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Effects of cigarette smoke and chronic hypoxia on airways remodeling and resistance. Clinical significance

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ABSTRACT

Previously we have reported that association of cigarette smoke (CS) and chronic hypoxia (CH) interact positively to physiopathologically remodel pulmonary circulation. In present study we have exposed guinea pigs to CS smoke (four cigarettes/day; 3 months; CS) and to chronic hypoxia (12% O_2, 15 days; CH) alone or in combination (CSCH animals) and evaluated airways remodeling and resistance assessed as Penh (enhance pause). We measured Penh while animals breathe air, 10% O_2 and 5% CO_2 and found that CS and CH animals have higher Penh than controls; Penh was even larger in CSCH animals. A rough parallelism between Penh and thickness of bronchiolar wall and muscular layer and Goblet cell number was noticed. We conclude that CS and CH association accelerates CS-induced respiratory system damage, evidenced by augmented airway resistance, bronchial wall thickness and muscularization and Goblet cell number. Our findings would suggest that appearance of hypoxia would aggravate any preexisting pulmonary pathology by increasing airways resistance and reactivity.

1. Introduction

Animals, usually rodents, exposed to cigarette smoke (CS) are valuable models to study several types of lung and respiratory pathways pathology such as chronic obstructive pulmonary disease (COPD). Among those models, guinea pigs have proven to be very suitable (Wright and Churg, 1990, 2002a), as they tolerate prolonged smoke exposure without the very important and rapid weight loss encountered in other species (Finch et al., 1995; Chen et al., 2006). In an attempt to underscore the mechanisms involved in the genesis of the pathology found in COPD in humans most works have been centered on the study of lung parenchyma (Wright and Churg, 2002a; Ardite et al., 2006; Ferrer et al., 2009). Findings indicating that even if the pathology of the smoking lung in guinea pigs does not completely match that encountered in humans (in particular is not evident the centrilobular predominance of the smoke-induced human emphysema; Wright and Churg, 1990), the laboratory model reproduces the main morphological and functional traits of the human emphysema. Thus, after three months of smoking ten cigarettes a day it is evident an increase in the air-space size that affects alveoli and alveolar ducts, a decrease in the length of elastic fibers as well as increases in residual volume, functional residual capacity and total lung capacity. The severity of the alterations progresses with the duration of the smoke exposure (Wright and Churg, 1990) and with the extent of smoke exposure, as Ferrer et al. (2009) found that smoking seven cigarettes a day causes no enlargement of air spaces until six months of exposure.

Alterations in pulmonary vessel structure and function are highly prevalent in patients with COPD which commonly develop hypertension, which in turn represents one of the principal factors associated with reduced survival in COPD patients (Peinado et al., 2008). Importantly enough, although the reduction of capillary bed seems smaller in the experimental model than in humans (Yamato et al., 1997; Wright and Churg, 2002b), smoking guinea pig model has also been found to be adequate to reproduce pathological and functional alterations encountered in COPD patients. Thus, in two recent studies Ferrer et al. (2009, 2011) found a decrease in the lung expression of endothelial nitric oxide synthase after three months of CS exposure (seven cigarettes/day), preceding the appearance of lung emphysema; they also found that CS increased smooth muscle cell proliferation in small arteries leading to pulmonary arterial hypertension and causing right ventricle hypertrophy.

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Interestingly, association of CS (5 cigarettes/day, 3 months) and sustained hypoxia (last two weeks of CS), as it frequently occurs at some stage of evolution in COPD patients further exaggerated the alterations of pulmonary circulation leading to conclude that hypoxemia represents a critical step in the progression of pulmonary vascular impairment that amplifies the initial effects of CS.

