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Effect of nitric oxide on *in vitro* responsiveness of bovine bronchus and pulmonary vessels

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ABSTRACT: Experiments were conducted in bovine isolated bronchi and pulmonary vessels to test whether nitric oxide (NO) could reduce carbachol and hypoxia or KCl (120 mM) induced contraction.

Segments of bronchus or pulmonary vessels were slipped around a water-filled balloon connected to a pressure transducer, and mounted in 3 ml thermostated chamber filled with Krebs-Henseleit solution equilibrated with different gas mixtures. NO-CO_2-N_2 mixtures containing 10, 50 or 100 ppm NO were prepared. The effect of methylene blue on intrinsic tone and the bias effect of residual red blood cells were assessed.

The results demonstrate that NO has no obvious effect on the intrinsic tone, the force generated by carbachol stimulation, or the spontaneous relaxation after removal of carbachol, in bronchi, with the exception of 100 ppm which increased the relaxing rate in small bronchi. By contrast, 50 and 100 ppm NO caused 53 and 61% decrease in the hypoxia-induced pulmonary arterial contraction, respectively. One hundred ppm NO caused 40, 38, 50 and 66% decrease in the KCl-induced contraction in pulmonary artery (PA), small pulmonary artery (SPA), small pulmonary vein (SPV) and pulmonary vein (PV), respectively. Sodium nitroprusside (10^{-5} M) and isoproterenol (10^{-5} M) reduced the carbachol-induced increase in bronchial pressure by 80% and nearly 100%, respectively. The residual concentration of haemoglobin in the chamber cannot explain the lack of effect of NO on the bronchi.

In the concentration range studied, NO had no relaxing effect on spontaneous relaxation of bronchi. In contrast, NO decreased the arterial and pulmonary venous contraction induced by hypoxia and/or KCl, the effect being more potent on pulmonary veins. This latter finding may have implications in NO inhalation therapy. *Eur Respir J., 1995, 8, 755–761.*

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Nitric oxide (NO) is known as an endothelium-derived relaxing factor (EDRF) in vascular smooth muscle. It stimulates the soluble guanylate cyclase and elevates intracellular cyclic guanosine monophosphate (GMP) [1, 2]. An inhibitory nonadrenergic noncholinergic (i-NANC) mediated response of the bronchus, *i.e.* a neurally-mediated bronchodilator response unaffected by adrenergic or cholinergic antagonists, has recently been demonstrated in a variety of species, including humans [3, 4]. The possible mediators for i-NANC are vasoactive intestinal peptide (VIP) [5], and/or NO [6]. Whether or not NO is an epithelium-derived relaxing factor is still controversial [7].

Studies of NO-induced relaxation, in order to mimick the effect of EDRF in the pulmonary vascular bed, are relatively scarce compared to those performed in the systemic circulation [8]. Recent studies have demonstrated that NO has a dilatory effect in the pulmonary artery both *in vivo* [9], and *in vitro* [10]. However, results concerning the effect of hypoxia on EDRF activity remain conflicting. In some studies, the synthesis of EDRF was suppressed during hypoxia and this inhibition increased its vasoconstricting effect, whereas in others, hypoxia appeared to stimulate EDRF activity [11–13].

Although the effect of inhaled NO on pulmonary vessels has been well-documented in mammals, the local effect of NO on pulmonary arteries (PA) and pulmonary veins (PV) of different sizes is unclear. Meanwhile, the effect of NO on bronchi at similar concentrations, *i.e.* less than 100 ppm (10^{-4}) in the gas phase remains poorly investigated. DUPUY *et al.* [14] found only a slight bronchodilating effect of NO *in vivo*. The action of NO on *in situ* bronchial smooth muscle (BSM) is still unclear. Inhaled NO reaches BSM after passing through the mucosa and a remarkably dense sheet of capillaries. Therefore, it may be speculated that the amount of NO reaching the BSM is very small compared to that in solution in the mucosa, as most of the NO should be converted into derivates in red blood cells. On the other hand, NO could act indirectly on nerve endings in the mucosa and decrease BSM tone.

