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Chronic exposure to hypoxia attenuates contractile responses in rat airways in vitro: a possible role for nitric oxide

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Abstract

We investigated the effect of chronic hypoxia (10% O_2 for 14 days) on airway responsiveness in rats. Chronic hypoxia significantly ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) attenuated contractions evoked by methacholine (10^{-9}–3×10^{-4} M), endothelin-1 (10^{-10}–3×10^{-7} M) and potassium chloride (10^{-3}–7×10^{-2} M) in rat isolated trachea. To investigate this attenuation, we studied the effect of epithelial removal, indomethacin (3×10^{-6} M), and L-nitro arginine methyl ester (L-NAME, 10^{-4} M), on contractile responses in control and chronically hypoxic rat trachea. Indomethacin did not alter contractions evoked by methacholine or endothelin-1 in control or hypoxic rats. In contrast, epithelial removal and L-NAME both significantly potentiated responses to methacholine and endothelin-1 in trachea from control and chronically hypoxic rats. In separate experiments, tracheal rings were first contracted with methacholine (10^{-6} M) and then relaxed, either by the nitric oxide donor sodium nitroprusside or by the β_2-adrenoceptor agonist, salbutamol. Sodium nitroprusside was significantly ($P < 0.001$) more effective at reversing induced tone in tracheal rings from chronically hypoxic than control rats. Salbutamol, however, was equally effective in chronically hypoxic and control rats. These results suggest that, in trachea from both control and chronically hypoxic rats, contractile responses to methacholine and endothelin-1 are inhibited by nitric oxide, probably released from the epithelium. The attenuation of contractile responses in airways from chronically hypoxic rats may be due to an enhanced guanylyl cyclase activity and hence, an increased response to nitric oxide.

Keywords: Airway reactivity; Methacholine; Endothelin-1; Epithelium; Indomethacin; Nitric oxide (NO)

1. Introduction

The effect of chronic hyperoxia on airway responsiveness has been extensively studied. For example, chronic hyperoxia increases the responsiveness of the airways to bronchoconstrictor stimuli in vivo and in vitro in immature and adult rats (Hershenson et al., 1992a,b, 1994; Szarek et al., 1995). In contrast, little is known about the effect of long-term hypoxia on airway responsiveness. Acute exposure to hypoxia induces an increase in airway responsiveness to inhaled spasmogens in sheep in vivo (Ahmed and Marchette, 1985). Furthermore, we have shown that acute hypoxia potentiates contractile responses to methacholine in isolated bovine bronchi (Clayton et al., 1996). Interestingly, a recent study showed that airways isolated from patients with Eisenmenger's syndrome (which results in severe and prolonged hypoxemia) exhibit a markedly enhanced contractile response to cholinergic stimulation (McKay et al., 1998).

Given that prolonged hypoxia can be a factor in several pulmonary diseases, we investigated the effects of chronic hypoxia on airway responsiveness. We compared responses to the spasmogens methacholine, endothelin-1 and potassium chloride in isolated trachea from control and chronically hypoxic rats. Our initial studies indicated that the contractile responses evoked by each of these agonists were significantly reduced in trachea from chronically hypoxic rats, therefore, further experiments were carried out in an attempt to elucidate the mechanisms underlying this attenuation. Several studies indicate that chronic hy-

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peroxia increases airway epithelial and smooth muscle layer thickness (Hershenson et al., 1992a,b, 1994; Szarek et al., 1995) and that this increase in airway wall thickness correlates with the increase in airway reactivity (Hershenson et al., 1992a). More recently, however, Szarek et al. (1995) showed that in hyperoxic rats, the increase in airway reactivity occurs before the remodelling process develops, indicating that other mechanisms are also involved in the development of hyperoxia-induced airway hyperresponsiveness. Interestingly, Hershenson et al. (1994) showed that pretreatment with the cyclooxygenase inhibitor indomethacin or removal of the epithelium reversed hyperoxia-induced airway hyperresponsiveness in immature rats in vitro. This indicates that epithelium-derived prostanoids contribute to the development of hyperoxia-induced enhancement of airway responsiveness. In this present study, we sought to examine if the release of (inhibitory) cyclooxygenase metabolites could also be responsible for the attenuation of contractile responses caused by exposure to chronic hypoxia.

Alternatively, several studies indicate that nitric oxide synthase expression is upregulated in the lungs of chronically hypoxic rats (Xue et al., 1994; Shaul et al., 1995). To test the hypothesis that release of nitric oxide was responsible for the attenuation of contractile responses in chronically hypoxic rat airways, we also studied the effect of the nitric oxide synthase inhibitor, L-nitro arginine methyl ester (L-NAME), on responses to methacholine and endothelin-1 in trachea from control and chronically hypoxic rats.

2. Methods

2.1. Development of chronic hypoxia

Rats were made chronically hypoxic according to the method of MacLean et al. (1995). Briefly, 28–30-day-old male Wistar rats (specific pathogen free) were placed in a hypobaric chamber which was depressurised over two days to 550 mbar (leading to a reduction of the oxygen concentration to 10%). The chamber was maintained at 21°C–22°C and ventilated with air at approximately 45 l/min. Rats were reared in these hypoxic/hypobaric conditions for two weeks and sacrificed immediately after removal from the chamber. Age matched control rats were reared alongside the hypobaric chamber but were maintained in room air throughout this time.