Airways obstruction in the COPD patients has two different components, the volume-dependent airway obstruction due to a loss of the lung recoil and the airways narrowing component due to pathological alterations of the bronchial walls (Hogg et al., 2004). However, as pointed out by Wright and Churg (2002a) and Churg and Wright (2007) investigations on smoking animal models of COPD have paid little attention to or even ignored (Churg et al., 2011) airways alterations. In smoking guinea pigs it has been found that after three months of ten cigarettes/day smoking there is an increase in the staining for neutral and acid mucins suggestive of Goblet cell hyperplasia/metaplasia (Wright et al., 1992; Wright and Churg, 2002a). In early works no changes were found in small airways wall thickness even after six and twelve months of CS exposure (Wright et al., 1992; Tron et al., 1987), but in more recent studies after six months of smoking seven cigarettes/day a thickening of wall of membranous bronchiole was seen (Churg et al., 2007). From a different perspective Joad and coworkers have reported in a series of studies that exposure of young guinea pigs to CS from weeks 1 to 6 of age causes airways C-fiber as well as brainstem neurons neurokinin-dependent sensitization setting the basis for exaggerated irritant induced bronchoconstriction and cough, as encountered in infants raised in homes with smokers (Mutoh et al., 1999; Joad et al., 2004; see also Kwong et al., 2001). The increased bronchoconstriction was evidenced by an increase in maximal Penh values (enhanced pause) after a citric acid aerosol application.

In present study we have used guinea pigs to study the effects of CS exposure on airways structure (bronchiolar wall thickness, Goblet cell number and bronchiolar muscle thickness) and airways resistance to flow assessed as enhanced pause (Penh). Since from the clinical point of view the interaction between cigarette smoke and hypoxia are very important as hypoxemia represents a critical step in the progression of pulmonary vascular impairment that amplifies the initial effects of CS. as in middle to advanced stages of the human COPD (Ferrer et al., 2011; see also Nizet et al., 2005; Le Jemtel et al., 2007), it would be desirable to know if the association of both factors, hypoxia and CS, accelerates the appearance or progression of the airways alterations. As a consequence we have followed the experimental paradigm used in our previous study (Ferrer et al., 2011) to study the effects of CS and sustained hypoxia isolated and in association on airways parameters.

2. Materials and methods

2.1. Animals

A group of 48 male Hartley guinea pigs (seven weeks of age) were purchased to Harlam Iberica and housed (3/cage) in an *ad hoc* room of the vivarium in a light–dark cycle of 12 h at a room temperature of 20–23 °C. Animals were provided standard chow and water supplemented with vitamin C (Farma Bayer S.A., España; 1 g/l) *ad libitum*. After one week of adaption (i.e., at 8 weeks or 2 months of age), animals were randomly distributed in 2 groups of 24 animals and divided in subgroups (Fig. 1). Twenty four animals were exposed to cigarette smoke for 3 months (12 weeks): 8 of them were maintained in normal atmosphere (smoking animals, CS) and 16 were exposed to a hypoxic atmosphere the last two smoking weeks (smoking hypoxic animals, CSCH). The other group was similarly divided: 8 animals remained in normal atmosphere for the entire 12 weeks or 3 months (control animals, Control) and 16 were exposed to hypoxia for the last two weeks (chronic hypoxic animals, CH). Body weight was measured weekly. Animal protocols were approved by the University of Valladolid Institutional Committee for Animal Care and Use following international laws and policies (Guide for the Care and Use of Laboratory Animals, National Institutes of Health, 85–23, 1985).

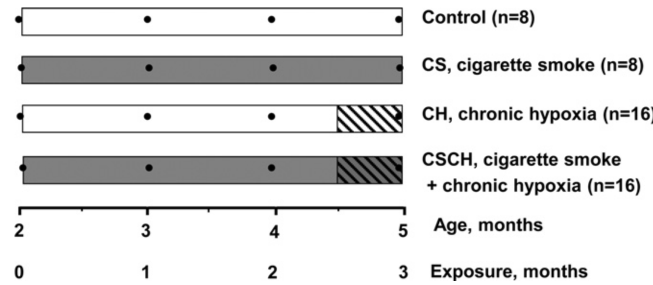

Fig. 1. Experimental groups and protocols. The schema shows, groups of guinea pigs used in present study (rectangular boxes), including duration of exposure to tobacco smoke in CS group, exposure to chronic hypoxia in CH group and co-application of both stressors in CSCH group. In every case ventilatory parameters were measured monthly as indicated by dots in each box. In analyzing the data and figure drawing in the entire study, data obtained in control group and in CH group up to 4 months were combined. An identical combination was made with data of CS group and CSCH group up to 4 months.

