Mechanisms for lung function impairment and airway hyperresponsiveness following chronic hypoxia in rats

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Habre W, Jánosi TZ, Fontao F, Meyers C, Albu G, Pache JC, Peták F. Mechanisms for lung function impairment and airway hyperresponsiveness following chronic hypoxia in rats. Am J Physiol Lung Cell Mol Physiol 298: L607–L614, 2010. First published February 5, 2010; doi:10.1152/ajplung.00222.2009.—Although chronic normobaric hypoxia (CH) alters lung function, its potential to induce bronchial hyperreactivity (BHR) is still controversial. Thus the effects of CH on airway and tissue mechanics separately and changes in lung responsiveness to methacholine (MCh) were investigated. To clarify the mechanisms, mechanical changes were related to end-expiratory lung volume (EELV), in vivo results were compared with those in vitro, and lung histology was assessed. EELV was measured plethysmographically in two groups of rats exposed to 21 days of CH (11% O2) or to normoxia. Total respiratory impedance was measured under baseline conditions and following intravenous MCh challenges (2–18 μg·kg−1·min−1). The lungs were then excised and perfused, and the pulmonary input impedance was measured, while MCh provocations were repeated under a pulmonary capillary pressure of 5, 10, and 15 mmHg. Airway resistance, tissue damping, and elastance were extracted from the respiratory impedance and pulmonary input impedance spectra. The increases in EELV following CH were associated with decreases in airway resistance, whereas tissue damping and elastance remained unaffected. CH led to the development of severe BHR to MCh (206 ± 30 vs. 95 ± 24%, P < 0.001), which was not detectable when the same lungs were studied in vitro at any pulmonary capillary pressure levels maintained. Histology revealed pulmonary arterial vascular remodeling with overexpression of α-smooth muscle actin antibody in the bronchial wall. These findings suggest that, despite the counterbalancing effect of the increased EELV, BHR develops following CH, only in the presence of intact autonomous nervous system. Thus neural control plays a major role in the changes in the basal lung mechanics and responsiveness following CH.

pulmonary hypertension; bronchial hyperreactivity; hypoxemia; end-expiratory lung volume

CHRONIC ALVEOLAR HYPOXIA is often encountered in chronic pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) (19) and sleep apnea syndrome (15). The pulmonary hypertension developing during chronic hypoxia (CH) (6, 8, 20) induces deleterious changes in the pulmonary microvasculature, which then affect the viscoelastic properties of the parenchyma (16, 17). This pulmonary vascular and bronchial remodeling further deteriorate the lung function and, therefore, initiate a positive cascade mechanism, leading to a vicious circle with a consequent severe impairment in gas exchange. Despite the considerable progress made toward a better understanding of the pathophysiological background of CH, the mechanisms responsible for the adverse pulmonary consequences are still poorly understood.

Although impairment in the basal lung function has been consistently reported in the presence of CH (4, 13, 28), all previous studies used global lung function parameters, such as end-expiratory lung volume (EELV) (4, 28) or total lung resistance (13) to characterize the lung functional changes, ultimately precluding the identification of which lung compartment is primarily affected by the CH. The importance of the distinguished role of the airway and lung tissue compartments in the pathogenesis of CH stems from the results of previous in vitro findings demonstrating adverse mechanical changes in airway preparations (5, 7, 21). These in vitro results, however, are difficult to extrapolate to an in vivo condition, and thus they have limited advancement in the understanding of the underlying pathophysiological mechanisms, due to the key role of the altered neural control of the airway and lung parenchymal compartments in the presence of CH (4, 6, 29).

The discrepancy between the in vivo and in vitro studies is even more remarkable, as concerns the altered lung responsiveness subsequent to CH, with studies reporting opposite findings even under similar experimental conditions. In vitro studies performed on isolated airway rings or bronchial smooth muscle reported either enhancement of the contractile response (5), no change (21), or an attenuation (7). Similar controversy is present for results obtained in vivo, with some studies reporting no change (13), a reduction (2), or an enhancement (6) of the lung responsiveness.