2.2. Tissue collection and preparation

Rats were killed by overdose of sodium pentobarbital and the lungs carefully dissected out and placed in oxygenated Krebs–Henseleit solution of the following composition (mM), NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. The trachea were excised and dissected free of adhering connective tissue in oxygenated Krebs–Henseleit solution and cut into intact cylindrical rings without damage to the epithelium. Each ring preparation was approximately 4 mm long with an internal diameter of ~ 2 mm. Each rat normally yielded four such tracheal ring preparations. Where appropriate, removal of the epithelium was achieved by gentle rubbing of the intimal surface.

2.3. Measurement of contractile responses

Contractile responses were measured from rings of trachea in vertical organ baths (10 ml) at 37 ± 0.5°C in oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit solution. Initial experiments indicated that, for both control and hypoxic animals, the optimum level of applied tension was 1.0 g wt. Tension was applied via two stainless steel wires inserted into the lumen. One wire was anchored and the other attached to a force displacement transducer (Grass FT03T). Tissues were allowed to equilibrate for 45 min, during which time tension was reapplied where necessary. Cumulative concentration–response curves were constructed to methacholine (10^{-10}–3×10^{-4} M), endothelin-1 (10^{-11}–3×10^{-7} M) and potassium chloride (10^{-3}–7×10^{-2} M). Results are expressed in mg wt.

To test whether exposure to chronic hypoxia might induce the release of cyclooxygenase metabolites or nitric oxide from the tracheal epithelium, we examined responses evoked by both methacholine and endothelin-1 in intact and epithelium-denuded tracheal rings in the presence and absence of (1) the cyclooxygenase inhibitor indomethacin (3×10^{-6} M) and (2) the nitric oxide synthase inhibitor L-NAME (10^{-4} M). Responses to methacholine are expressed as a percentage of the first curve (methacholine alone) maximum. On each experimental day, one tissue was subjected to two consecutive concentration–response curves to methacholine alone to ensure that responses did not alter with time.

Due to tachyphylaxis, it is not possible to perform consecutive concentration–response curves to endothelin-1, therefore, tissues were initially stimulated with a maximal concentration (10^{-4} M) of methacholine and responses to endothelin-1 expressed as a percentage of this response. Removal of the epithelial layer from tracheal preparations was verified by light microscopy.

In a separate series of experiments, tracheal rings were preconstricted with a single concentration of methacholine (3×10^{-7} M for control rat trachea and 10^{-6} M for hypoxic rat trachea). The concentrations used were approximately the EC_{30} for methacholine in trachea from control and chronically hypoxic rats. Once contractions had plateaued, cumulative concentration–response curves were constructed to salbutamol (10^{-9}–10^{-4} M) and sodium nitroprusside (10^{-9}–10^{-4} M). Results are expressed as percent reversal of the initial methacholine contraction. On

each experimental day, one tissue acted as a time control to ensure that the methacholine contraction was sustained.

2.4. Materials

The following chemicals were used; endothelin-1 (Novabiochem), indomethacin (Sigma), L-NAME (Sigma), methacholine chloride (Sigma), potassium chloride (BDH), (α-[(*t*-butylamino)methyl]-4-hydroxy-*m*-xylene-*a*,*a*′-diol hemisulphate salt) (salbutamol, Sigma) and sodium nitroprusside (Sigma). Concentrations in the text refer to the salts, with the exception of salbutamol, which is expressed as the base. Stock solutions of drugs were prepared in distilled water and subsequent dilutions made in Krebs–Henseleit solution, with the exception of indomethacin which was dissolved in ethanol. In experiments where indomethacin was used, one tissue acted as a vehicle control whereby an appropriate volume of ethanol was added to the bath.

2.5. Analysis of results

Results are expressed as means ± S.E.M. Statistical significance between data sets was tested by two-way analysis of variance. Significance between maximum responses and pD_2 values (the negative log of the concentration evoking 50% of the maximum response) was calculated using Student's *t*-test. A probability level of $P < 0.05$ was considered significant. Number of observations (n) refers to the number of animals used.