2.2. Smoking protocol

Animals were daily exposed to the smoke of 4 cigarettes (2R4F; Kentucky University Research; Lexington, KY, USA, 11 mg tar, 0.8 mg nicotine per cigarette), 5 days/week, using a nose-only inhalation system (Protowerx Design Inc; Langley, British Columbia, Canada). Control animals were equally sham-exposed.

2.3. Exposure to chronic hypoxia

Animals were introduced into glass chambers (125 l × 50 h × 50 w cm; 8 animals/chamber) continuously fluxed with a gas mixture (12% O_2 in N_2; PO_2 ≈85 mmHg; equivalent to ≈4300 m) and kept for two weeks. Accumulation of CO_2 was prevented by the continuous flow of the gas mixtures (3 l/min) and by a carbon dioxide absorbent (soda lime, Analema Vorquímica S.L., Spain). Guinea pigs remained in this atmosphere except for 30–40 min/day during routine maintenance and smoking session.

2.4. Plethysmography

The plethysmographic system allows recording pressure fluctuations within the chamber with a high gain differential transducer. Ideally, the frequency of pressure fluctuations would correspond to breathing frequency (f_{resp}, breaths/min), but there are spurious fluctuations due to animal movements that were electronically rejected. The tidal volume (V_T, ml) is provided by the software of the system from the integration of the inspiratory curve; the system was calibrated automatically by software after steady injection into the chamber of 5 ml air. This integration was made at the recording conditions of temperature, pressure and water vapor saturation. Then, to express ventilatory volumes in BTPS conditions data were corrected for the body temperature of the guinea pig (39 °C; Schwenke et al., 2007) and water vapor saturation as well atmospheric pressure in Valladolid.

Ventilatory parameters were measured in conscious, freely moving guinea pigs by whole body plethysmography. The system (Emka Technologies, Paris, France with software IOX version 1.8.9.4) consisted of 5 l methacrylate chambers continuously fluxed

(2 l/min), with temperature inside of 22–24 °C. Animals were placed in the plethysmographic chamber flushed with compressed air for an initial period of around 30 min until they adapted to the chamber ambient and acquired a standard resting behaviour. Thereafter, we initiated the recording while flushing the plethysmographic chamber with 3 different gas mixtures: normoxia (fraction of inspired oxygen ($F_{I_{O_2}}$ of 0.21)), hypoxia ($F_{I_{O_2}}$ 0.10 in N_2) and hypercapnia (fraction of inspired carbon dioxide ($F_{I_{CO_2}}$ of 0.05 in air)). Each recording period lasted 10 min. At the outlet of one of the plethysmographic chambers we have an oximeter (Dräger, Sensor Oxydig, Lubbeck, Germany) to verify the PO_2. Usually in 3–4 min the desired atmosphere is attained and therefore the analysis time is usually restricted to the last 6 min of exposure. Since in present study we were mostly interested in comparing respiratory parameters measured in identical conditions in all experimental groups, potential modifications in body temperature of the animals while breathing in the different atmospheres were not taken into account in those measurements. Parameters measured or computed include breathing frequency f_{resp}; tidal volume (V_T); minute ventilation ($\dot{V}_E$); inspiratory time (T_I); expiratory time (T_E); respiratory duty cycle (T_I/T_{tot}); mean inspiratory flow (V_T/T_I); mean expiratory flow (V_T/T_E); and enhanced pause (Penh). Penh was automatically computed by the software of the equipment and quantified according to the following formula:

$$\text{Penh} = \left(\frac{\text{PEF}}{\text{PIF}}\right) \times \left[\left(\frac{T_E}{T_{E_{65}}}\right) - 1\right]$$

where PIF = Peak Inspiratory Height in the plethysmographic recording; PEF = Peak Expiratory Height in the plethysmographic recording; T_E = Expiratory Time; $T_{E_{65}}$ = Time to expire 65% of the "volume"; Pause = $T_E/T_{E_{65}} - 1$; Penh = PEF/PIF × Pause.

