Effects of endogenous superoxide anion and nitric oxide on cholinergic constriction of normal and hyperreactive guinea pig airways

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SUMMARY

In a guinea pig model of allergic asthma, we have recently established that a deficiency of nitric oxide (NO) contributes to the increased ex vivo responsiveness of isolated perfused tracheae to methacholine after the early asthmatic reaction, at 6h after inhalational challenge of the animals with ovalbumin aerosol. Since this deficiency could be caused by a reaction of NO with enhanced levels of inflammation-induced superoxide anion ($O_2^-$), we examined the effect of endogenous $O_2^-$ on the regulation of methacholine-induced constriction by NO of intact perfused tracheal tube preparations from unchallenged (control) guinea pigs and from animals 6h after ovalbumin challenge. In the presence of the NO synthase (NOS) inhibitor $N\omega$-nitro-L-arginine methyl ester (L-NAME; 100 µM), tracheae obtained from unchallenged guinea pigs showed a 1.7-fold increase in the maximal response to intraluminally-applied methacholine ($p<0.05$). By contrast, the maximal airway response to methacholine was significantly decreased in the presence of the $O_2^-$ scavenger superoxide dismutase (SOD; 100 U/ml), by approximately 45% ($p<0.01$). The SOD-induced decrease in responsiveness to methacholine was reversed by L-NAME. Tracheal preparations obtained at 6h after allergen challenge showed a 1.8-fold increased responsiveness to intraluminally-applied methacholine compared to controls ($p<0.001$), which was not further enhanced in the presence of L-NAME. SOD had neither an effect on the increased responsiveness, nor did it restore the potentiating effect of L-NAME. These results indicate that (1) in normoreactive tracheal preparations, the regulatory role of NO is partially counteracted by endogenous $O_2^-$, and (2) the deficiency of NO in hyperreactive tracheae obtained at 6h after ovalbumin challenge is not caused by its reaction with $O_2^-$, but rather to decreased cNOS activity.

INTRODUCTION

Several lines of evidence indicate that NO is importantly involved in the regulation of airway smooth muscle tone. In vitro, stimulation of the inhibitory non-adrenergic, non-cholinergic (i-NANC) nerves causes relaxation of airway smooth muscle from various species, including guinea pigs and man, which can at least partially be blocked in the presence of NOS inhibitors (1-3). Furthermore, NO causes a concentration dependent relaxation of guinea pig tracheal strips precontracted with carbachol (4), while in the presence of the NOS inhibitors L-NAME and $N\omega$-monomethyl-L-arginine (L-NMMA), muscarinic agonist and histamine-induced constriction of intact perfused guinea pig tracheal tube preparations is enhanced (5,6). In addition, the endothelin-1 and bradykinin-induced epithelium-dependent relaxation of guinea pig trachea in vitro was shown to be mediated by NO (7,8).

In vivo, inhaled NO reverses histamine and methacholine-induced bronchoconstriction in guinea pigs (9), dogs (10) and humans (11), while in the guinea pig administration of L-NAME causes enhanced bronchoconstriction in response to both allergens (12) and contractile agonists (5, 13).
Airway hyperreactivity to pharmacological stimuli such as histamine and muscarinic agonists, is a hallmark of allergic asthma. Using a guinea pig model of allergic asthma, characterised by allergen-induced early and late asthmatic reactions, airway inflammation and airway hyperreactivity to histamine and methacholine after these reactions (14,15), we have recently demonstrated that a deficiency of NO contributes to the hyperresponsiveness of intact perfused tracheae to histamine and methacholine, as observed after the early asthmatic reaction (6).

The mechanism of the NO deficiency after the early asthmatic reaction is presently unknown. Since allergic asthma both in guinea pigs and in man is associated with enhanced production of $O_2^-$ by a variety of inflammatory cells (16-21), it can be hypothesized that inactivation of NO by increased levels of $O_2^-$ is involved. Thus, $O_2^-$, like NO being a radical, is a potent chemical inactivator of NO by its fast reaction with this bronchodilator to form peroxynitrite (ONOO$^-$) (21). Evidence for an NO scavenging role of $O_2^-$ in the regulation of smooth muscle contraction has been found previously in the vasculature (22-24) and in the gastrointestinal (25) and urogenital (26) tract.