3. Results

3.1. The effect of chronic hypoxia on agonist responses

Methacholine, endothelin-1 and potassium chloride each evoked concentration-dependent contractions of tracheal rings isolated from control rats. The threshold concentration for contraction in each case was between 3×10^{-10} and 3×10^{-9} M for methacholine, between 10^{-10} and 3×10^{-10} M for endothelin-1 and between 10^{-3} and 10^{-4} M for potassium chloride. Exposure to chronic hypoxia significantly attenuated contractile responses to methacholine ($P < 0.05$ for data sets, $n = 32$), endothelin-1 ($P < 0.01$ for data sets, $n = 16$) and potassium chloride ($P < 0.01$ for data sets, $n = 24$) (Fig. 1). Chronic hypoxia evoked a small but statistically significant decrease in the sensitivity of the tissue to methacholine (pD_2 values for methacholine in control rat trachea; 5.99 ± 0.08 and for methacholine in hypoxic rat trachea; 5.68 ± 0.10, $P < 0.05$ for data points, $n = 32$). In contrast, there was no change in the sensitivity to endothelin-1 (pD_2 values for endothelin-1 in control rat trachea, 8.10 ± 0.09 and endothelin-1 in hypoxic rat trachea, 7.98 ± 0.12, $n = 8$). Maximum responses to methacholine were not altered by chronic hypoxia (maximum response to methacholine in control rat trachea; 1224.5 ± 113.6 mg wt and maximum response to methacholine in hypoxic rat trachea; 1067.4 ± 121.3 mg wt), however, the maximum responses to both endothelin-1 and potassium chloride were significantly attenuated by chronic hypoxia (maximum response to endothelin-1 in control rat trachea; 1412.9 ± 161.3 mg wt and maximum response to endothelin-1 in hypoxic rat trachea; 929.3 ± 129.7, $P < 0.05$ for data points, $n = 8$. Maximum response to potassium chloride in control rat trachea; 789.3 ± 49.3 mg wt and maximum response to potassium chloride in

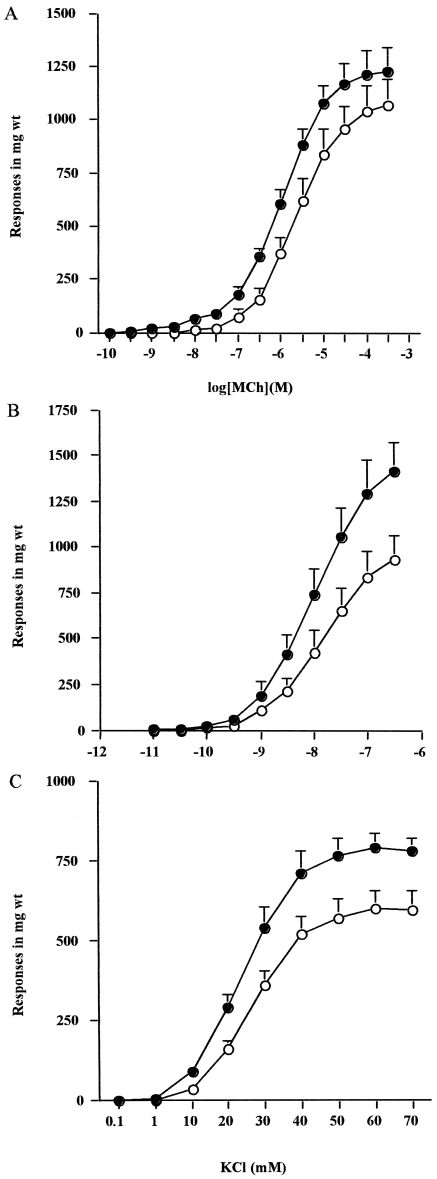

Fig. 1. Cumulative concentration–response curves to (A) methacholine, (B) endothelin-1 and (C) potassium chloride in tracheal rings from control (●) and chronically hypoxic (○) rats. Chronic hypoxia significantly attenuated responses to methacholine ($P < 0.05$ for data sets, $n = 32$), endothelin-1 ($P < 0.01$ for data sets, $n = 16$) and potassium chloride ($P < 0.01$ for data sets, $n = 24$).

hypoxic rat trachea; 600.1 ± 56.1 mg wt, $P < 0.05$ for data points, $n = 24$.).

3.2. The effect of epithelial removal on agonist responses

Consecutive concentration–response curves to methacholine alone were not significantly different from each other, indicating that methacholine responses did not alter with time (data not shown). In trachea from control rats, removing the epithelial layer significantly ($P < 0.05$ for data sets, $n = 8$) enhanced responses to methacholine (Fig. 2A). Removing the epithelium enhanced the sensitivity of the tissue to methacholine (pD_2 values for methacholine in intact control rat trachea; 6.32 ± 0.08 and pD_2 values for methacholine in denuded control rat trachea; 6.61 ± 0.15, $P < 0.05$ for data points, $n = 8$) but did not alter the maximum contractile response (maximum response to methacholine in intact control rat trachea; 99.9 ± 0.2% and maximum response to methacholine in denuded control rat trachea; 115.2 ± 14.7%, $n = 8$). Removing the epithelium had a similar effect on methacholine responses in trachea from chronically hypoxic rats. Responses to methacholine were significantly ($P < 0.05$ for data sets, $n = 8$) enhanced in epithelial-denuded tracheal rings from hypoxic rats (Fig. 2B). Again, removing the epithelium increased the sensitivity of the tissue to methacholine (pD_2 values for methacholine in intact hypoxic rat trachea; 6.04 ± 0.06 and pD_2 values for methacholine in denuded hypoxic rat trachea; 6.26 ± 0.08, $P < 0.05$, $n = 8$) without altering the maximum response to methacholine (maximum response to methacholine in intact hypoxic rat trachea; 99.9 ± 1.1% and maximum response to methacholine in denuded hypoxic rat trachea; 111.6 ± 13.9%, $n = 8$).