Therefore, the present study was set out to characterize the separate roles of the airway and tissue compartments in the respiratory mechanical changes following CH by applying low-frequency forced oscillation method. In an attempt to elucidate the mechanisms for the altered lung function following CH, 1) airway and tissue mechanical parameters were related to the absolute lung volume changes to establish the role of the altered EELV; 2) the lung function and responsiveness were compared under in vivo and in vitro conditions to characterize the importance of the altered neural control and to establish whether the altered pulmonary hemodynamics per se affect lung responsiveness; and 3) histological evaluations were performed to assess the influence of the pulmonary vascular and bronchial remodeling. We hypothesized that the pulmonary hypertension resulting from CH may lead to bronchial hyperreactivity (BHR), and the autonomous nervous system plays an important role in its manifestation.
METHODS

Hypoxia Exposures

Following Institutional Ethics Committee and Animal Well Care approvals, studies were performed on two groups of Sprague-Dawley rats, weighing 276–436 g. Animals in group H (n = 9) were placed in a Plexiglas normobaric hypoxic chamber for 21 days, where the oxygen (O2) concentration was maintained during the entire exposure at ∼11%. The gas mixture from the chamber continuously circulated through a system that absorbed CO2 and controlled temperature and humidity. Rats involved in group C underwent identical exposure, except these animals were allowed to breathe room air (n = 7).

Study Protocol

In vivo experiments. Experiments were started by measuring the EELV, as detailed below. The rats were then anesthetized and mechanically ventilated. When stable respiratory mechanical and systemic hemodynamic conditions have been established, four to six recordings of the input impedance of the respiratory system (Zrs) were collected to establish the baseline. Increasing doses of methacholine (MCh) were then infused through the jugular venous line at doses of 2, 6, and 18 μg·kg⁻¹·min⁻¹. A period of 6 min was allowed after the onset of each MCh perfusion, and the collection of Zrs was started in each minute thereafter, until a steady-state contraction had developed. Zrs data were then collected during each infusion level under the steady-state condition [i.e., airway resistance (Raw)] values were within 5%] to assess the in vivo lung responsiveness. After the recording of the dose response curve was completed, a 20-min period was allowed for the rat to recover. A polyethylene tube (0.58 mm inner diameter, 0.96 mm outer diameter, Portex) was then introduced via the right jugular vein and advanced under continuous hemodynamic monitoring to the main pulmonary artery to record the pulmonary arterial pressure (Ppa) in vivo.

In vitro experiments. After completing the in vivo experiments, the heart-lung blocks were excised and perfused −1 h later, as detailed below. Perfusion of the isolated lungs was started with the reservoirs set at a physiological level of Ppa (17.5 mmHg) and left atrial pressure (Pla = 7.5 mmHg) in both groups of lungs. A 15-min period of lung perfusion was necessary to establish steady-state conditions before the start the protocol on the isolated lungs. After the steady-state conditions had been reached, three levels of pulmonary capillary pressure (Pc) were established in random order (5, 10, and 15 mmHg), while a constant level of pulmonary blood flow (Qp) was maintained. This was achieved by elevating the reservoir supplying the pulmonary artery with simultaneous lowering of the outflow of the pulmonary venous catheter. At each Pc level, baseline four to six recordings of the pulmonary input impedance (ZL) were collected. Constrictor responses of the ex vivo lungs were provoked by infusing MCh into the catheter supporting the pulmonary artery by a constant-flow infusion pump (ID2S, model ID 2 ET, Asnieres, France) at doses of 2, 6, and 18 μg·kg⁻¹·min⁻¹ (body weight). The infusion rate was set from 0.5 to 4.5 ml/h (ranging from 0.08 to 1.5% of the total Qp) from a MCh solution containing 1 mg/ml MCh. Each infusion level lasted for ∼20–25 min, which resulted in the administration of 2- to 2.5-ml MCh solution in total.