The aim of the present study was to investigate, in

identical *in vitro* conditions, the effect of NO on both carbachol-induced bovine bronchial contraction and hypoxia- or KCl-induced contraction of pulmonary vessels, the concentration of haemoglobin in the experimental chamber being measured.

Material and methods

Tissue preparation

Adult bovine (6–7 year old) lung lobes were obtained from a local abattoir. The specimens were transported in ice-cold (4°C) Krebs-Henseleit solution (KH) (composition in mM: 118.4 NaCl, 4.7 KCl, 2.5 $CaCl_2.2H_2O$, 1.2 $MgSO_4.7H_2O$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 11.1 D-glucose) to the laboratory and dissected in ice-cold and oxygenated KH less than 1 h after the death of the animals. Bronchial segments of both 3rd order (3–5 mm in diameter, 10 mm in length) and greater than 5th order (1 mm in diameter, 8 mm in length) were isolated. Pulmonary arteries and veins both of large (PA and PV) and small (SPA and SPV) sizes were isolated. The size of the segments were similar to those of the bronchi. Segments were maintained in 50 ml KH bubbled with a 5% CO_2 in O_2 gas mixture at room temperature for 30 min, and then kept at 4°C.

Experimental recordings

The experimental set-up has been described in detail previously [15]. A 3 ml thermostated chamber (Strathkelvin Instruments, Glasgow, Scotland), was adapted to insert a self-made latex water-filled balloon (latex, from Adam Technique, Paris, France) connected with a pressure transducer (P23DB 10V, Statham Inc., Oxnard, CA, USA) near the bottom of the chamber. The chamber was filled with 3 ml KH at 37°C and then closed by a rubber stopper. The gas mixture entered the chamber through a tube near the bottom and left it *via* a tube passing through the stopper. Balloon pressure was recorded using a Labtech Software (Laboratory Technologies Corporation, Wilmington, MA, USA). Small bronchial and pulmonary vascular segments were mounted in the same chamber but a smaller balloon (1 mm in diameter) was used. Three concentrations (10, 50 and 100 ppm) of NO were obtained by mixing the gas provided by a tank filled with 2,000 ppm NO in N_2 with CO_2 and N_2 from another tank through a gas volumeter (Gallus 6/20 D, Schlumberger, France). The mixture was stored for the time of the experiment in a 50 L latex balloon (Bognier Burnet Boyer, Puteaux, France) and was passed through the chamber by means of a pump (Rena 301, Annecy, France). NO concentrations were checked with a chemiluminescence NO analyser (Model -10, Thermo Environmental Instruments Inc., USA). Throughout all of the experiments, NO_2 concentration was not detectable with the analyser (*i.e.* NO_2 concentration was less than 1 ppm in 10, 50 and 100 ppm NO atmosphere).

Protocol

Bronchial segments. Each bronchial segment, weighing about 400 mg for the large ones and 50 mg for the small ones, was kept at 4°C in a 50 ml solution for one day. It was then transferred to a 100 ml oxygenated KH solution at room temperature for 10 min. Finally, the bronchus was slipped around the balloon in the organ bath filled with 3 ml KH equilibrated with room air. The chamber was closed with a rubber stopper and the O_2-free gas mixture (5% CO_2 in N_2) flushed through to remove O_2 from the KH. The pressure in the balloon which served as preload was set at approximately 10 hPa (the pressure that stretches this type of preparation at optimal length [15]), and the tissue was allowed to equilibrate for approximately 30 min. After 5 min of baseline pressure recording, the bronchus was challenged with carbachol (10^{-5} M) under hypoxic control conditions and the pressure was recorded for 25 min. The increase in pressure above baseline is referred to as "active pressure". Thereafter, carbachol was removed from the chamber by wash-out with O_2-free KH solution, and the rate of spontaneous relaxation after removal of carbachol was recorded during 30 min. A second carbachol-induced contraction was then elicited, either in the same control condition or in the test condition, *i.e.* in the presence of a given concentration of NO in the gas mixture (10, 50 or 100 ppm).