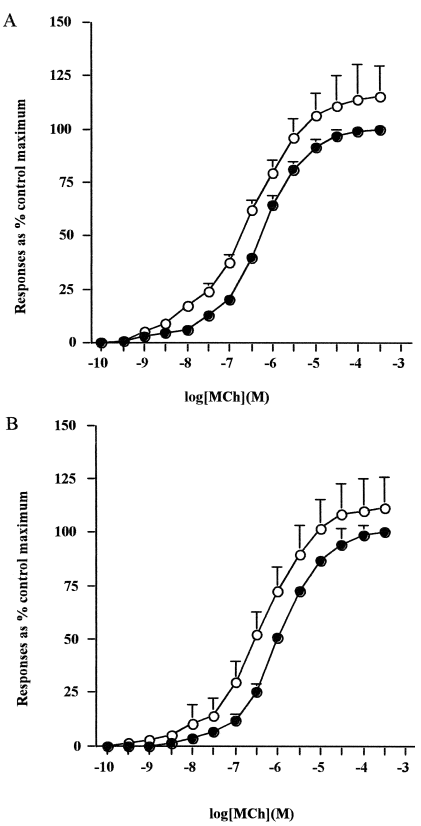

Fig. 2. Cumulative concentration–response curves to methacholine (10^{-10}–3×10^{-4} M) in intact (●) and epithelial-denuded (○) tracheal rings from (A) control rats and (B) chronically hypoxic rats. Removing the epithelium significantly ($P < 0.05$ for data sets, $n = 8$) enhanced responses to methacholine in both control and chronically hypoxic rats.

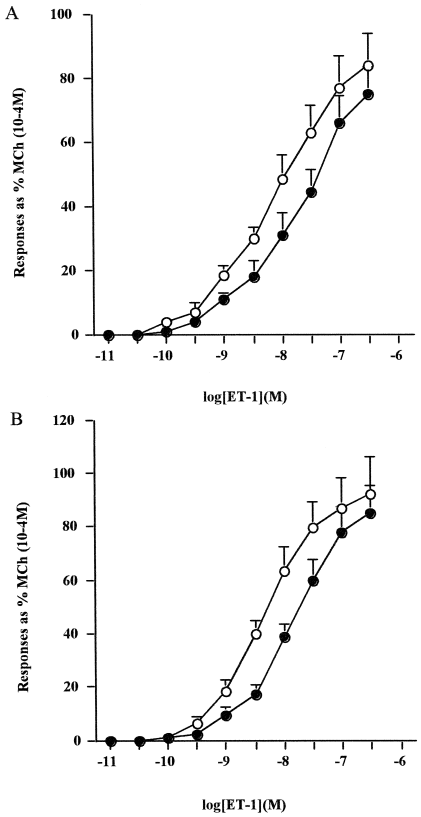

Fig. 3. Cumulative concentration–response curves to endothelin-1 (10^{-11}–3×10^{-7} M) in intact (●) and epithelial-denuded (○) tracheal rings from (A) control rats and (B) chronically hypoxic rats. Removing the epithelium significantly ($P < 0.05$ and $P < 0.01$, respectively, for data sets, $n = 8$) enhanced responses to endothelin-1 in both control and chronically hypoxic rats.

In epithelium-denuded tracheal rings from control rats, contractile responses to endothelin-1 were also significantly ($P < 0.05$ for data sets, $n = 8$) enhanced (Fig. 3A). Removing the epithelium evoked a significant increase in the sensitivity of the tissue to endothelin-1 (pD_2 values for endothelin-1 in intact control rat trachea; 7.89 ± 0.09 and pD_2 values for endothelin-1 in denuded control rat trachea; 8.19 ± 0.12, $P < 0.05$ for data points, $n = 8$), albeit without altering the maximum response (maximum response to endothelin-1 in intact control rat trachea; 74.8 ± 8.5% and maximum response to endothelin-1 in denuded control rat trachea; 84.1 ± 9.9%, $n = 8$). In chronically hypoxic rats, removing the epithelium again significantly ($P < 0.01$, for data sets, $n = 8$) enhanced responses to

endothelin-1 (Fig. 3B). Removing the epithelium increased the sensitivity of the tissue to endothelin-1 (pD_2 values for endothelin-1 in intact hypoxic rat trachea; 7.98 ± 0.11 and pD_2 values for endothelin-1 in denuded hypoxic rat trachea; 8.42 ± 0.13, $P < 0.01$ for data points, $n = 8$), but did not alter the maximum response (maximum response to endothelin-1 in intact hypoxic rat trachea; $84.7 \pm 10.6\%$ and maximum response to endothelin-1 in denuded hypoxic rat trachea; $92.4 \pm 13.7\%$, $n = 8$).

