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The Role of the Inhibitory Nonadrenergic Noncholinergic System in Antigen-Induced Pulmonary Hypersensitivity

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Abstract

To study the role of the inhibitory nonadrenergic noncholinergic (i-NANC) system in regulating bronchial reactivity during antigen challenge, we first tested a blocker of the i-NANC system (oxyhemoglobin, HbO_2, 2.5 μm) on the relaxation response of guinea pig tracheal strips (n=6) *in vitro* to electrical field stimulation (ES) in the presence of atropine (1 μg/ml) and propranolol (2 μg/ml). Fresh HbO_2 significantly inhibited 35.3±4.5% ($P<0.001$) of the NANC relaxation response. Secondary, 26 anesthetized, ovalbumin-sensitized animals were divided into three groups: antigen challenged (n=10), pretreated with HbO_2 (13 mg/kg) and challenged (n=9), and treated with HbO_2 only (n=7). Pulmonary resistance (R_L) and dynamic compliance (Cdyn) were measured 15-20 min prior to (baseline) and up to 30 min after antigen or HbO_2 injection. Antigen challenge alone induced early maximal respiratory changes: R_L increased 1646±115% above baseline (2 min) whereas Cdyn decreased 42±10% below baseline (4 min). These changes returned to baseline within 15 min. Pretreatment with HbO_2 increased peak respiratory responses induced by antigen [R_L, 3728±1680% above baseline; Cdyn, 69±7% below baseline ($P<0.05$)]. HbO_2 delayed significantly ($P<0.05$) the time for recovery of R_L and Cdyn. HbO_2 alone had little effect on respiratory parameters. We conclude that HbO_2 may antagonize the i-NANC system in the airway and this antagonism may accentuate pulmonary hypersensitivity during acute antigen challenge.

Key Words: asthma, airway reactivity, bronchoconstriction, oxyhemoglobin

Introduction

For years change in or malfunction of the inhibitory nonadrenergic noncholinergic (i-NANC) system has been postulated as a possible cause for hyperreactive airways, such as asthma (1). Bowman et al. (5) demonstrated that oxyhemoglobin (HbO_2) reversibly blocks the i-NANC in the bovine retractor penis muscle *in vitro*. Relaxation of the muscle could be elicited by either electrical field stimulation of the inhibitory system (24) or by the inhibitory factor isolated from the same tissue by Ambache et al. (2). Jin et al. (13) and Selemidis et al. (23) demonstrated that HbO_2 acts as the scavenger for nitric oxide (NO) which is the transmitter for the i-NANC system (4, 7).

Previously, we showed that HbO_2 plus transmural stimulation blocked the late relaxation following antigen challenge in the guinea pig tracheal pouch (15). Similarly, Miura et al. (22) demonstrated that antigen exposure induces impairment of neural NO-mediated relaxation in the guinea pig airways *in vitro*. According to the above results, we can further

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examine the relationship between the malfunction of the i-NANC system and hyperreactive airways *in vivo*. In this study, we first demonstrated again the blocking action of HbO_2 on the i-NANC system in the guinea pig trachea *in vitro*. Subsequently, we applied HbO_2 to the anesthetized antigen-challenged guinea pig (*in vivo*). We used changes in pulmonary resistance (R_L) and dynamic compliance (Cdyn) to assess the degree of bronchial hypersensitivity.

Materials and Methods

In Vitro Study

Six spirally cut tracheal strips (4 cm stretched) from normal control male Hartley strain guinea pigs weighing 273±12 g were used to examine if HbO_2 would directly block the inhibitory NANC system in the airway. Animals were anesthetized with sodium pentobarbital (33.3 mg/kg, ip) and exsanguinated via the abdominal aorta. The trachea was immediately excised and placed in warm physiological salt solution (PSS) (24) where blood and connective tissue were removed, spirally cut and mounted between two parallel platinum electrodes (7 mm apart). Following this 5 min preparation period, the tracheal strip was immersed, with electrodes, into an organ bath filled with PSS, and maintained at 37°C, and bubbled with a gas mixture of 95% O_2 and 5% CO_2. The PSS composition was (in mmol/l): NaCl 117.50; KCl 5.37; $CaCl_2$ 2.52; $MgCl_2$ 2.10; NaH_2PO_4 1.17; $NaHCO_3$ 25.50; and glucose 11.00. The pH of the solution was about 7.42. The trachea strip was attached by string to an isometric transducer (Grass FT 03 C) and resting tension was adjusted to 3.0±0.1 g initially for half an hour equilibration period. Electrical stimulation (ES) (Grass S48 Stimulator) was at 40 volts with 40 pulses per sec of 2 msec duration for 10 sec. ES was repeated only after tension had returned to the baseline level or had leveled off (approximately 8-10 min).