Supplemental experiments. To characterize the changes in EELV during the MCh provocation tests, supplemental experiments were performed in six rats (3 control and 3 exposed to 21-day hypoxia). These animals were anesthetized with intraperitoneal chloral hydrate (350 mg/kg), since this anesthesia regimen permitted both the forced oscillatory measurements to be performed during short apneic periods, while the animals were also able to perform inspiratory efforts in the closed plethysmograph.

EELV Measurements

All animals were anesthetized on day 22 with isoflurane (1.4%) by a face mask and intubated with a polyethylene cannula (14-gauge, Braun, Melsungen, Germany) using sterile techniques. EELV measurements were accomplished in both groups by a body plethysmograph, as detailed previously (14). Briefly, the trachea was occluded at end expiration until three to four spontaneous inspiratory efforts were generated by the animal in the closed box. An end-expiratory pressure of 2.5 cmH2O was applied during these maneuvers to be in accordance with the subsequent impedance measurements. Changes in tracheal pressure and plethysmograph box pressure were recorded during these maneuvers, and EELV was calculated by applying Boyle’s law to the relationship between tracheal pressure and box pressure after correcting for the box impedance (14).

Animal Preparations

After completing the EELV measurements, the rats were mechanically normoventilated with a tidal volume of 7 ml/kg body wt. A positive end-expiratory pressure of 2.5 cmH2O was applied, a respiratory rate of 70–80 breaths/min with a constant volume-cycled rodent ventilator (model 683, Harvard Apparatus, South Natick, MA). Anesthesia was maintained with pentobarbital administered intravenously every 40 min (5 mg/kg). The femoral artery was cannulated (Abocath 22G) and attached to a pressure transducer (model 156 PCE 06-GW2, Honeywell, Zürich, Switzerland) for continuous blood pressure monitoring. An arterial line was also used for blood-gas analysis (model 505, Acid Base Laboratory, Copenhagen, Denmark). The femoral vein was also cannulated in an identical manner for drug delivery. The airway pressure, arterial pressure, ECG, and rectal temperature were continuously monitored by a data collection and acquisition system (Biopac, Santa Barbara, CA). Fentanyl was administered intra-arterially (15 μg/kg) to ensure adequate analgesia.

In Vitro Preparations

After the in vivo measurements were completed, the lungs were excised and perfused in an identical manner detailed previously (17). Briefly, heparin (1.5 IU/g) was administered intravenously for complete anticoagulation of the blood. Thirty-five milliliters of arterial blood were next gently withdrawn, while the collected blood was continuously replaced by the intravenous infusion of colloidal solution (hydroxyethyl-starch 6%). This maneuver maintained a constant intravascular volume and a mean systemic blood pressure >50 mmHg, and thus minimized the risk of ischemic lesions in the lungs. The collected diluted blood was centrifuged (4,000 rpm for 10 min), and 17 ml of plasma were extracted. The resulting reconstituted blood with a hematocrit level of ∼35% served as priming perfusate.

The chest was widely retracted following a midline sternotomy, and a polyethylene catheter (14-gage, Braun, Melsungen, Germany) was placed into the main pulmonary artery via the right ventricular outflow track, advanced until it was immediately proximal to the bifurcation, and next connected to medical grade silicone tubing (1.47 mm inner diameter, Ulrich, St. Gallen, Switzerland). The animals were then completely exanguinated by widely opening the left ventricle and the left atrium. To minimize the warm ischemic time period until reperfusion, the lungs were immediately flushed via the pulmonary artery cannula with 30 ml of cold (10°C) hydroxyethyl-starch 6% solution from a height of 30 cm. Through the left ventricular bifurcation, another catheter was placed into the left ventricle, into which a Combitfix7-Adapter (Braun, Melsungen, Germany) was tightly fixed and connected to medical grade silicone tubing. Finally, a third catheter (polyethylene tubing, inner diameter 0.88 mm, Portex, Hythe, UK) was introduced directly into the left atrium for measurement of Pla. The lungs and the heart were excised in a single block, dissected free of adjacent tissue, and weighed. The heart-lung block was suspended from an isometric force displacement transducer (Grass...
FT03, Quincy, MA) in a thermostabilized, humidified Plexiglas chamber. The lungs were ventilated with air mixed with 5% CO₂ and a respiratory rate of 50 breaths/min, a tidal volume of 7 ml/kg, and a positive end-expiratory pressure of 2.5 cmH₂O were maintained. A series of hyperinflations (peak pressure of 25–30 cmH₂O) were applied by occluding the expiratory port of the ventilator until the atelectatic areas were completely abolished.