3.3. Effect of cyclooxygenase blockade

Indomethacin (3×10^{-6} M) did not alter responses to methacholine in tracheal rings from either control (Maximum response to methacholine in control rat trachea; $99.6 \pm 0.3\%$ and maximum response to methacholine in control rat trachea pretreated with indomethacin; $111.6 \pm 16.6\%$, $n = 8$. pD_2 values for methacholine in control rat trachea; 6.23 ± 0.08 and pD_2 values for methacholine in control rat trachea pretreated with indomethacin; 6.07 ± 0.12, $n = 8$. Figure not shown) or chronically hypoxic rats (Maximum response to methacholine in hypoxic rat trachea; $99.1 \pm 4.7\%$ and maximum response to methacholine in hypoxic rat trachea pretreated with indomethacin; $95.3 \pm 14.4\%$, $n = 8$. pD_2 values for methacholine in hypoxic rat trachea; 6.21 ± 0.06 and pD_2 values for methacholine in control rat trachea pretreated with indomethacin; 6.15 ± 0.13, $n = 8$. Figure not shown.).

In addition, indomethacin did not alter responses evoked by endothelin-1 in tracheal rings from control rats (Fig. 4A, maximum response to endothelin-1 in control rat trachea; $71.5 \pm 8.2\%$ and maximum response to endothelin-1 in control rat trachea pretreated with indomethacin; $77.3 \pm 10.2\%$, $n = 8$. pD_2 values for endothelin-1 in control rat trachea; 7.91 ± 0.07 and pD_2 values for endothelin-1 in control rat trachea pretreated with indomethacin; 7.78 ± 0.12, $n = 8$) or chronically hypoxic rats (Fig. 4B, maximum response to endothelin-1 in hypoxic rat trachea; $87.8 \pm 8.2\%$ and maximum response to endothelin-1 in hypoxic rat trachea pretreated with indomethacin; $87.9 \pm 7.6\%$, $n = 8$. pD_2 values for endothelin-1 in hypoxic rat trachea; 7.86 ± 0.08 and pD_2 values for endothelin-1 in hypoxic rat trachea pretreated with indomethacin; 7.72 ± 0.10, $n = 8$).

3.4. Effect of nitric oxide synthase blockade

In tracheal rings from control rats, addition of the nitric oxide synthase blocker L-NAME to the organ bath resulted in a small (< 200 mg wt) but sustained contractile response in three from 16 tissue preparations. In tissue from chronically hypoxic rats, the contractile response to L-NAME was of a similar magnitude, but was present in five from 16 preparations.

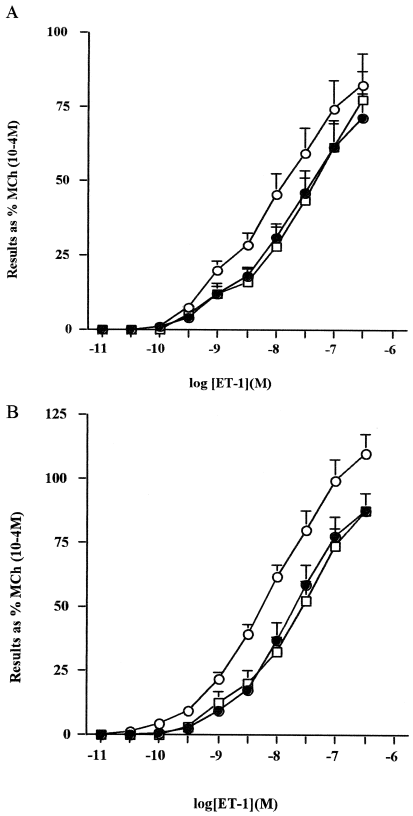

Fig. 4. Cumulative concentration–response curves to endothelin-1 (10^{-11}–3×10^{-7} M) alone (●), or in the presence of either 10^{-4} M L-NAME (○) or 3×10^{-6} M indomethacin (□) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. Pretreatment with indomethacin did not alter responses to endothelin-1 in either control or chronically hypoxic rats. In contrast, pretreatment with L-NAME significantly ($P < 0.05$ and $P < 0.01$, respectively, for data sets) enhanced responses to endothelin-1 in trachea from both control and hypoxic rats.

L-NAME (10^{-4} M) significantly ($P < 0.05$ for data sets, $n = 8$) potentiated methacholine responses in tracheal rings from control rats (Fig. 5A, pD_2 values for methacholine in control rat trachea; 6.29 ± 0.10 and pD_2 values for methacholine in control rat trachea pretreated with L-NAME; 6.62 ± 0.14, $P < 0.05$ for data points, $n = 8$), albeit without altering the maximum response (maximum response to methacholine in control rat trachea; $99.9 \pm 0.1\%$ and maximum response to methacholine in control rat trachea pretreated with L-NAME; $116.2 \pm 14.6\%$, $n = 8$).