In the present study, we examined the effect of inactivation of $O_2^-$ by SOD on the efficacy of endogenous NO in inhibiting methacholine-induced contraction of perfused guinea pig tracheal preparations. This was performed in normoreactive airways obtained from unchallenged control animals, as well as in hyperreactive airways from guinea pigs obtained at 6h after allergen provocation, in order to investigate the hypothesis that NO-scavenging by $O_2^-$ contributes to allergen-induced airway hyperresponsiveness after the early asthmatic reaction.

**METHODS**

**Animals**

Outbred specific pathogen free guinea pigs (Charles River SAVO, Kiszlegg, Germany), weighing 500 - 800 g, were used in this study. All animals were actively IgE-sensitized to ovalbumin (OA) at three weeks of age as described by Van Amsterdam et al. (27). In short, 0.5 ml of an allergen solution containing 100 µg/ml ovalbumin and 100 mg/ml Al(OH)$_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck. The animals were used experimentally in weeks 4 to 8 after sensitization. The animals were group-housed in individual cages in climatized animal quarters and given water and food *ad libitum*, while a 12-h on/ 12-h off light cycle was maintained.

All protocols described in this study were approved by the University of Groningen Animal Health Committee.
Chapter 3

Allergen provocation

Ovalbumin provocations were performed by inhalation of aerosolized solutions. The provocations were performed in a specially designed animal cage, in which the guinea-pigs could move freely (14). The volume of the cage was 9 l, which ensured fast replacement of the air inside the cage with aerosol and vice versa. A DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, USA) driven by an airflow of 8 l/min provided the aerosol required, with an output of 0.33 ml/min.

Allergen provocations were performed by inhalation of increasing aerosol concentrations of 0.5, 1.0, 3.0, 5.0 and 7.0 mg/ml ovalbumin in saline for 3 min, separated by 10-min intervals. Allergen inhalations were discontinued when the first signs of respiratory distress were observed. No anti-histaminic was needed to prevent the development of anaphylactic shock. Previous studies measuring pleural pressure changes in ovalbumin sensitized, permanently instrumented, unrestrained guinea pigs have indicated that the allergen-induced early asthmatic reaction induced by this procedure is maximal within 20 min and lasts for up to 5 h (14,15).

Tracheal perfusion

Six hours after ovalbumin challenge, the guinea pigs were sacrificed. Non challenged IgE-sensitized animals were used as controls. The animals were killed by a sharp blow on the head and exsanguinated. The tracheas were rapidly removed and placed in Krebs-Henseleit (KH) solution (37°C) of the following composition (mM): NaCl 117.50, KCl 5.60, MgSO₄ 1.18, CaCl₂ 2.50, NaH₂PO₄ 1.28, NaHCO₃ 25.00, D-glucose 5.50; gassed with 5% CO₂ and 95% O₂; pH 7.4.

The tracheas were prepared free of serosal connective tissue and cut into two halves of approximately 17 mm before mounting in a perfusion setup, as described previously (6). To this aim, the tracheal preparations were attached at each side to stainless steel perfusion tubes fixed in a Delrin perfusion holder. The holder with the trachea was then placed in a water-jacketed organ bath (37°C) containing 20 ml of gassed KH (the serosal or extraluminal (EL) compartment). The lumen was perfused with recirculating KH from a separate 20 ml bath (mucosal or intraluminal (IL) compartment) at a constant flow rate of 12 ml/min. Two axially centred side-hole catheters connected with pressure transducers (TC-XX,Viggo-Spectramed B.V., Bilthoven, The Netherlands) were situated at the distal and proximal ends of the trachealis to measure hydrostatic pressures (P_{outlet} and P_{inlet}, respectively). The signals were fed into a differential amplifier to obtain the difference between the two pressures (ΔP = P_{inlet} - P_{outlet}), which was plotted on a flatbed chart recorder (BD 41, Kipp en Zonen, Delft, The Netherlands). ΔP reflects the resistance of the tracheal segment to perfusion and is a function of the mean diameter of the trachea between the pressure taps (28). The transmural pressure in the trachea was set at 0 cm H₂O. At the perfusion flow rate used, a baseline ΔP of 0.1 to 1.0 cm H₂O was measured, depending of the diameter of the preparation.

After a 45 min equilibration period with three washes with fresh KH (both IL and EL), 1
μM isoprenaline was added to the EL compartment for maximal smooth muscle relaxation to assess basal tone. After three washes during at least 30 min, the trachea was exposed to EL 40 mM KCl in KH to obtain a receptor-independent reference response. Subsequently, the preparation was washed 4 times with KH during 45 minutes until basal tone was reached and a cumulative concentration response curve (CCRC) was made with IL methacholine. When used, SOD (100 U/ml, occasionally 1000 U/ml) was applied to the IL or EL reservoir, 30 min prior to agonist-addition. L-NAME (100 μM) was added to the IL reservoir, 45 min prior to agonist addition.