A steady state response to ES in the presence of a cholinergic blocker atropine (1 μg/ml) and a β-adrenergic blocker propranolol (2 μg/ml) was obtained after 4-8 ES over a time period of 0.5 to 1.5 hr. The PSS with atropine and propranolol was changed every 0.5-1 hour and care was taken to avoid exposure of tissue to air during washes. Fresh oxyhemoglobin was prepared as described by Bowman et al. (5) with slight modifications. Commercially available hemoglobin (Sigma, human hemoglobin, twice crystallized, type IV) was dissolved in saline (15 mg/ml) and then sodium dithionite (2 mg/ml) was added to reduce the hemoglobin. Dithionite was removed immediately by passing the solution through a Sephadex (G-25) column. Oxyhemoglobin concentration in the effluent was measured by a cyanmethemoglobin method. The solution was used within 20 min of its preparation. Following a period with stable response to ES, HbO_2 was then added to the organ bath at 0.16 mg/ml. Four to five ES were completed in the presence of HbO_2 before the bath was again changed. ES was continued until active relaxation response had recovered to pre-HbO_2 level.

In Vivo Study

Twenty-six male Hartley strain guinea pigs weighing 341±10 g were sensitized and divided into three groups: antigen challenged (n=10), pretreated with HbO_2 and challenged (n=9), and treated with HbO_2 without antigen challenge (n=7). All animals were sensitized with 200 mg ovalbumin (Sigma, St. Louis) in 1 ml saline (ip injection) 10 days prior to the experiment. Animals were initially anesthetized with ketamine (44 mg/kg, im) and sodium pentobarbital (12 mg/kg, ip) followed by supplements of pentobarbital as needed. Challenged animals were injected (iv) with 40 μg ovalbumin in 1 ml saline. A full description of the experimental setup and justification for the methods have been made previously (16). Briefly, the trachea, jugular vein, and carotid artery were cannulated. The animal breathed via bias flow and changes in the flow (detected by a pneumotachograph) were used to determine flow ($\dot{V}$) and tidal volume (V_T). Esophageal pressure (Pes) was measured by a 0.5F Mikro-tip pressure sensor. Outputs of V_T, $\dot{V}$, and Pes were adjusted to be in phase so that the method of Amdur and Mead (3) could be used to determine R_L and Cdyn.

Fifteen to thirty min prior to antigen injection, baseline values were repeatedly determined 4-6 times and 3 breaths were analyzed each time. Time at antigen injection was designated as zero min and data were collected at each min for the first 6 min, then at 10, 15, 20, and 30 min. At each sample period, three breaths were analyzed for respiratory mechanical parameters, R_L and Cdyn. For the HbO_2 treated groups, fresh HbO_2 (13.4 mg/kg) was intravenously injected 2 min before the antigen or designated zero time. HbO_2 was made as described above. Mean values obtained prior to zero time were used as baseline values (100%) for each individual animal. After the zero time, respiratory changes were expressed as percentage of this baseline.

Statistical Analysis

All values are given as means±SE. Paired Student's t-test was used to establish significant *in vitro* differences. For *in vivo* studies, linear correlations were used to establish significance where

Table 1. Baseline Data for the Three Groups of Anesthetized Guinea Pigs.

Group	n	Body wt. g	R_L $cmH_2O/ml/s$	Cdyn ml/cmH_2O
Antigen challenged	10	358	0.21	0.59
		±10	±0.03	±0.06
Antigen + HbO_2	9	343	0.17	0.93*
		±11	±0.02	±0.12
HbO_2 alone	7	317	0.17	0.78
		±30	±0.03	±0.06

Values are means ± SE. n, number of animals; R_L, pulmonary resistance; Cdyn, dynamic lung compliance; and HbO_2, oxyhemoglobin. *Significant difference ($p<0.05$) between the two antigen challenged groups.