In trachea from chronically hypoxic rats, L-NAME significantly ($P < 0.01$ for data sets) enhanced responses to methacholine, in this case with a concomitant increase in the maximum response (Fig. 5B, maximum response to methacholine in hypoxic rat trachea; $99.9 \pm 1.1\%$ and maximum response to methacholine in hypoxic rat trachea; $126.2 \pm 11.9\%$, $P < 0.05$ for data points, $n = 8$) as well as an increase in the sensitivity of the tissue to methacholine (pD_2 values for methacholine in hypoxic rat trachea;

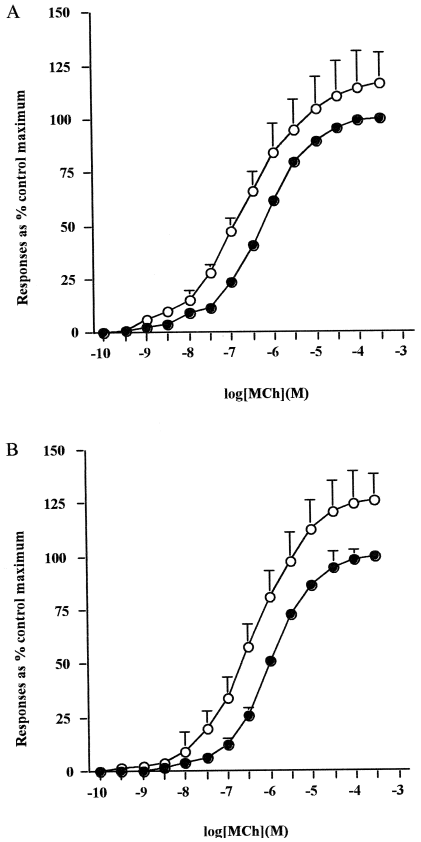

Fig. 5. Cumulative concentration–response curves to methacholine (10^{-10}–3×10^{-4} M) alone (●), or in the presence of 10^{-4} M L-NAME (○) in tracheal rings from (A) control rats and (B) chronically hypoxic rats. Pretreatment with L-NAME significantly ($P<0.05$ and $P<0.01$, respectively, for data sets, $n=8$) enhanced responses to methacholine in both control and chronically hypoxic rats.

5.98 ± 0.09 and pD_2 values for methacholine in hypoxic rat trachea pretreated with L-NAME; 6.49 ± 0.11, $P<0.01$ for data points, $n=8$).

In addition, L-NAME (10^{-4} M) significantly ($P<0.05$ for data sets, $n=8$) potentiated responses to endothelin-1 in tracheal rings from control rats (Fig. 4A, pD_2 values for endothelin-1 in control rat trachea; 7.91 ± 0.07 and pD_2 values for endothelin-1 in control rat trachea pretreated with L-NAME; 8.20 ± 0.11 $P<0.05$ for data points, $n=8$), albeit without altering the maximum response (maximum response to endothelin-1 in control rat trachea; 71.5 ± 8.2% and maximum response to endothelin-1 in control rat trachea pretreated with L-NAME; 82.3 ± 10.5%, $n=8$).

In trachea from chronically hypoxic rats, L-NAME significantly ($P<0.01$ for data sets) enhanced responses to endothelin-1, in this case with a concomitant increase in the maximum response (Fig. 4B, maximum response to endothelin-1 in hypoxic rat trachea; 87.8 ± 8.2% and maximum response to endothelin-1 in hypoxic rat trachea; 110.2 ± 7.2%, $P<0.05$ for data points, $n=8$) as well as an increase in the sensitivity of the tissue to endothelin-1 (pD_2 values for endothelin-1 in hypoxic rat trachea; 7.86 ± 0.08 and pD_2 values for endothelin-1 in hypoxic rat trachea pretreated with L-NAME; 8.16 ± 0.09, $P<0.05$ for data points, $n=8$).

3.5. Effect of chronic hypoxia on relaxatory responses to salbutamol and sodium nitroprusside

Salbutamol and sodium nitroprusside both reversed methacholine-induced contractions in a concentration dependent manner. Salbutamol initiated responses at concentrations of 10^{-8} M in control rat bronchi and between 10^{-8} and 3×10^{-8} M in hypoxic rat bronchi (Fig. 6A). Chronic hypoxia did not alter the ability of salbutamol to reverse methacholine-induced contraction (mean maximal inhibitions, at the 10^{-4} M level; 36.6 ± 6.0% in control rat bronchi and 40.7 ± 6.3% in bronchi from chronically hypoxic rats).

Sodium nitroprusside was of similar potency to salbutamol, initiating responses at concentrations of 3×10^{-8} M in control rat bronchi and 10^{-8} M in bronchi from hypoxic rats (Fig. 6B). In contrast, chronic hypoxia significantly ($p<0.001$ for data sets, $n=8$) enhanced responses evoked by sodium nitroprusside, albeit without altering the maxi-