According to VAN DEN BRINK [16], the efficacy of a relaxant agonist is inversely related to the amount of functional antagonist present. Because 10^{-5} M carbachol corresponds to the plateau of the concentration-response curve and, hence, may be a supramaximal concentration of agonist, experiments were also conducted using 10^{-6} M carbachol, which corresponds to the concentration producing half the maximal effect (EC_{50}). The concentration of haemoglobin in the solution was measured using Labstix (sensitivity 6.05–25 nM $Hb \cdot L^{-1}$, Bayer Diagnostics, Miles Inc., USA).

In order to assess whether NO had an inhibitory effect on intrinsic tone, methylene blue (10^{-4} M) was added to the solution in the preload state.

In order to check the effects of the stimulators of GMP cyclase and adenosine monophosphate (AMP) cyclase, sodium nitroprusside (10^{-5} M) or isoproterenol (10^{-5} M) were added during carbachol (3×10^{-6} M)-induced maintained contraction.

Pulmonary vascular segments. The protocol for pulmonary vascular segments was similar to that for bronchial segments except for the preload, which was set between 20–30 hPa as determined in preliminary experiments. For PA, the contraction was induced by bubbling the KH solution with an O_2-free mixture containing 5% CO_2 in N_2 gas mixture. When the pressure developed by the arterial segment plateaued, either NO mixture (50 or 100 ppm NO with 5% CO_2 in N_2) or oxygenated mixture (5% CO_2 in O_2) was added. Since we also intended to study the effect of NO on veins, which do not respond to hypoxia, in another set of experiments, vessels (PA, SPA, PV and SPV) were contracted with a K^+ rich (120

mM) solution obtained by substituting KCl for NaCl in equal molar amount. In control groups, the contraction of the different types of vessel were recorded for 20 min. In tested groups, NO was given when the maximum contraction was reached or 5 min after it plateaued for the veins and arteries, respectively.

Contraction of PA with bronchial segments. As the capillary network in the bronchial preparation is dense, it could be suggested that some remaining red blood cells in the solution could bias the response of the bronchus to NO. In order to investigate this hypothesis, a bronchial and a PA segment were set together in the chamber and the PA was challenged with the hypoxic gas mixture with and without NO, assuming that if some haemoglobin was present in the bronchial preparation, the effect of NO on the PA preparation would be hindered.

Data analysis

Each experimental condition was tested in five or six segments of either bronchi or blood vessels isolated from the lungs of different animals. The results are expressed as mean±standard error (SEM). Data are given as either raw or relative values. Relative values refer to the percentage change in the pressure developed during the tested condition with respect to the control condition, *i.e*. the ratio of the value during the 2nd contraction (test contraction C2) to that during the 1st (control contraction C1). Statistical analysis was performed with paired Student's t-tests and Wilcoxon's nonparametric tests to compare mean values of maximal pressures during control and test contractions, as well as mean values of residual pressures after 30 min of relaxation between control and test relaxation. As nonparametric tests and Student's t-tests gave consistent results, only the latter are reported. Unpaired Student's t-tests were used to compare the mean values among different experimental groups. Statistical significance was set at a value of p less than 0.05.

Drugs used

Carbachol, sodium nitroprusside, isoproterenol and methylene blue were purchased from Sigma (La Verpillière, France). NO mixture was purchased from Messer Griesheim (Saint Denis, France).

Results

Effect of NO, sodium nitroprusside and isoproterenol on bronchi

In 3rd order bronchi, none of the three NO concentrations used (10, 50 or 100 ppm) induced a significant change in active pressure or spontaneous relaxation rate of bronchi contracted with 10^{-5} M carbachol (fig. 1). For 10^{-6} M carbachol, the active pressure and relaxation rate in bronchi treated with 100 ppm NO were 7.50±2.38 and 0.40±0.09 hPa·min^{-1}, respectively. These figures were not significantly different from those obtained in control bronchi, *i.e*. in the absence of NO (8.08±1.98 and 0.4±0.09 hPa·min^{-1}, respectively).

In small bronchi, 100 ppm NO had no effect on carbachol-induced contraction (data not shown), but a twofold increase was observed in the spontaneous relaxing rate, from 0.08±0.04 hPa·min^{-1} in the absence of NO to 0.15±0.06 hPa·min^{-1} in the presence of NO ($p<0.05$).