2.5. Measurements of airway morphometry and Goblet cells

Explanted lungs of 32 animals were inflated with 4% formaldehyde at a constant pressure of 25 cm H_2O during 24 h, and then embedded in paraffin. Histological examination was performed in 4 μm sections stained with hematoxylin–eosin (H&E) and Alcian Blue for airway morphometry and Goblet cells quantification, respectively. The external and the internal limits of the muscular layer were outlined and both total and lumen area were computed using an image analysis system (ImagePro Plus, Media Cybernetics, Inc., Bethesda, MD). A total 230 sections of membranous bronchioles from the 32 animals (8 animals/group) were analyzed. External and internal areas were determined and bronchiolar wall thickness estimated as the difference between them. The area of the muscular layer of the bronchioles was estimated as the difference between the total and internal areas. The presence of Goblet cells was expressed as the number of cells per perimeter of bronchiolar epithelium (cells/mm). Additional methodological details can be found in Ferrer et al. (2009).

2.6. Data presentation and statistics

Data were evaluated using a Graph Pad Prism Software, version 4 (GraphPad Software Inc., San Diego, CA, USA) and were presented as mean ± SEM. The significance of the differences between the means was calculated by one and two-way analysis of variance (ANOVA) for repeated measures (when applicable) with Newman–Keuls and Bonferroni multiple comparison tests, respectively. Potential interactions between CS and CH also were statistically assessed. Specific Statistical p values of 0.05 or less were considered to represent significant differences.

3. Results

3.1. Animal's general status. Evolution of body weight and hematocrit

The general status of the smoking animals was normal in appearance and the motility and playing behaviour among cage mates was not obviously different from controls. Fig. 2A shows the evolution of the body weight of animals. Weight gain in all groups ran parallel up to the 3 months of age. Thereafter, guinea pigs exposed to cigarette smoke (CS and CSCH groups) gain less weight than controls so that by the end of the 3 months exposure experimental animals weighed 19.5% less than control animals.

Hematocrit was measured at the end of the study and found to be increased in all experimental groups: CS (48 ± 2; $p < 0.01$), CH (45 ± 2; $p < 0.05$) and CSCH (51 ± 1; $p < 0.001$) vs. Control (40 ± 1). Hematocrit difference between CH and CSCH groups was statistically different ($p < 0.001$) and between CS and CSCH was marginally significant ($p = 0.058$). However, when statistical analysis for interaction was performed, it did not support a positive interaction indicating a simple additive effect between both stimuli ($p > 0.05$).

3.2. Ventilatory effects of acute hypoxia and hypercapnia in control animals

Fig. 3A shows sample plethysmographic recordings obtained in the same animal in the three different atmospheres, normoxia, hypoxia and hypercapnia. In Fig. 3B we present mean values obtained in Control guinea pigs at all ages (2–5 months) in the three studied atmospheres: normoxia (air), hypoxia (10% O_2) and hypercapnia (5% CO_2 in air).

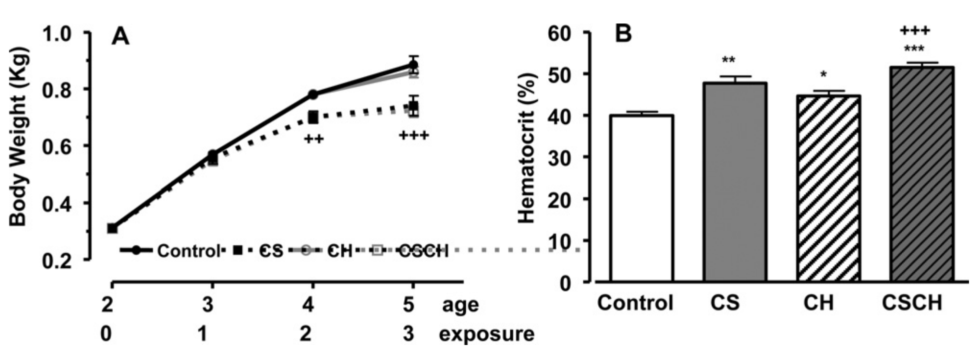

Fig. 2. Body weight and hematocrit values in Control, cigarette smoke exposed (CS), chronic hypoxia exposed (CH) and both cigarette smoke and hypoxia exposed (CSCH) guinea pigs. (A) Body weight evolution during the experiments (3 months) in the four groups of guinea pigs (from 2 to 5 months of age). $^{++}p < 0.01$ and $^{+++}p < 0.001$ CS and CSCH vs. Control and CH (two way ANOVA with age and treatment as independent factors). (B) Hematocrit in the four groups of animals as label in the figure. Data are means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ every group vs. Control; $^{+++}p < 0.001$ CSCH vs. CH (one way ANOVA). In every case, sample sizes as depicted in Fig. 1.