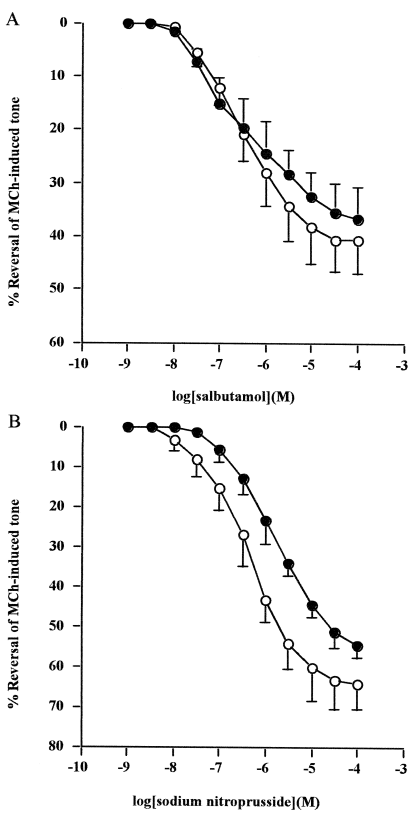

Fig. 6. Reversal of methacholine-induced tone in tracheal rings from control (●) and chronically hypoxic rats (○) by cumulative addition of (A) salbutamol (10^{-9}–10^{-4} M) and (B) sodium nitroprusside (10^{-9}–10^{-4} M). Responses to sodium nitroprusside, but not salbutamol, were significantly ($P<0.001$ for data sets, $n=8$) greater in trachea from chronically hypoxic rats.

mum responses (mean maximal inhibitions, at the 10^{-4} M level; $54.6 \pm 3.1\%$ in control rat bronchi and $64.1 \pm 6.4\%$ in bronchi from chronically hypoxic rats).

4. Discussion

Our initial results showed that responses evoked by the bronchoconstrictors methacholine and endothelin-1 were significantly attenuated in tracheal rings from chronically hypoxic rats compared to controls. These results are perhaps in keeping with the finding that chronic hyperoxia potentiates contractile responses in rat airways both in vivo and in vitro (Hershenson et al., 1992a,b, 1994; Szarek et al., 1995). A number of possible mechanisms exist which could explain the attenuation, which we found in our present study. Both methacholine and endothelin-1 bind to and activate membrane-bound receptors which are coupled to G proteins and both agonists are thought to initiate contraction of airway smooth muscle via generation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (Grandordy et al., 1986; Mattoli et al., 1991). This led us to speculate that chronic hypoxia may inhibit some part of the signalling process which occurs between receptor activation and initiation of contraction. For example, in cardiac tissue, chronic hypoxia has been shown to cause down-regulation of β-adrenoceptors (Voelkel et al., 1981) as well as uncoupling of these receptors from their regulatory G proteins (Richalet, 1990). Whether this occurs with airway muscarinic and endothelin-1 receptors is unknown, however, chronic hypoxia has been shown to cause a down-regulation in cholinergic responsiveness in rat pulmonary arteries (Orton et al., 1988). Furthermore, it has also been demonstrated that lung endothelin-1 levels are elevated by chronic hypoxia (Elton et al., 1992), which would perhaps be expected to result in down-regulation of endothelin receptors.

Our results with potassium chloride, however, would suggest that the attenuation of contractile responses is due to some reason other than alterations in receptor number or impairment of signal transduction processes. Potassium chloride evokes contraction of smooth muscle by causing depolarisation of the smooth muscle membrane, resulting in the stimulation of 'L'-type voltage operated Ca^{2+} channels. Contractions evoked by potassium chloride therefore represent a direct stimulation of airway smooth muscle which does not involve receptor activation and generation of second messengers. The results for potassium chloride followed a similar pattern to methacholine and endothelin-1, namely a significant impairment of contractile responses in trachea from chronically hypoxic rats. This indicates that some other mechanism(s) must be responsible for the attenuation of agonist-induced contractions.

A previous study by Hershenson et al. (1994) demonstrated that hyperoxia-induced airway hyperresponsiveness was substantially reduced by either epithelial removal or treatment with the cyclooxygenase inhibitor indomethacin. This strongly suggests that epithelium-derived cyclooxygenase metabolites contribute to the increased airway reactivity in rats exposed to chronic hyperoxia. This led us to postulate that release of cyclooxygenase metabolites (perhaps inhibitory prostaglandins or prostacyclin) could be responsible for the attenuation of contractile responses in chronically hypoxic rat airways. In this present study, removing the epithelium enhanced responses to methacholine and endothelin-1 in tracheal rings from both control and chronically hypoxic rats, suggesting that the epithelium is indeed releasing an inhibitory factor in both of these preparations. Indomethacin, however, did not alter responses evoked by these agonists in either control or hypoxic rats, indicating that this inhibitory factor is not a cyclooxygenase metabolite. Our results suggest that this inhibitory factor may be nitric oxide or a nitric oxide-like substance, since the nitric oxide synthase inhibitor L-NAME enhanced contractions evoked by methacholine and endothelin-1. Chronic hypoxia increases nitric oxide synthase expression in the rat lung and induces de novo nitric oxide synthase expression in the rat pulmonary artery endothelium (Xue et al., 1994), supporting the suggestion of an increased basal release of nitric oxide in the chronically hypoxic rat lung. More recently Shaul et al. (1995) demonstrated that expression of nitric oxide synthase I, the isoform of nitric oxide synthase localised to the airway epithelium in rats (Schmidt et al., 1992), is increased in rats exposed to chronic hypoxia. This suggests that an increased release of nitric oxide may be responsible for the attenuation in contractile responses in airways from chronically hypoxic rats. Perhaps in keeping with the finding that nitric oxide synthase is expressed in the airway epithelium of both control and chronically hypoxic rats (Xue et al., 1994), we found that L-NAME not only potentiated contractions in chronically hypoxic trachea, it also enhanced responses in control tissue. It would appear, therefore, that the hyporesponsiveness of chronically hypoxic rat trachea is not simply due to the release of nitric oxide, since nitric oxide is also released from control rat trachea. This led us to speculate that the responsiveness of the airway smooth muscle to nitric oxide may be altered by chronic hypoxia. To this end, we conducted experiments whereby the ability of the nitric oxide donor sodium nitroprusside to reverse methacholine-induced tone was compared in trachea from control and chronically hypoxic rats. As shown in Fig. 6, responses to sodium nitroprusside were significantly greater in trachea from chronically hypoxic rats. This does not appear to represent a non-specific enhancement of bronchodilator responses, since responses to the β_2-adrenoceptor agonist salbutamol, which acts via stimulation of adenylyl cyclase (Rinard et al., 1983) as well as opening of membrane potassium channels (Miura et al., 1992), were not altered by chronic hypoxia. Mindful of the reduced contractile response to methacholine in hypoxic rat trachea, we used concentrations of methacholine which pro-