Bronchial intrinsic tone was not significantly altered by methylene blue. Sodium nitroprusside and isoproterenol reduced the carbachol-induced active pressure by 80 and 99%, respectively (fig. 2).

Effect of NO on pulmonary vessels

Effect of NO on hypoxia-induced contraction in the pulmonary artery. Fifty ppm NO induced a partial relaxation of hypoxic preconstricted pulmonary artery (fig. 3a). In the hypoxia group (not challenged with either NO or O_2), the active pressure was maintained, *i.e*. the

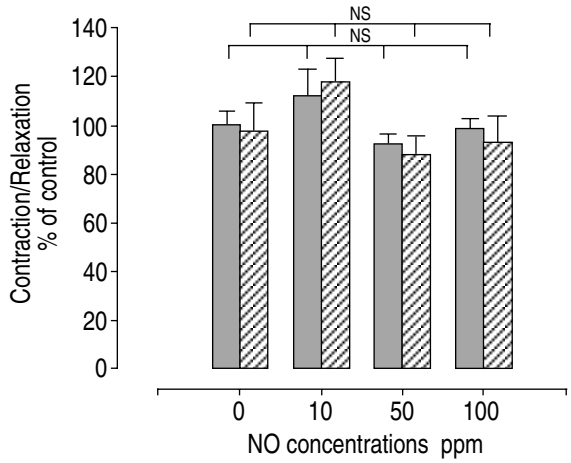

Fig. 1. – Effect of NO on bronchial active pressure and spontaneous relaxing rate of carbachol-induced contraction in bovine isolated bronchial segments. Mean relative values of contraction (▬), and spontaneous relaxing rate (▭) are plotted *versus* NO concentration. Data are given as means for six bronchial segments in each group. Vertical bars indicate SEM. NS: not significant.

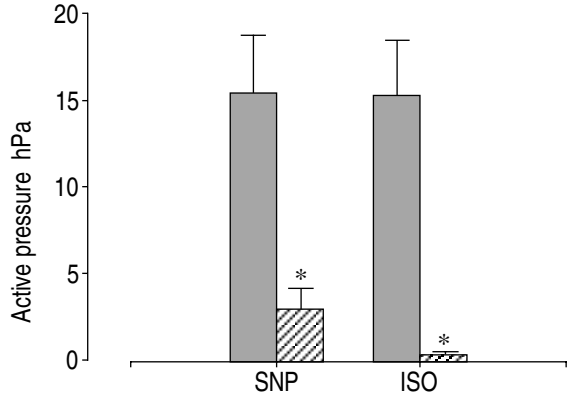

Fig. 2. – Effect of sodium nitroprusside (SNP) and isoproterenol (ISO) on bronchi contracted with carbachol. The maximal active pressure induced by carbachol (3×10^{-6} M; ▬) and the residual active pressure after the administration of sodium nitroprusside (10^{-5} M) or isoproterenol (10^{-5} M) (▭) were plotted. Data are given as means for seven bronchial segments. Vertical bars indicate SEM. *: $p<0.01$.