**Data analysis**

To correct for differences in baseline ΔP and in ΔP changes in response to contractile stimuli due to variation in resting internal diameter of the preparations used, IL responses of the tracheal tube preparations to methacholine were expressed as a percentage of the response induced by EL administration of 40 mM KCl. The contractile effect of 10 mM MeCh (highest concentration) was defined as $E_{\text{max}}$. Using this $E_{\text{max}}$, the sensitivity to MeCh was evaluated as $pEC_{50}$ (-log $EC_{50}$) value.

The results are expressed as means ± SEM. Statistical analysis was performed using the Student's t-test for unpaired observations. Differences were considered statistically significant at p<0.05.

**Chemicals**

Histamine hydrochloride, oval-bumin (grade III), aluminum hydroxide, (-)-isoprenaline hydro-chloride, superoxide dismutase (isolated from bovine erythrocytes) and L-$N^\omega$-nitro arginine methyl ester (L-NAME) were obtained.
from Sigma Chemical Co. (St. Louis, MO, USA) and methacholine chloride from Aldrich (Milwaukee, WI, USA).

**RESULTS**

In perfused tracheal preparations from unchallenged guinea pigs, the NOS inhibitor L-NAME (100 µM, IL) caused a marked 1.7-fold potentiation of the $E_{\text{max}}$ value of IL methacholine ($p<0.05$), without an effect on the sensitivity ($pEC_{50}$) to this agonist (Figure 1A; Table 1). By contrast, inactivation of $O_2^-$ with SOD (100 U/ml) resulted in a significant decrease in the $E_{\text{max}}$ of the tracheal preparations to IL methacholine by approximately 45% ($p<0.01$), irrespective of the route of administration of SOD (IL or EL; Figure 1B; Table 1). In addition, a small but significant decrease in sensitivity to the agonist was observed in the presence of SOD (Table 1). Inhibition of NOS with L-NAME reversed the SOD-induced decrease in responsiveness to IL methacholine (Figure 1C; Table 1).

In the ovalbumin-challenged group of animals, the absolute $\Delta P$ response to KCl was unchanged compared to the control group (5.93 ± 1.18 cm H$_2$O vs. 6.55 ± 2.19 cm H$_2$O, respectively, NS). However, the $E_{\text{max}}$ to IL methacholine was significantly increased by 1.8-fold ($p<0.001$), without a change in $pEC_{50}$ (Figure 2A; Table 1). This increase was not further enhanced in the presence of L-NAME (Figure 2A; Table 1). In addition, the enhanced responsiveness to methacholine was not reversed in

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**Figure 2.** Methacholine (MeCh) cumulative concentration response-curves of perfused tracheal tube preparations obtained from unchallenged control guinea pigs and guinea pigs at 6h after allergen challenge. (A) Preparations from unchallenged controls in the presence of buffer (open circles; n=12; same data as presented in Figure 1) and from ovalbumin-challenged guinea pigs in the presence of buffer (open diamonds; n=11) and 100 µM L-NAME (IL; closed circles; n=6). (B) Preparations from ovalbumin-challenged guinea pigs in the presence buffer (open diamonds; n=12), 100 U/ml SOD (EL; closed diamonds; n=6, or IL; closed triangles; n=6) and 1000 U/ml (EL; closed squares; n=3). (C) Preparations of ovalbumin-challenged guinea pigs in the presence of buffer (open diamonds; n=12), 100 U/ml SOD (EL; closed diamonds; n=6), and a combination of 100 µM L-NAME (IL) and 100 U/ml SOD (EL; closed squares; n=4). Each data point is the mean ± SEM. See Table 1 for $E_{\text{max}}$ and $pEC_{50}$ values of the methacholine concentration-response curves.
the presence of both IL or EL SOD, at a concentration of 100 U/ml (Figure 2B; Table 1). A similar result was obtained, when using a ten-fold higher concentration (1000 U/ml, EL) of the \( \text{O}_2^\cdot \) scavenger (Fig. 2B). Furthermore, there was no effect of SOD on the tracheal responsiveness to methacholine in the presence of L-NAME (Figure 2C; Table 1). Both in the control preparations and in preparations from the ovalbumin-challenged animals, L-NAME and SOD, as well as the combination of these agents, had no effect on basal and KCl-induced tone (data not shown).