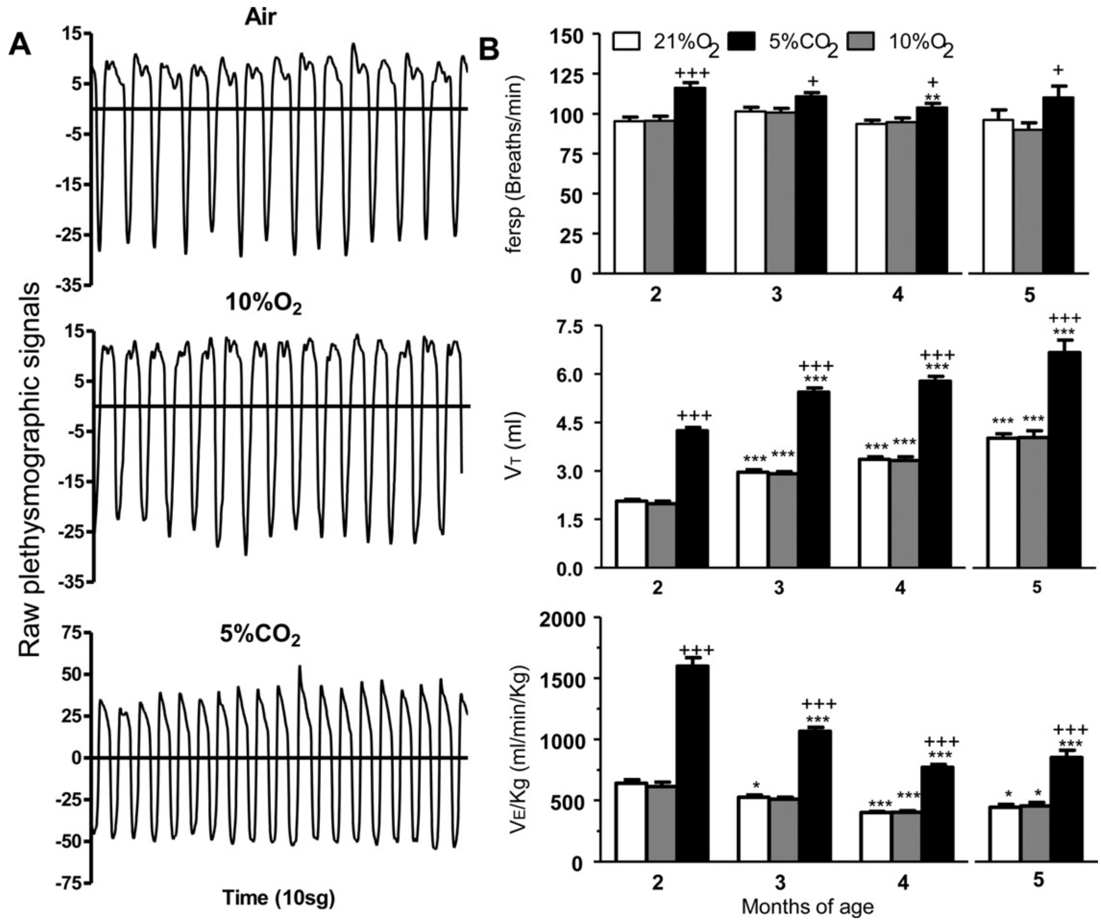

Fig. 3. Age-dependent breathing pattern in control guinea pigs while breathing in air, $F_{I_{O_2}}$ 0.10 and $F_{I_{CO_2}}$ 0.05 atmospheres. (A) Sample rough plethysmographic recordings. (B) Top, breathing frequency (f_{resp}). $^{+}p<0.05$ $^{+++}p<0.001$, $F_{I_{CO_2}}$ 0.05 vs. air. Middle, tidal volume (V_T) $^{***}p<0.001$ at 3, 4 and 5 months vs. 2 months $^{+++}p<0.001$ 5% CO_2 vs. air. Bottom, minute ventilation/kg of body weight $^{***}p<0.001$ $^{**}p<0.01$ at 3, 4 and 5 months vs. 2 months $^{+++}p<0.001$ 5% CO_2 vs. air. Data are means ± SEM (statistical significances were obtained using a two-way ANOVA for repeated measures, with age and breathing atmospheres as independent factors). In every case, sample sizes as depicted in Fig. 1.

Normoxia (*room air, during 10 min*). Under this condition, the ventilatory pattern varied with age (Fig. 3B, empty bars). f_{resp} at a $F_{I_{O_2}}$ of 0.21 did not change between 2 and 5 months of age (96 ± 3 and 96 ± 6 breaths/min, respectively), while V_T increased two fold in this period of time from 2.06 ± 0.06 ml at two months up to 4.15 ± 0.14 ml at five months ($^{***}p<0.001$ at all ages vs. two months). As a consequence, $\dot{V}_E$ also augmented with age, but the increase in V_T and $\dot{V}_E$ run behind body weight gain so that $\dot{V}_E$/kg of body weight decreased with age from 642 ± 27 at two months down to 446 ± 23 ml/kg at five months ($^{***}p<0.001$ at all ages vs. 2 months; Fig. 3 empty bars).

Hypoxia ($F_{I_{O_2}}$ *of 0.10, during 10 min*). Acute hypoxia did not alter the f_{resp}, V_T or $\dot{V}_E$/kg encountered in normoxic conditions at any age (Fig. 3B, grey bars), indicating that guinea pigs do not hyperventilate in response to acute hypoxic hypoxia.