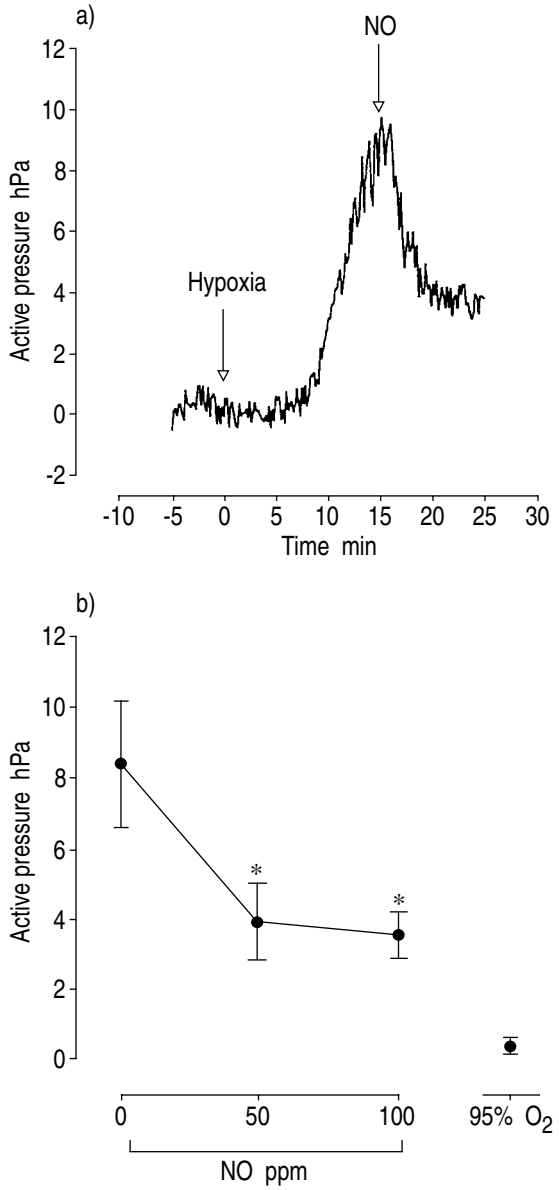

Fig. 3. – NO and reoxygenation in pulmonary artery preconstricted with hypoxia. a) Original trace from a single bovine isolated pulmonary artery segment showing the change in active pressure upon stimulation with hypoxic gas mixture (5% CO_2 in N_2 indicated by arrow) and 50 ppm NO. b) Each preparation was challenged with hypoxia during the first 15 min and then exposed to 50 and 100 ppm NO or 95% oxygen during the following 10 min. Data are given as means for six pulmonary artery segments. Vertical bars indicate SEM. *: $p<0.05$ (compared with NO).

mean pressure remained constant after 10 or 30 min of exposure to hypoxia (8.4±1.8 hPa). In the group receiving 5% CO_2 with O_2, *i.e.* upon reoxygenation, the active pressure decreased sharply from 8.0±1.6 to 0.4±0.2 hPa after 5 min O_2 exposure. In the hypoxia group to which NO was added, 50 and 100 ppm NO decreased the active pressure from 8.4±1.8 to 3.9±1.1 hPa and from 9.2±2.1 to 3.6±0.7 hPa after 5 min of NO exposure, respectively ($p<0.05$) (fig. 3b).

Effect of NO on KCl-induced pulmonary vascular contractions. NO decreased KCl-induced contraction in all

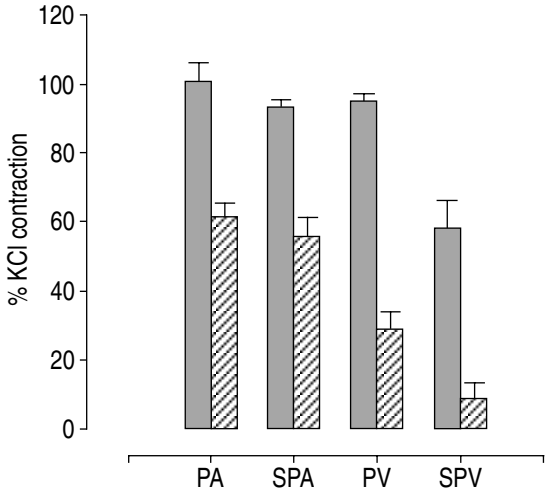

Fig. 4. – Effect of NO (100 ppm) on KCl (120 mM) induced pulmonary vascular contraction in pulmonary vessels. Mean relative values of active pressure (*i.e.* P15/Pmax×100%) induced by KCl (120 mM) in the presence (▬) or absence (▭) of 100 ppm NO. Data are given as means for 6–7 vascular segments. Vertical bars indicate SEM. PA: pulmonary artery; SPA: small pulmonary artery; PV: pulmonary vein; SPV: small pulmonary vein; P15: residual pressure 15 min after the maximal contraction was reached; Pmax: maximal pressure.

of the vessels (PA, SPA, PV and SPV) studied. The vasodilating effect of NO was stronger on veins than on arteries (fig. 4).

Effect of NO on bronchi and pulmonary arteries. When PA segments were tested in combination with bronchi, the results were similar to those when PA segments were tested alone, *i.e.* the presence of the bronchial tissue did not impair NO-induced decrease in hypoxic active pressure (active pressure 7.5±0.8 hPa during hypoxia and 3.7±0.5 hPa after 50 ppm NO).