The Ppa and Pla values were recorded continuously (Honeywell, model 156-PC 06-GW2), and a transit-time flowmeter (T-201 CDS, Transonic Systems, Ithaca, NY) situated between the perfusion reservoir and the catheter cannulating the main pulmonary artery measured the Q˙p continuously. Pc was estimated by applying the Gaar equation [Pc = Pla + 0.44 × (Ppa – Pla)] (9) and was used to assess the capillary filling pressure before the maneuvers. Airway pressure, Ppa, Pla, Pc, Q˙p, and the lung weight were recorded and stored on a microcomputer at a sampling rate of 50 Hz via an analog/digital interface converter (Biopac, Santa Barbara, CA).

**Measurement of Airway and Tissue Mechanics**

To characterize the airway and tissue mechanics, the input Zrs in the in vivo experiments and that of the lungs (Zl) in the in vitro preparations were measured by using the forced oscillatory technique, as described in detail previously (10, 18). Briefly, the tracheal cannula was connected from the respirator to a loudspeaker-in-box system at end expiration. The loudspeaker generated a small-amplitude pseudorandom signal with frequency components between 0.5 and 21 Hz through a polyethylene wave tube (length = 100 cm, inner diameter = 2 mm). Two identical pressure transducers (model 33NA002D, IC Sensors, Milpitas, CA) were used for measurement of the lateral pressures at the loudspeaker and at the tracheal end of the wave tube. Zrs or Zl was calculated as the load impedance of the wave tube (27). To separate the airway and tissue parameters, a model was fitted to the Zrs or Zl. spectra by minimizing the relative differences between the measured and modeled impedance values. The model contained a frequency-independent Raw and airway inertance, in series with a frequency-dependent Raw and airway inertance, in series with a constant-phase tissue compartment characterized by the coefficients of tissue damping (G) (Grs and Gl for the Zrs and Zl, respectively) and elastance (H) (Hrs and Hl for the Zrs and Zl, respectively) (11). The impedance of the tracheal cannula and the connecting tubing was also determined, and the Raw and airway inertance values were corrected by subtracting the instrumental resistance and inertance values from them. Specific Raw (SRaw) was calculated by multiplying Raw by the corresponding EELV value.

**Histological Preparations**

After the in vitro experiments were completed, the lungs were fixed by instilling 4% formalin into the trachea at a hydrostatic pressure of 20 cmH₂O. Transhilar horizontal sections (perpendicular to the longitudinal axes of the lung from the hilum) were included in paraffin. Two 5-μm sections were prepared in each lung specimen and were stained first with the Miller stain, and then with the monoclonal anti-SMA antibody (clone 1A4, Dako, CA) and then with the monoclonal antibody was estimated by measuring the area of the stained cells in 119 and 162 readings from the control and hypoxic animals, respectively. Since the border of the labeled cells was distinct around the bronchial wall normalized to a circumferential length of 10 μm measured on the basal membrane. The histological measurements were performed by using an image analysis system (Q550-iiw Quantimet, Leica) connected to a DMRBE microscope (Leica) via a video camera (Sony DCC 930p tri ccd). Image acquisition was accomplished via a ×63 dry objective (NPL-Fluothar 63/0.9) and a ×10 lens. Four to six color images were randomly selected from each histological preparation, and three to four bronchi were identified and analyzed on each section by means of the interactive measurement facilities of the QWin software (Leica).