Hypercapnia ($F_{I_{CO_2}}$ *of 0.05 in air, during 10 min*). f_{resp} in hypercapnia was statistically identical at all ages. Hypercapnia caused a moderate but significant increase in the f_{resp} as compared to normoxia at all ages but 5 months (+++ and +, $p<0.001$ and $p<0.05$, respectively; Fig. 3B, black bars). V_T in hypercapnic atmosphere increased with age being at 3, 4 and 5 months statistically higher than at 2 months ($^{***}p<0.001$). At every age V_T in hypercapnic atmosphere was nearly doubled than in normoxic conditions ($^{+++}p<0.001$; Fig. 3). $\dot{V}_E$/kg of body weight in hypercapnia declined with age being maximum at 2 months and minimum at 5 months ($^{***}p<0.001$ at all ages vs. two months), but at any age it was nearly double that $\dot{V}_E$ encountered in normoxic atmosphere ($^{+++}p<0.001$). In other words, hypercapnia about doubled $\dot{V}_E$ at any studied age (Fig. 3, black bars).

Table 1 shows T_I, T_E, T_I/T_{tot}, V_T/T_I, and V_T/T_E parameters in control animals at different ages. Results are comparable to those previously reported in young guinea pigs (Wiester et al., 1988).

3.3. Penh

Penh is a unit-less index which can be obtained in laboratory animals using whole body plethysmography. It is the unique indicator of airway responsiveness and airways resistance that can be obtained in conscious unrestrained animals and therefore is freed from the bias imposed by anesthesia or restraining stress. Fig. 4A and B show, respectively, Penh values in Control and CS animals

Table 1
Ventilatory times and flows in control guinea pigs.

Age (months)	T_I (ms)	T_E (ms)	T_I/T_{tot} (%)	V_T/T_I (ml/s)	V_T/T_E (ml/s)
2	262.9 ± 7.3	397.3 ± 12.1	39.8	7.83	5.18
3	244.9 ± 7.0	372.3 ± 10.3	39.5	12.08	7.95
4	265.7 ± 7.0	402.1 ± 9.9	39.7	12.60	8.33
5	256.6 ± 14.9	409.8 ± 25.4	38.5	16.17	10.12

Inspiratory time (T_I), expiratory time (T_E), % time of the respiratory cycle occupied by inspiration (T_I/T_{tot} (%), inspiratory flow (V_T/T_I) and expiratory flow (V_T/T_E)).