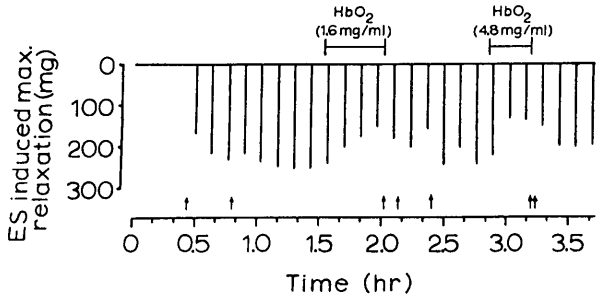

Fig. 1. *In vitro* peak relaxation response (reduction in tension) for one tracheal strip to repeated electrical field stimulation (ES) in the presence of atropine and propranolol, with and without oxyhemoglobin in the bath. ↑ indicates wash. Baseline tension was 3.0±0.1 g.

appropriate. Statistical significance was assumed for $p<0.05$. Comparisons of *in vivo* differences between all three groups at each time point were made by using analysis of variance. Multiple *t*-test (one tailed) was performed if the analysis of variance resulted in significance (6). This procedure may lead to concluding statistical significance due only to multiple sampling. To ensure against this multiple sampling problem, we calculated the probability of this type error (29). We accepted a probability of this type error of 5% or less. For example, we accepted significant differences for each variable (i.e., R_L and Cdyn) in the case where three or more times out of the ten time points (1-30 min) were significant ($P<0.05$) by t-test because the calculated probability was 1% or less.

Results

In Vitro Study

Fig. 1 shows an example of maximal active relaxation to ES before, during, and after HbO_2 (1.6 and 4.8 mg/ml) in the presence of atropine (1 μg/ml) and propranolol (2 μg/ml). Propranolol at 2 μg/ml completely blocked β-adrenergic relaxation effect of Levophed (2.5 μg/ml) in preliminary studies. Prior to HbO_2 and in the presence of atropine and propranolol, the six tracheal strips had an average steady state response to ES of 322±33 mg of active relaxation. This relaxation was significantly ($P<0.01$) reduced to 208±33 mg (35% reduction) during incubation with HbO_2 (1.6 mg/ml). The maximal inhibition occurring 26±5 min after HbO_2 was added to the bath. Repeated washing returned active relaxation back to near original levels.

Higher doses of HbO_2 (1.4 - 3.0 times) were tested on four occasions, as in Fig. 1, and greater suppression of the NANC relaxation was shown in all cases. Our studies also showed that propranolol (1×10^{-6} M and 6.8×10^{-6} M) reduced the total relaxation due to ES by 41±4% (n=5), similar to findings of Southrada et al. (24, 25).

In Vivo Study

Mean respiratory values of the baseline period (before antigen or HbO_2) for each group are given in Table 1. Cdyn was statistically lower in the antigen challenged group compared to the HbO_2 pretreated, antigen challenged group.

Fig. 2 presents temporal changes in RL and Cdyn (as % of baseline) for all animals. Three of the animals in the antigen challenged group died (arterial blood pressure < 10 Torr and respiratory arrest) 3-6 min after antigen infusion (zero time), while in the HbO_2 pretreated and challenged group three animals died 8-18 min after antigen infusion. Thus, the number of animals decreased during the 30 min observation period. Also, the values of 10500% R_L and 0% Cdyn were used when an animal attempted to breath (ie, alteration in Pes) but there was no air flow in or out of the lungs. This was because 10,493% R_L and 3% Cdyn were the largest actual changes ever measured with air flow.

Antigen challenge increased mean R_L to a

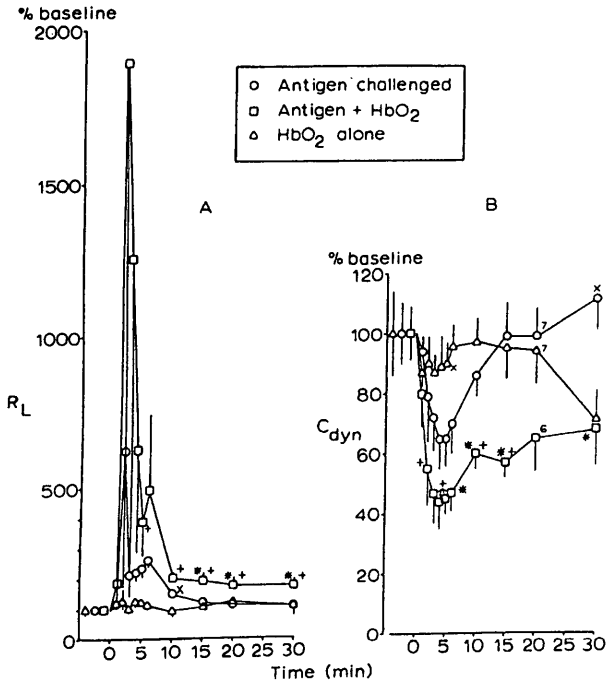

Fig. 2. Temporal changes in pulmonary resistance (R_L) and dynamic compliance (Cdyn) values in antigen challenged, oxyhemoglobin (HbO_2) pretreated + antigen, and HbO_2 alone groups. Bars during the baseline period represent ± 1 SE of intra-group variation. Statistical differences ($P<0.05$) between groups: *, antigen challenge vs. HbO_2 pretreated and antigen; +, HbO_2 pretreated and antigen vs. HbO_2 alone; x, antigen challenge vs. HbO_2 alone. We accepted significant differences for each variable only where three or more times out of the ten time points (1 - 30 min) were significant by t-test (see Methods).