Analysis of haemoglobin concentration

Haemoglobin concentration after the 2nd contraction was similar in the bronchi to that in PA, 0.067±0.010 and 0.080±0.007 µM, respectively. The amounts of haemoglobin in the chamber (masses) were 3×10^{-3} × molar concentration, *i.e.* 0.20±0.03 and 0.24±0.02 nmol.

Discussion

In the present study, the effect of NO was examined in contracted bovine isolated bronchi and pulmonary vessels. The results show that NO at concentrations up to 100 ppm has no obvious effect on carbachol-induced bronchial contraction, whereas at identical concentrations it significantly decreases both hypoxia and KCl-induced pulmonary vascular contraction. The lack of effect of NO on bronchial contraction is not due to its inactivation by haemoglobin present in residual red blood cells in the bronchial tissue.

NO has recently been identified as an important endothelium-derived relaxing factor, that causes relaxation of

vascular smooth muscle [9]. It has also been suggested that NO, or some related compounds, may be the epithelium-derived relaxing factor (EpDRF) [17, 18]. In this respect, GUPTA and PRASAD [19] have demonstrated that, in rabbits, an inhibitor of NO synthesis, L-N^G-monomethyl arginine (L-NMMA), significantly reduces H_2O_2-induced tracheal smooth muscle relaxation, suggesting that NO may be an intrinsic airway smooth muscle relaxing factor. Moreover, DUPUY *et al*. [14] found that, in guinea-pigs, NO inhibits methacholine-induced bronchoconstriction in a concentration-dependent manner between 5–300 ppm [14]. In contrast, HÖGMAN *et al*. [20] have shown that NO inhaled at 80 ppm has no significant effect on airway tone in healthy human volunteers. Furthermore, WATSON *et al*. [21] reported that neither NO nor VIP appears to be released during the stimulation of the preganglionic cervical nerve, which induces a contraction of guinea-pig tracheal smooth muscle, but both are released during transmural stimulation, NO being released independently of the epithelium. The difference in NO-induced effect in airways may be due to the differences in species. Bovine samples were chosen in this study, as they seem to have biological characteristics, among other species, close to those of humans, in that they have an intrinsic tone [22] as well as excitation-contraction coupling mechanisms similar to those in humans [23].

In our study, carbachol-induced bronchial contractions were unaltered by NO at concentrations of 10–100 ppm. However, a twofold increase in spontaneous relaxation was observed in small bronchi. According to other results [24], the concentrations we used should be sufficient to elicit a bronchodilatory effect. Taking into account the solubility of NO in KH at 37°C, under the equilibrium conditions, the concentration of NO in the liquid phase are 0.02 and 0.2 µM for 10 ppm and 100 ppm in the gas phase, respectively. It is worth noting that in many experiments on smooth muscle, higher concentrations were used, about 20 times higher than those used in the present study [25]. Such high concentrations correspond to NO concentrations of 200–2,000 ppm in the gas phase, which are highly toxic and even lethal when inhaled [26]. Possible reasons for this discrepancy could be as follows: 1) bronchi from different species may display different responses to NO, we used bovine bronchial segments whereas HÖGMAN *et al*. [24] used rabbit trachea; 2) exogenous NO may have a different effect from the NO radical synthesized by epithelium; 3) the slight bronchodilator effect observed *in vivo* in humans [20] could be due to the stimulation of nervous receptors in the mucosa; and 4) the different orders of bronchi may display a different responsiveness to agonists or inhibitors of the contraction.

To comprehensively address the issue of the effect of NO in airways, the following methodological considerations deserve further discussion. Firstly, NO reacts with O_2 in a time and concentration-dependent manner. As a consequence, in the present study, gas mixtures used in presence of NO were O_2-free. As shown previously using the same method in the same preparation, hypoxia has no significant effect on active pressure during carbachol-induced bronchial contraction [15]. Therefore, in both control and tested groups, experiments were performed under hypoxic conditions to minimize the effect of NO-O_2 reaction.