**Statistical Analysis**

Scatters in the parameters are expressed in SE values. The paired t-test was utilized to estimate the effects of CH on the respiratory mechanical parameters. Two-way repeated-measures ANOVA was used with variables CH and MCh dose to establish the effects of CH on the in vivo lung responsiveness. Another two-way repeated-measures ANOVA was used with variables Pc and MCh dose to assess the effects of Pc on the in vitro lung responsiveness. The Student-Newman-Keuls multiple-comparison procedure was employed to compare the lung mechanical parameters under different conditions. Wilcoxon signed-rank test was used to compare the histological findings between the protocol groups. Pearson product-moment correlation test was applied to estimate the associations between the variables. In each test, a significance level of P < 0.05 was applied.

**RESULTS**

Exposure of the animals to hypoxia led to statistically significant increases in Ppa (15.6 ± 3.9 vs. 26.8 ± 3.1 mmHg, P < 0.05).

Figure 1 summarizes the results of the lung volume and respiratory mechanical measurements obtained in the control and hypoxic rats under the baseline conditions. CH induced marked and statistically significant elevations in the EELV (P < 0.001). The hypoxia-induced EELV increases were associated with significant decreases in Raw (P = 0.01), while SRaw exhibited no significant change (P = 0.31). No changes were observed in the basal values of the respiratory tissue parameters following CH (P = 0.15 and P = 0.46 for Grs and Hrs, respectively).

The effects of CH on the lung responsiveness to MCh are demonstrated in Fig. 2 in the in vivo experiments. In both groups of rats, MCh induced increases mainly in the Raw, while the alterations in the respiratory tissue parameters were markedly smaller. For Raw, the two-way ANOVA revealed significant interactions between the CH and MCh (P = 0.017), indicating that CH exposure has a significant effect on the altered airway tone during MCh challenges. The CH did not affect the MCh-induced changes in the other mechanical parameters (P = 0.064 and P = 0.23 for Grs and Hrs, respectively). As concerns the MCh-induced percent changes relative to the baseline, Raw exhibited markedly and statistically significantly greater increases in group H (P = 0.016) than in the animals of group C, while the MCh-induced relative changes in the other parameters were not affected by CH exposure. Furthermore, significant correlations were found between the EELV and the MCh-induced Raw elevations (R = 0.66, P = 0.01). The results of the supplemental experiments performed on the six rats revealed similar changes in BHR (210 ± 45 vs. 551 ± 15% increases in Raw after MCh 18 μg·kg⁻¹·min⁻¹ in control and hypoxic rats, respectively). However, the MCh provocations did not affect EELV, with mean changes of 0.4 ± 2.4 and 0.6 ± 3.8% in the control and hypoxic rats after the highest dose of MCh, respectively.

Unlike the results obtained in vivo, the airway and parenchymal mechanical parameters under the baseline conditions were comparable between the isolated, perfused lung obtained from the rats enrolled in groups C and H at each Pc level (Raw: 0.44 for Grs and 0.46 for Hrs, respectively).
24.4 ± 1.5 vs. 25.1 ± 1.9 cmH₂O·s·l⁻¹, G: 223 ± 42 vs. 235 ± 13 cmH₂O/l, and H: 1,760 ± 181 vs. 1,526 ± 49 cmH₂O/l for groups C and H, respectively, at Pc = 10 mmHg). The results obtained during the in vitro MCh challenges are summarized in Fig. 3. The MCh-induced elevations in Raw were associated with similar increases in G, while H was not affected. In contrast to the in vivo results, no difference was detectable between the two groups of rats in the MCh-induced increases in the airway and parenchymal mechanical parameters under the in vitro experimental setting (P = 0.55, P = 0.15, and P = 0.77 for Raw, GL, and HL, respectively). Furthermore, maintenance of low or high Pc in the pulmonary capillaries had no effect on the lung reactivity to MCh (P = 0.77, P = 0.76, and P = 0.53 for Raw, GL, and HL, respectively).