maximum of 1746±115% of the baseline value at two min and decreased mean Cdyn to a minimum of 58±10% of the baseline value at four min. Both parameters had returned to near baseline values by fifteen minutes. Pretreatment with HbO2 increased R_L: peak at 3828±1680% of baseline at 2 min post antigen, but this increase was significant only at 15-30 min. On the other hand, Cdyn was significantly decreased starting from three min and maximal change (31±7%) occurred at 4 min. HbO_2 treatment alone caused slight and insignificant changes in Cdyn and R_L. Compared to the HbO_2 + antigen group, HbO_2 alone had higher Cdyn ($p<0.05$) at 1-15 min and lower R_L ($p<0.05$) at 15-30 min. Evaluating the respiratory responses solely from animals that survived during the entire thirty min showed similar temporal changes in both R_L and Cdyn to those described above for all animals as a whole, although changes in R_L and Cdyn were not as severe during the early phase (0-10 min). Antigen challenge increased R_L and decreased Cdyn maximally to 621±488% (2 min) and 79±15% (4 min) of baseline, respectively. Pretreatment with HbO_2 increased R_L to 1895±1720% (2 min) and decreased Cdyn to 44±9% (6 min) of baseline post antigen.

Discussion

Oxyhemoglobin, at the concentration used, blocked 35% of the inhibitory NANC system *in vitro*. Although not statistically significant, HbO_2 pretreatment tended to increase R_L, yet significantly to decrease Cdyn in the early responses (0-10 min) to antigen challenge. HbO_2 significantly delayed the time for recovery of RL and Cdyn (from 10 min to > 30 min). Thus, these data obtained from this blocking agent for the inhibitory NANC system suggest that the NANC system may play an important role in the pulmonary hypersensitive reaction to antigen, at least in enhancing recovery.

In the *in vitro* preparation, we were able to demonstrated only a partial (35%) but significant blockage of the ES induced NANC relaxation in the tracheal strip by a concentration of HbO_2 that gave a 100% blockage in the retractor penis muscle (5). The difference in the blocking response may be due to differences in tissue or in activity of the HbO_2. As in the earlier *in vitro* study with HbO_2 (5), blockage of the i-NANC system took time to develop and was reversible with repeated washes. Higher dosage of HbO_2 indicated that the inhibitory NANC system was not maximally blocked by the level of HbO_2 used. HbO_2 did not seem to directly affect muscle tone since resting tone returned to near original level after each ES (Fig. 1). In the *in vitro* system, Miura et al. (22) showed that a NO synthase inhibitor, N^w-nitro-L-orginine methyl ester, blocks while antigen treatment attenuates the i-NANC responses.

The mechanism by which HbO_2 blocks the i-NANC system may be related to its scavenging NO, which is the transmitter of the i-NANC system. The fact that only the hemoglobins with ferrous iron (oxyhemoglobin and carboxyhemoglobin) are effective in blocking NANC relaxation due to both electrical field stimulation and NANC inhibitory factor (5) suggest that HbO_2 acts either at the receptor site or directly on the inhibitory neural transmitter. The greater activity of the ferrous hemoglobin form is also seen in the ability of hemoglobin to constrict cerebral arteries (27). Configurational change which may occur between ferrous and ferric states may modify hemoglobin's blocking ability. Alternatively, superoxide radicals released during auto-oxidation of HbO_2 to methemoglobin (20, 28) might cause co-oxidation and inactivation of the inhibitory neural transmitter for the NANC system.

The relaxant actions of NO, NO-donors, EDRF and the nitrergic transmitter in some tissues can be inhibited by oxyhemoglobin (5, 12, 18). The effect is due to the affinity of the haem group for NO (11) and

the subsequent oxidation of the NO/oxyhemoglobin complex to methemoglobin and NO_3^+ (14). In the case of NO-donors, it can be assumed that oxyhemoglobin has a higher affinity for NO than the original ligand.