**Table 1** Effects of L-NAME (100 µM, IL), SOD (100 U/ml, IL or EL), and L-NAME (100 µM, IL) + SOD (100 U/ml, EL) on the responsiveness to methacholine (IL) of intact perfused tracheae from unchallenged and ovalbumin-challenged guinea pigs.

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<th>Unchallenged</th>
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<tr>
<td></td>
<td>pEC(_{50}) (-log M)</td>
<td>( E_{\text{max}} ) (%)</td>
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<tr>
<td>Vehicle</td>
<td>3.18 ± 0.10</td>
<td>62.3 ± 5.1</td>
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<td>SOD (IL)</td>
<td>2.62 ± 0.13(^{+})</td>
<td>34.9 ± 7.7(^{+})</td>
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<td>SOD (EL)</td>
<td>2.73 ± 0.06(^{+})</td>
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<td>L-NAME (IL)</td>
<td>3.20 ± 0.09</td>
<td>109.0 ± 16.8(^{+})</td>
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<td>L-NAME (IL) + SOD (EL)</td>
<td>3.47 ± 0.41</td>
<td>84.2 ± 3.0(^{+})</td>
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<th>Ovalbumin-challenged</th>
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<tr>
<td></td>
<td>pEC(_{50}) (-log M)</td>
<td>( E_{\text{max}} ) (%)</td>
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<tr>
<td>Vehicle</td>
<td>3.40 ± 0.12</td>
<td>112.0 ± 5.9(^{+})</td>
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<tr>
<td>SOD (IL)</td>
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<td>106.4 ± 10.3</td>
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<tr>
<td>SOD (EL)</td>
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<tr>
<td>L-NAME (IL)</td>
<td>3.37 ± 0.30</td>
<td>113.3 ± 8.5</td>
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<tr>
<td>L-NAME (IL) + SOD (EL)</td>
<td>3.76 ± 0.36</td>
<td>95.5 ± 11.2</td>
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**Definition of abbreviations:** EL = extraluminal; IL = intraluminal; L-NAME = \( \text{N}^\omega \)-nitro-L-arginine methyl ester; SOD = superoxide dismutase; pEC\(_{50}\) = -log EC\(_{50}\); \( E_{\text{max}} \) = maximal effect obtained in the presence of 10 mM MeCh. Values are expressed as mean ± SEM of 4 to 12 experiments. * \( E_{\text{max}} \) is expressed as a percentage of the increase in \( \Delta P \) induced by EL administration of 40 mM KCl. \(^{†}\) \( p < 0.001 \) compared with the unchallenged control group. \(^{‡}\) \( p < 0.05 \) compared with the vehicle control group. \(^{§}\) \( p < 0.01 \) compared with the vehicle control group. \(^{º}\) \( p < 0.001 \) compared with the SOD (EL)-treated group.

**DISCUSSION**

Using perfused tracheal tube preparations obtained from unchallenged ovalbumin-sensitized control guinea pigs, it was demonstrated that endogenous NO counteracts IL methacholine-induced airway narrowing and that this regulation is partially under negative control of endogenous \( \text{O}_2^\cdot \). This is indicated by the observation that the NOS inhibitor L-NAME potentiates methacholine-induced tracheal contraction, while the \( \text{O}_2^\cdot \) scavenger SOD reduces the contractile agonist-induced response, presumably by preventing the reaction of the superoxide anion with endogenous NO to form ONOO\(^{-} \) (21). Evidence for an NO-scavenging role of endogenous \( \text{O}_2^\cdot \) in the regulation of smooth muscle tone was first documented in the vasculature, where endothelium-dependent relaxations mediated by NO were potentiated in the presence of SOD (22,23), while these relaxations were reduced in the presence of \( \text{O}_2^\cdot \) generating systems (23), or when endogenous SOD was inhibited (24). Similar observations were done with respect to iNANC nerve- or exogenous NO-mediated relaxation of gastrointestinal (25) and urogenital (26) smooth muscle preparations.

The role of \( \text{O}_2^\cdot \) as NO scavenger in the unchallenged airway preparations was confirmed
by the observation that the SOD-induced reduction of methacholine-induced tone was reversed by the NOS-inhibitor L-NAME. The reversal also suggests that the effect of $O_2^-$ on the tracheal responsiveness to methacholine is exerted only via inactivation of methacholine-induced NO production. The absence of a direct effect of endogenous $O_2^-$ on tracheal smooth muscle is supported by the observation that SOD had no effect on basal tone. The observation that IL and EL SOD inhibited methacholine-induced tracheal constriction to the same extent suggests that maximal inactivation of $O_2^-$ was obtained under both conditions used.