Figure 4 depicts the histological findings in the rats in groups C and H. CH induced a significant thickening of the pulmonary arteries (P < 0.001), whereas it had no statistically detectable effect on the bronchial wall thickness (P = 0.15). Moreover, CH led to a proliferation of smooth muscle cells stained with α-SMA antibody in the lungs, which was manifested in an increase in the peribronchial surface area (P < 0.001).

DISCUSSION

The present study addressed the effects of chronic normobaric hypoxia on the basal respiratory mechanics and attempted to clarify the mechanisms leading to subsequent changes in the lung responsiveness to exogenous constrictor stimuli. The findings of the present study consistently demonstrate the development of pulmonary hypertension to CH. This alteration in the pulmonary hemodynamics was associated with marked increases in the EELV. The separate assessment of the mechanical properties of the airways and the respiratory tissues revealed that these lung volume changes resulted in significant decreases in Raw, whereas no changes were observed in the damping and elastic properties of the respiratory tissues. Lung provocation with MCh revealed an enhanced airway responsiveness following the chronic exposure to CH in the intact animals, whereas this phenomenon was not detectable when the same lungs were investigated in vitro. Examining the structural background of these functional changes revealed pulmonary arterial vascular remodeling with overexpression of actin in the smooth muscle cells stained with α-SMA antibody in the bronchial wall.

Consistent with previous studies applying similar CH exposures (1, 26), the adopted experimental model in the present study led to systematic elevations in the Ppa, which subsequently resulted in pulmonary vascular and bronchial remodeling. Thus this model is appropriate to investigate the effects of CH on the basal lung function and the airway responsiveness to exogenous constrictor stimuli. As far as we are aware, this is the first study in which the effects of CH on the EELV were combined with separate measures of the changes in the airway and tissue mechanics, and responsive to the same animal both under in vivo and in vitro conditions. The importance of such investigation stems from the great deal of controversy that appears in the literature concerning the effects of CH on the basal lung mechanics and responsiveness to constrictor stimuli.
In agreement with previous findings, we obtained marked increases in the EELV following CH (4, 22, 28). This increase may be attributed to reflex mechanisms by which the rats compensated the hypoxia by activating the chest wall muscles (4) to actively maintain an elevated EELV. Despite the extensive assessment of altered EELV following CH, no data are available for the changes in the airway mechanical properties following CH. Our low-frequency forced oscillatory measurements performed in vivo after CH reveal marked decreases in the Raw (Fig. 1). The Raw decreases are subsequent to the actively elevated EELV rather than changes in the airway smooth muscle tone for the following reasons: these changes are no longer detectable after normalization to lung volume (i.e., in SRaw), the differences in the basal Raw values were not different between groups C and H when the same lungs were assessed excised, and no structural alterations were detectable in bronchial walls (Fig. 4). In addition, the primary role of the increased EELV following CH in the decreased Raw can be further substantiated from the similar magnitude of Raw decrease observed in the present study than that obtained previously in rats after passively elevating the EELV with similar amount observed after CH (12).

Concerning the respiratory tissue mechanical changes following CH, G and H were comparable between the two groups, both under in vivo and in vitro conditions. This finding suggests that the pulmonary vascular remodeling obvious from the histological sections was not strong enough to be manifested in the altered damping or elastic properties of the respiratory (in vivo) or in the lung (in vitro) tissues. The lack of change in the lung tissue mechanics following CH is in agreement with previous findings reporting no change in the lung compliance (4, 13, 22) or in the pressure-volume curve (4, 22). During
MCh infusions, the increases in G are likely a consequence of enhanced ventilation heterogeneities, which is confirmed by the fact that the increases in G are more pronounced in the isolated lungs when the agonist is delivered to the pulmonary artery and reaches preferably the lung periphery.