Our baseline values for R_L and Cdyn (Table 1) are similar to those obtained by Mills and Widdicombe (19) of 0.19 $cmH_2O/ml/s$ and 0.54 ml/cmH_2O and to our previous study (16) of 0.19 $cmH_2O/ml/s$ and 0.72 ml/cmH_2O, respectively, in the anesthetized tracheotomized guinea pig. Although some respiratory parameters (ie, $\dot{V}_E$, V_T, and f) were correlated with the changes in R_L and Cdyn, and it has been shown that changes in lung volumes (ie, FRC) and dynamic factors can alter R_L and Cdyn (9), this does not prove to be the cause and effect relationship here. We have previously shown (1) that changes of respiratory values, in the order of magnitude measured here, do not contribute significantly to alterations in R_L and Cdyn. It is possible that concurrent changes in respiratory patterns, R_L and Cdyn, are induced by a common factor.

Antigen challenge in this study caused an increase in R_L and a decrease in Cdyn which lasted less than fifteen minutes, a result similar to our previous findings (16). The degree of maximal change in R_L and Cdyn in the present study were not significantly different (*t*-test, $P>0.05$) from the previous study in which R_L increased to 231±28 % and Cdyn decreased to 72±10% of baseline, with all animals surviving for 30 min. Unlike the earlier study, where peak changes in R_L and Cdyn occurred simultaneously at 5 min, R_L peaked at 2 min in this study. This shift in maximal response time and magnitude was largely influenced by the animal that did not survive the antigen challenge.

Pretreatment with HbO_2 was demonstrated *in vitro* to block the inhibitory NANC system and thus should enhance the respiratory responses to antigen challenge, assuming that this NANC system was at least partially active in the sensitized guinea pig. Souhrada et al. (25) have shown *in vitro* that airway smooth muscle from sensitized guinea pigs, chronically exposed to antigen, demonstrated a decrease of only 20% in overall relaxation (including the NANC system). Ko and Lai (15) showed also that HbO_2 plus transmural stimulation blocked the late relaxation following antigen challenge *in vivo*. Similarly, Miura et al. (21) showed that antigen exposure causes dysfunction of the *in vivo* i-NANC system in cats. HbO_2 did indeed enhance the decrease in Cdyn during most of the hypersensitive period. The reason for the insignificant increase in R_L by HbO_2 during the first ten min may have been related to one of several reasons: 1) The wide animal variation in response to antigen with and without HbO_2 pretreatment may have obscured any real difference. 2) Alternatively, the NANC system may be more active in the smaller, more peripheral airways which influence Cdyn more than R_L (10). Since the NANC system is reported to travel through the vagus (8) and morphological studies (17) indicate vagal innervation primarily in the upper airways, this explanation is doubtful.

Another significant finding of this study is that the NANC system is shown to play an active role in respiratory recovery from a hypersensitive state *in vivo*. This is evidenced from the fact that pretreatment of HbO_2 at least doubled the time of recovery for both R_L and Cdyn following antigen.

Comparison of the results from the *in vitro* and *in vivo* studies raises two questions: 1) It is not clear why the latent period of HbO_2 is much longer *in vitro* than *in vivo*. 2) Whether information obtained from the *in vitro* studies can be applied to the *in vivo* condition. The explanation for the first question might be related to diffusion distances. In the *in vitro* preparation, HbO_2 needs to diffuse through the epithelium and interstitial space to reach the neuromuscular junction (possible location of receptors) with long diffusion distance. *In vivo* capillary distribution reduces this diffusion distance and may accelerate the suppression of the NANC system. Diffusion distances were also speculated as a possible cause for differences between *in vivo* and *in vitro* airway response to antigen challenge (16).

For the second question, the *in vitro* study used only part of the airway, the trachea. Using the *in vitro* data, we make a rough estimation for the *in vivo* total airway system here. HbO_2 *in vitro* produced a maximum 35% inhibition of the NANC system. Since R_L, with laminar flow, is inversely related to airway radius to the fourth power, the predicted increase in R_L with pretreatment of HbO_2 would be 5.6 times that produced by antigen challenge alone. We found only 2.3 times increase due to HbO_2 pretreatment in this study. This difference is not surprising since the predicted increase is based on several unconfirmed assumptions as follows: 1) Air flow remains laminar. 2) HbO_2 has the same effect on all other airways as the trachea. 3) The length-tension relationship of airways is linear. Actually, the relationship is not linear (26), direct comparison requiring equal resting tension *in vitro* and in vivo. For these reasons and others, in vitro results are difficult to directly compare to in vivo studies in quantitative terms but they are similar qualitatively.

In summary, we demonstrated that HbO_2 significantly attenuated the i-NANC system *in vitro*. HbO_2 pretreatment significantly decreased Cdyn in the early responses to antigen challenge and delayed

the time for recovery of R_L and Cdyn. Our data suggest that the i-NANC system may play an important role in enhancing recovery of the pulmonary hypersensitive reaction to antigen.

Acknowledgments

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