Secondly, as the experiments lasted 30 min in the presence of NO, it could be argued that this period was not long enough for NO to diffuse through the sample. Several arguments fail to support this hypothesis: 1) NO is a small molecule and, hence, its diffusion should be faster than that of any other pharmacological compound; 2) NO rapidly dilates pulmonary artery under the same experimental conditions; and 3) since bronchus samples contain some red blood cells, it could be suggested that some haemoglobin released by the capillary network reacts with NO to form NO-Hb, and, therefore, less NO molecules or even no NO, reach the smooth muscle. This is unlikely because in a first step the sample was in a 50 ml solution for one day. It can be expected that red cells would be diluted in the solution after this delay. Moreover the sample was transferred into a 100 ml KH solution at room temperature before the experiment for 10 min and was finally placed in the experimental chamber which contained 3 ml KH. These two latter operations should dilute the remaining red cells to a negligible concentration. However, the amount of haemoglobin needed to significantly decrease the concentration of NO is also very small. The amount of NO flowing through the chamber is: $V' \cdot F_{NO} \cdot t$, where V' is the gas flow passing through the chamber (2.2 mmol·min^{-1}), F_{NO} the fraction of NO in the gas phase and t the duration of the bubbling phase. For 10, 50 and 100 ppm NO, the amounts of NO passing through the chamber, during 15 min, were 0.3, 1.5 and 3 µmol, respectively. Therefore, to avoid any bias in the experiments, owing to the tetrameric structure of the haemoglobin molecule, the amount of haemoglobin in the chamber should be less than 0.075 µmol for 10 ppm NO and less than 0.75 µmol for 100 ppm NO. Indeed, the amount of haemoglobin was less than these figures in all cases. Furthermore, as PA segments reacted in a similar way whether or not bronchial samples were present, and as the amounts of haemoglobin were not different in the bronchus and PA groups, these results strongly suggest that the amount of residual haemoglobin provided by the bronchial capillary network was not a confounding variable in the present experiments.

Sodium nitroprusside (10^{-5} M), a GMP cyclase stimulator, relaxed the carbachol-induced bronchial contraction by 80%. Therefore, the absence of relaxing effect of NO on bronchi should be due to the low concentration of NO provided by a 100 ppm concentration in the gas phase (2×10^{-7} M in the liquid phase), *i.e*. the amount of NO coupled with GMP cyclase was not sufficient to produce a relaxation. However, this low concentration was sufficient to increase the spontaneous relaxing rate after carbachol-induced contraction in small bronchi.

Our results on PA agree with those of other investigators [27, 28]. Fifty ppm NO decreased hypoxia-induced active pressure to about 53% (fig. 3b). Several investigators have hypothesized that the hypoxia-induced PA contraction was, at least in part, due to the depressed synthesis of EDRF by endothelium [10, 28–30]. As a

consequence, direct administration of NO should be able to reverse, at least in part, the hypoxia-induced contraction caused by decreased EDRF as observed in the present study.

To assess the local effect of NO on the pulmonary vascular bed, veins and arteries were contracted with KCl to make the results comparable. This set of experiments revealed that the relaxant effect of NO on small vessels was similar to that on PA; 100 ppm NO caused a decrease of 40–50% in the active pressure induced by KCl. The relaxant effect of NO was significantly greater in large pulmonary veins, in which 100 ppm NO induced about 66% decrease in active pressure induced by KCl. As the effect of NO on the different segments of the pulmonary vessels is not well-documented, this finding is not comparable with previous studies. Although the mechanism responsible for the stronger effect of NO in veins than arteries is not clear, this finding may have clinical implications when inhaled NO is used in patients with pulmonary hypertension or chronic obstructive pulmonary disease (COPD). Due to the more potent relaxant effect of NO on large PV and SPV than on PA, NO could reduce blood retention in pulmonary circulation. A preliminary study of pulmonary haemodynamics in patients with acute respiratory disease syndromes suggests that pulmonary venous resistance decreases more than arterial resistance when 35 ppm NO is inhaled [31].

In conclusion, unlike its effect in pulmonary vessels, NO has little effect on airway contraction *in vitro*. The more potent effect of NO on pulmonary veins than on pulmonary arteries requires further investigation, since it may have implications in the clinical use of NO inhalation therapy.

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