As in a previous study (6), we demonstrated that tracheal preparations obtained from guinea pigs after the early asthmatic reaction at 6h after allergen challenge had an increased responsiveness to methacholine. This finding seems to be in contrast with a number of other previous studies using human or animal isolated airway ring or strip preparations, demonstrating that (allergen-induced) airway hyperreactivity to contractile agonists \textit{in vivo} is associated with a normal or even decreased reactivity of the \textit{ex vivo} preparations to these agonists (for review see ref. 29). However, since the perfused tracheal preparations used in the present study retained structural airway integrity, it may be concluded that allergen-induced airway hyperreactivity is mainly caused by geometric and/or epithelial changes and not by changes of airway smooth muscle function, which is the main determinant of contraction in airway strip or ring preparations. Since the observed \textit{ex vivo} hyperreactivity was closely mimicked by the administration of L-NAME to control preparations of unchallenged guinea pigs, while, in addition, the hyperreactive airway preparations were unresponsive to the NOS-inhibitor, it can be concluded that a deficiency of contractile agonist-induced NO in the airways is a major determinant of the observed hyperreactivity. Very recently, similar results were obtained \textit{in vivo}, by the use of inhaled L-NAME (13).

The source of NO in our perfused control preparations is unknown. However, the NO was most likely produced by a constitutive isotype of NOS (cNOS), since only the agonist-induced constriction and not KCl-induced constriction or basal tone was increased after inhibition of NOS activity by L-NAME. cNOS in the airways is basally expressed in the neuronal, endothelial and epithelial cells (30). Since the epithelium appears to be an important source of contractile agonist-induced NO production in the guinea pig trachea (5,8) and allergen challenge may cause epithelial damage due to inflammation of the airways (31), we previously hypothesized that the deficiency of NO in the trachea of hyperreactive guinea pigs was due to epithelial shedding. However, no damage of the airway epithelium was observed in tracheal preparations of ovalbumin-challenged guinea pigs after the EAR, which makes this possibility unlikely (6).

Nevertheless, bronchoalveolar lavage studies have indicated that allergen-induced airway hyperreactivity after the early asthmatic reaction in our guinea pig model is associated with a marked influx of (activated) eosinophils and neutrophils in the airways (15,32). Since these cells (16,20,33) as well as alveolar macrophages (19) and the airway epithelium (34) have the capacity to generate reactive oxygen species in response to proinflammatory stimuli, scavenging of NO by inflammation-induced enhanced $O_2^-$ production would be an alternative mechanism underlying the allergen-induced deficiency of biological action of this molecule and subsequent airway hyperreactivity. However, SOD, even up to a concentration of 1000 U/ml, had no effect on the increased responsiveness to methacholine
in the tracheal preparations obtained at 6h after allergen challenge and it did not restore the potentiating effect of L-NAME on methacholine-induced tone in these preparations either, which indicates that inactivation of NO by $O_2^-$ was not involved.

Our study does not exclude the possibility that in allergic asthma enhanced production of $O_2^-$ after allergen challenge is involved in the development of airway hyperreactivity by other mechanisms. Thus, reactive oxygen species in the lung may enhance the inflammatory response and airway reactivity by provoking mediator release and chemotaxis, inhibition of epithelial neutral endopeptidase activity, airway secretion and enhanced vascular permeability (reviewed by Barnes, see ref. 18). Indeed, direct evidence that $O_2^-$ may be involved in the allergen-induced airway hyperreactivity was recently reported by Ikata et al. (35), who showed inhibition of ovalbumin-induced airway hyperreactivity to acetylcholine by a polyoxyethylene modified long-acting SOD in guinea pigs after repeated allergen challenge. In addition, enhanced $O_2^-$ production by polymorphonuclear leukocytes has also been implicated in the airway hyperreactivity of patients with chronic airway obstruction (33).

In conclusion, our study demonstrated that under basal conditions, the inhibitory effect of endogenous NO on cholinergic airway tone is under negative control of $O_2^-$. However, enhanced $O_2^-$ production, by its reaction with NO, may not be the cause of the deficiency of NO in hyperreactive tracheae obtained after the allergen-induced early asthmatic reaction, suggesting that this deficiency is rather due to a reduced cNOS activity.

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REFERENCES


