to ACH concentrations below 2%. The constrictor response to 4% ACH dropped significantly from 82.2 ± 8.7 to 57.3 ± 6.4 mmHg/dlV_T ($p < 0.05$). Additionally, the response to the 8% ACH solution decreased from 135.0 ± 24.0 to 91.2 ± 17.1 mmHg/dlV_T ($p < 0.0005$, Fig. 4).

Influence of the NOS Inhibitor L-NAME on AHR to ACH (Group E)

After the first ACH provocation tests, the animals inhaled L-NAME (1.2 mM) for approximately 40 min. Following this inhalation, the constrictor response to ACH was

Fig. 2. Influence of 300 ppm NO in air on the airway constriction induced by ACH infusion (0.09 ml/min).

measured again. Responses to all ACH concentrations were elevated compared with the initial control provocation test. E_{dyn} response to 2% ACH solution increased from 48.4 ± 3.3 to 87.9 ± 14.0 mmHg/dlV_T after L-NAME inhalation. After 30 min, the response to ACH inhalation was still significantly elevated (Fig. 5).

Influence of NO on APS-Induced AHR to ACH (Group D)

In this group the animals were exposed to 500 mg/m³ APS twice for 2 h. In previous investigations, we demonstrated that APS inhalation of an aerosol containing this concentration caused AHR [9]. The result was confirmed in this investigation (Fig. 6).

Following APS exposure twice for 2 h, the response to 1% ACH rose from 23.3 ± 1.0 to 59.5 ± 21.2 mmHg/dlV_T (N.S.). In the provocation test after 2 more h of APS exposure, the constrictor responses to ACH decreased under the influence of 300 ppm NO from 59.5 ± 21.2 to 41.0 ± 7.0 mmHg/dlV_T (N.S.).

After APS exposure, E_{dyn} increased significantly ($p < 0.05$) from 42.8 ± 4.0 to 119.1 ± 23.1 mmHg/dlV_T in the 2% ACH challenge test. In the provocation test after 2 further h of APS exposure, the constrictor responses to 2% ACH dropped significantly ($p < 0.05$) to 54.9 ± 4.1 mmHg/dlV_T under the influence of 300 ppm NO inhalation.

Discussion

The present study demonstrated the influence of NO inhalation on AR to ACH in normal and in hyperresponsive rabbits. NO showed significant attenuating effects on

Fig. 3. Responses of E_{dyn} to ACH without (■) and under the influence of 150 ppm NO (◆).

Fig. 4. Responses of E_{dyn} to ACH without (■) and under the influence of 300 ppm NO (◆).

the bronchoconstrictor response to ACH inhalation and infusion. This effect was especially evident for strong airway constrictions. No significant effects of 300 and 150 ppm NO were found following provocation with ACH concentrations less than 2%. However, constrictor responses resulting from 4 and 8% ACH could be effectively

Fig. 5. Response of E_{dyn} in ACH challenge tests before (■) and after inhalation of 1.2 mM of L-NAME for 40 min. (■) immediately after L-NAME inhalation; (◆) 45 min after L-NAME inhalation.

reduced to about 70% of the basic response without NO inhalation. More potent NO effects were found during airway constriction induced by ACH infusion. Although ACH infusion only resulted in an approximate doubling of the E_{dyn} baseline value, subsequent NO inhalation reduced E_{dyn} significantly. This effect may be the result of applied steady-state conditions. In addition to the effects on the short term constrictor response to ACH inhalation, NO seems to reduce airway constriction over a prolonged period.

Moreover, we tested if the inhibition of NOS by the well known NOS inhibitor L-NAME [10] alters the response to ACH. A significant increase in the constrictor responses to 2.0% ACH in the provocation test directly after L-NAME inhalation was observed. 45 min after L-NAME inhalation, the responses to 1.0 and 2.0% ACH concentrations were still significantly increased, although the amplitude of the responses is reduced compared with the previous provocation test. This observation can be explained by a decreased standard deviation. The exaggerated constrictor response to ACH due to reduced endogenous NO generation after L-NAME inhalation confirms the participation of NO in the regulation of AR.

In this study we also evaluated the effect of NO on AHR induced after acute APS exposure. The strong oxidant APS was chosen since previous investigations demonstrated the ability of APS to induce a pronounced AHR in our rabbit model within a few hours [13–15]. We found that NO reduced the constrictor response effectively. The ACH challenge test after 2 h of APS exposure demonstrated an elevated AR. However, under the influence of NO, the constrictor responses were reduced to nearly the same level as before APS inhalation.

Fig. 6. ACH challenge tests without and under the influence of 300 ppm NO after inhalation of APS.

The mechanisms of the APS-induced AHR have not been clarified yet. The reduced NO production in airway epithelial cells might explain the enhanced AR to ACH after acute APS exposure. Nijkamp et al. [17] presume a deficiency of NO production in respiratory tract cells to be related to the development of AHR and, therefore, enhanced constrictor responses after virus-induced AHR.

The NO concentrations leading to significant effects in our rabbit model were rather high.

Högman et al. [8] investigated the influence of NO on methacholine-induced bronchoconstrictions in a different rabbit model. According to their study, 80 ppm NO added to the inspired gas effectively modulated the airway tone response to nebulized methacholine.

The discrepancy between the NO concentrations examined by Högman et al. [8] and the concentrations used in the present study leading to an effectively reduced airway constriction may be due to different experimental procedures. In our rabbit model animals breathed spontaneously, whereas Högman et al. examined ventilated rabbits whose airways may have been opened by the ventilation pressure.

Our experiments confirm the protective effects of 300 and 150 ppm NO on ACH-induced bronchoconstrictions. However, the problem of NO toxicity cannot be ignored. The development of nitrates and peroxynitrites resulting in methemoglobinemia or nitrogen dioxide having a toxic effect on lung tissue may explain the toxic effect of higher NO concentrations [1, 3, 16]. For this reason, the application of higher NO concentrations should be limited to therapeutic use and only applied over short periods of time. Additionally, many studies demonstrate an effective therapeutic use of NO in lower concentrations [9, 10, 27], avoiding the toxic effects. For this reason, future

experiments should clarify in detail whether low and thus less toxic NO concentrations can be used effectively in the prevention or treatment of acute airway constrictions.

Conclusions

ACH-induced airway constrictions could be reduced effectively during inhalation of 300 ppm NO in our rabbit model. After inhibiting the NOS by using the potent NOS inhibitor L-NAME, constrictor airway responses to ACH increased significantly. Moreover, the constrictor responses to ACH in hyperresponsive rabbits decreased under the influence of exogenous NO. The present investigation confirms the modulating influence of NO on AR, especially after induction of AHR by exposure to an occupational irritant.

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