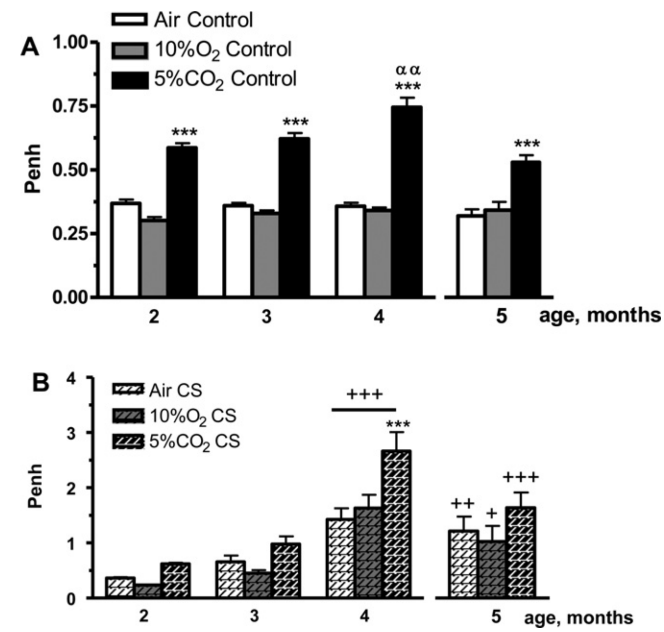

Fig. 4. Enhanced pause (Penh) values in Control and cigarette smoke exposed guinea pigs while breathing air, $F_{I_{O_2}}$ 0.10, and $F_{I_{CO_2}}$ 0.05. (A) shows data for control animals of different ages. $^{***}p < 0.001$ vs. air breathing. $^{\alpha\alpha}p < 0.01$ hypercapnic breathing at 4 months vs. all other ages. (B) shows data for animals exposed to cigarette smoke during different periods of time. Crosses label statistical differences from age matched controls (statistical significances were obtained using a two-way ANOVA for repeated measures, with age and breathing atmospheres as independent factors when comparing within group of smoking animals; when comparing smoking animals with age-matched controls the independent factors were breathing atmospheres and treatment). In every case, sample sizes as depicted in Fig. 1.

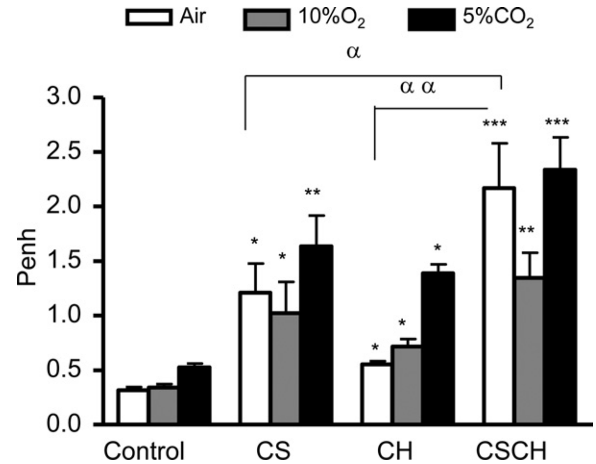

Fig. 5. Penh values in the three experimental groups (CS, CH and CSCH) at 5 months of age. In all experimental groups Penh values were higher than in Control in the three atmospheres ($^{*}p < 0.05$; $p < 0.01$ and $p < 0.001$ vs. Control and correspondent atmosphere). $^{\alpha}p < 0.05$ and $^{\alpha\alpha}p < 0.01$ air in CSCH vs. CS and CH groups, respectively (two-way ANOVA with treatment and atmosphere as independent factors). In every case, sample sizes as depicted in Fig. 1.

at all ages while breathing air, $F_{I_{O_2}}$ 0.10 or $F_{I_{CO_2}}$ 0.05. Notice first, that Penh values are nearly identical at all ages, secondly that Penh values are comparable in normoxia and hypoxia, and lastly that in hypercapnia Penh value was significantly higher, nearly double, than in the other two conditions ($^{***}p < 0.001$) and was maximum at 4 months (0.75 ± 0.04) and minimum at 5 months (0.53 ± 0.03); in fact Penh was significantly higher at 4 months in comparison to all other ages ($^{\alpha\alpha}p < 0.01$).