Characterization of the lung responsiveness to exogenous cholinergic constrictor stimuli (MCh) proved the enhancement of the airway responsiveness following normobaric CH (Fig. 2). The experiments on the isolated, perfused lungs obtained from the same animals revealed no systematic relationships between the pulmonary vascular pressure and the lung responsiveness (Fig. 3), indicating that the altered Ppa per se is not responsible for the observed BHR after CH. Interestingly, the in vitro experiments also revealed that the hyperreactivity in the rats with CH was no longer detectable when the same lungs were denervated, excised, and perfused (Fig. 3). This finding suggests the presence of the mechanism responsible for the BHR to CH that only exerts its effect in the intact animals. One possibility for such a mechanism would be the altered EELV, which was only detectable in vivo. However, an increased EELV does not explain the presence of enhanced airway reactivity to MCh, since an elevated lung volume counteracts the airway smooth muscle constriction (24). It is noteworthy, however, that the elevated EELV with subsequent decreases in the basal Raw result only in a more prominent manifestation of the airway hyperresponsiveness when the changes are expressed as percent changes from baseline (Fig. 2, left). While the elevated EELV counteracted the active smooth muscle
contraction at relatively low MCh concentrations (6 μg·kg⁻¹·min⁻¹), the higher MCh doses overwhelmed the blunting effect of the elevated EELV and resulted in a clear appearance of BHR. This finding indicates that assessment of BHR following CH requires a sufficient amount of constrictor agonist to compensate for the decreased Raw due to the elevated EELV. The insufficient level of constriction to reach this threshold in previous studies may explain the lack of the detectable BHR (2, 13), and thus this phenomenon may potentially contribute to the controversy in the literature.

To further characterize the role of the altered EELV in the altered lung responsiveness following CH, we also examined the possibility of lung volume changes during the MCh challenges in the supplemental experiments. Lung volume changes during the provocations would not only alter the airway diameter in an indirect manner, but may also affect the lung responsiveness via the altered tension applied to the airway smooth muscle. While the results of these supplemental experiments were in full agreement with those obtained in the main study groups, the EELV did not exhibit a tendency for a change following MCh challenges under any condition. This lack of changes in the lung volume following challenge confirms that the excessive changes in Raw following hypoxia are not related to indirect mechanisms secondary to altered lung volume.

Having excluded the active lung volume alterations, the other major difference between the in vivo and in vitro condition is the presence of neural control in the lungs in the latter. Indeed, an enhancement in the vagal tone has been demonstrated to be present following CH (4). Since the enhanced vagal tone augments the bronchoconstriction induced by cholinergic stimuli (24, 25), and the vagal tone increases further with increasing doses of MCh (30), it is plausible to conclude that increased vagal tone contributed greatly to the development of BHR after CH.

In addition, the exploration of the structural background of the CH in the lungs revealed overexpression of α-SMA-stained cells, around both the airways and the arteries, demonstrating pulmonary vascular and bronchial remodeling. Considering that α-SMA is responsible for the smooth muscle contraction, this increase suggests a development of smooth muscle thick-
ening, which, additionally, contributes to the BHR observed under the in vivo condition. This finding is in agreement with earlier results demonstrating an upregulation of the expression of α-SMA proteins following CH (23). In agreement with previous results demonstrating the presence of BHR in vivo and normal responsiveness in vitro (6), this remodeling in the present study was not manifested in the altered in vitro responsiveness to MCH after CH. This finding demonstrates the need for the neural control of the tracheo-bronchial tree, particularly that of the vagus to modulate the response of the airway smooth muscles to MCH following CH. The lack of this mechanism in the previous in vitro studies may explain, in part, their inability to detect BHR following CH (6, 7, 13, 21).

In summary, the results of the present study demonstrate that chronic exposure to normobaric hypoxia decreases markedly the basal Raw, which can be attributed to the elevated lung volume subsequent to reflex adaptation of the respiratory muscles to the low inhaled oxygen concentration in vivo. This change was not associated with detectable alterations in the pulmonary or respiratory tissue mechanics. Lung provocations with intravenous MCh revealed enhanced airway responsiveness following CH, which can be primarily attributed to the enhancement of the bronchial smooth muscle contractility that is modulated by the neural control of the airways. These findings indicate the potentially beneficial role of the anticholinergic treatment to prevent BHR that develops after CH in patients with COPD, sleep apnea, or prolonged exposure to high altitude.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the author(s).

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