duced approximately 30% of the maximum contractile response in trachea from both control and hypoxic rats. This, together with the finding that salbutamol responses were unaltered by chronic hypoxia, indicates that the enhancement of sodium nitroprusside responses is unlikely to be due to the inverse relationship between the level of airway tone and the potency of relaxant agonists (Van den Brink, 1973).

Interestingly, a recent study in Fisher rats, a strain of rats which exhibit airway hyperrresponsiveness, found results which are in direct contrast to our findings. Jia et al (1995) demonstrated that contractile responses to carbachol were potentiated in the Fisher rats while relaxant responses to sodium nitroprusside were attenuated. The authors speculated that the increased responsiveness to spasmogens in these rats is due to a decreased guanylyl cyclase response to nitric oxide. In favour of this hypothesis is the finding that sodium nitroprusside produces significantly less cGMP in airway smooth muscle from Fisher rats than controls (Jia et al., 1995). Our results with L-NAME indicate that airways of chronically hypoxic and control rats release nitric oxide or a nitric oxide-like substance which opposes contractile responses. It may be possible, therefore, that the decreased response to spasmogens and increased response to sodium nitroprusside which we found in chronically hypoxic rats in our study is due to an increased guanylyl cyclase response produced by chronic exposure to hypoxia. In future studies, we intend to measure cGMP production in airway smooth muscle from chronically hypoxic and control rats in an attempt to elucidate this.

Nitric oxide may be released from a number of potential sources within the airways, including airway smooth muscle (Xue et al., 1994), non-adrenergic non-cholinergic nerve terminals (Belvisi et al., 1991; Li and Rand, 1991), alveolar macrophages (Stuehr and Marletta, 1987) and inflammatory cells (McCall et al., 1989). Our results showed that removing the epithelium mimics the effect of L-NAME on contractile responses in both control and hypoxic rats, suggesting that nitric oxide or a nitric oxide-like substance is being released from the epithelial layer in our preparations. In dogs (Gao and Vanhoutte, 1993) and rabbits (Spina and Page, 1991), it has been proposed that the airway epithelium is not a major source for endogenous nitric oxide-like substances, however, previous studies have indeed confirmed nitric oxide synthase expression the airway epithelium of both control and chronically hypoxic rats (Xue et al., 1994). Soluble guanylyl cyclase, however, appears to be localised to the airway smooth muscle and not the epithelium (Rengasamy et al., 1994), indicating that nitric oxide performs a paracrine role in the respiratory system, being produced by the airway epithelium and activating soluble guanylyl cyclase in airway smooth muscle. The functional role of the nitric oxide synthase signalling pathway in the control of airway function remains controversial (Munakata et al., 1990; Spina and Page, 1991; Stuart-Smith, 1990; Nijkamp et al., 1993), but our results support physiological studies which suggest that endogenous nitric oxide may act as a bronchodilator (Alving et al., 1993). In our present study, adding L-NAME to the organ baths resulted in a small contraction in three from 16 tracheal rings from control rats and five from 16 from hypoxic rats. This indicates that there was basal release of nitric oxide in only a few tissue preparations, whereas L-NAME significantly potentiated contractions in almost all cases. This suggests that nitric oxide may be released in response to contraction of the airway smooth muscle and would then act to oppose the contractile response.

In summary, contractile responses to methacholine, endothelin-1 and potassium chloride were attenuated in isolated trachea from chronically hypoxic rats. Indomethacin did not alter responses to methacholine or endothelin-1, however, addition of L-NAME or removal of the epithelium significantly enhanced contractile responses in both control and chronically hypoxic rats. The ability of sodium nitroprusside to reverse methacholine-induced tone was enhanced in trachea from chronically hypoxic rats. These results show that, in both chronically hypoxic and control rats, contractile responses to methacholine and endothelin-1 are attenuated by nitric oxide which appears to be released from the airway epithelium. The attenuation of contractile responses in airways from chronically hypoxic rats may be due to an enhanced guanylyl cyclase activity and hence, an increased response to nitric oxide.

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