In CS animals (Fig. 4B) Penh increased in the three conditions with duration of the exposure to cigarette smoke, reaching maximum values after 2 months of exposure (4 months old; nearly 4× control values; $^{+++}p < 0.001$ vs. Control of 4 months of age); at 3 months of exposure (5 months old) Penh remained high in comparison to age-matched controls ($^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ vs. 5 month old Control).

3.4. Penh values and bronchiolar wall structure

Fig. 5 shows Penh values in 5 months old animals in Control, CS, CH and CSCH groups, measured while breathing in air, $F_{I_{O_2}}$ 0.10 and $F_{I_{CO_2}}$ 0.05 atmospheres. Note first, that Penh values in the three experimental groups (CS, CH and CSCH) were higher than in Control group in every condition (significance labeled by asterisks) and second, that Penh values were higher in CSCH than in CS and CH groups in the three atmospheres, differences reaching statistical significance while breathing air. The fact that in normal air atmosphere the differences in the values of Penh in the CSCH group vs. CH and CS groups reach statistical significance (significance labeled by alphas), would suggest a positive interaction between the two stressors (chronic hypoxia and cigarette smoke) to increase airways resistance, yet statistical analysis did not support it ($p > 0.05$).

Top four images of Fig. 6 show microphotographies of lung parenchyma evidencing a certain degree of alveolar walls thickening, as well as a higher level of inflammatory cell infiltration in sections from CS and CH animals. Both changes are more noticeable in the section of the CSCH where a clear neutrophilic–eosinophilic infiltration is evident. The four lower images are representative sections obtained from animals belonging to each of the four groups. It is apparent a thickening of the bronchiolar wall of CH and CS, being particularly noticeable in CSCH group; the thickening of the bronchiolar muscle layer is also evident. A parallel increase in Goblet cell number is also increasingly noticeable from control to CH, CS, and CSCH sections (labeled by arrows).

The thickness of the bronchiolar wall is shown in Fig. 7A. In CS and CH groups the area of the bronchiolar wall was larger but was not statistically different from controls. In CSCH group thickness of bronchiolar wall was nearly double than controls, being the differences highly significant ($^{***}p < 0.001$); similarly it was different from CS and CH groups ($^{\alpha\alpha}p < 0.01$). The same qualitative findings were attained if bronchiolar wall thickness is expresses as the ratio wall area/total bronchiole area. Also consistent with Penh values, are the thickness of bronchial muscle layer and number of Goblet cells/mm of bronchial epithelium which tend to be larger in CS and CH groups and were significantly larger in CSCH group ($^{***}p < 0.001$ CSCH vs. Control; $^{+}p < 0.05$ and $^{++}p < 0.01$ CS and CH vs. CSCH; interaction between tobacco and hypoxia was not statistically significant, $p > 0.05$; Fig. 7B).

4. Discussion

Our findings include: (1) cigarette smoke exposure produces a moderate time-dependent decrease in the body weight; both cigarette smoke and chronic hypoxia synergistically increase the hematocrit; (2) age-dependent (2–5 months) ventilatory pattern of control guinea pigs includes a constant f_{resp}, an increase in V_T with age and an age-dependent decrease in $\dot{V}_E$/kg of body weight; acute hypoxia does not alter the ventilatory pattern at any age, while acute hypercapnia causes a marked V_T-dependent increase in $\dot{V}_E$ at all ages; (3) in control animals Penh values in 0.05 $F_{I_{CO_2}}$ atmosphere is about double than in air and 0.10 $F_{I_{O_2}}$ atmospheres; (4) in CS group Penh increases significantly as exposure time progresses whether it is measured in air, 0.10 $F_{I_{O_2}}$, or 0.05 $F_{I_{CO_2}}$ atmospheres; (5) exposure to sustained hypoxia (CH group) also increases Penh

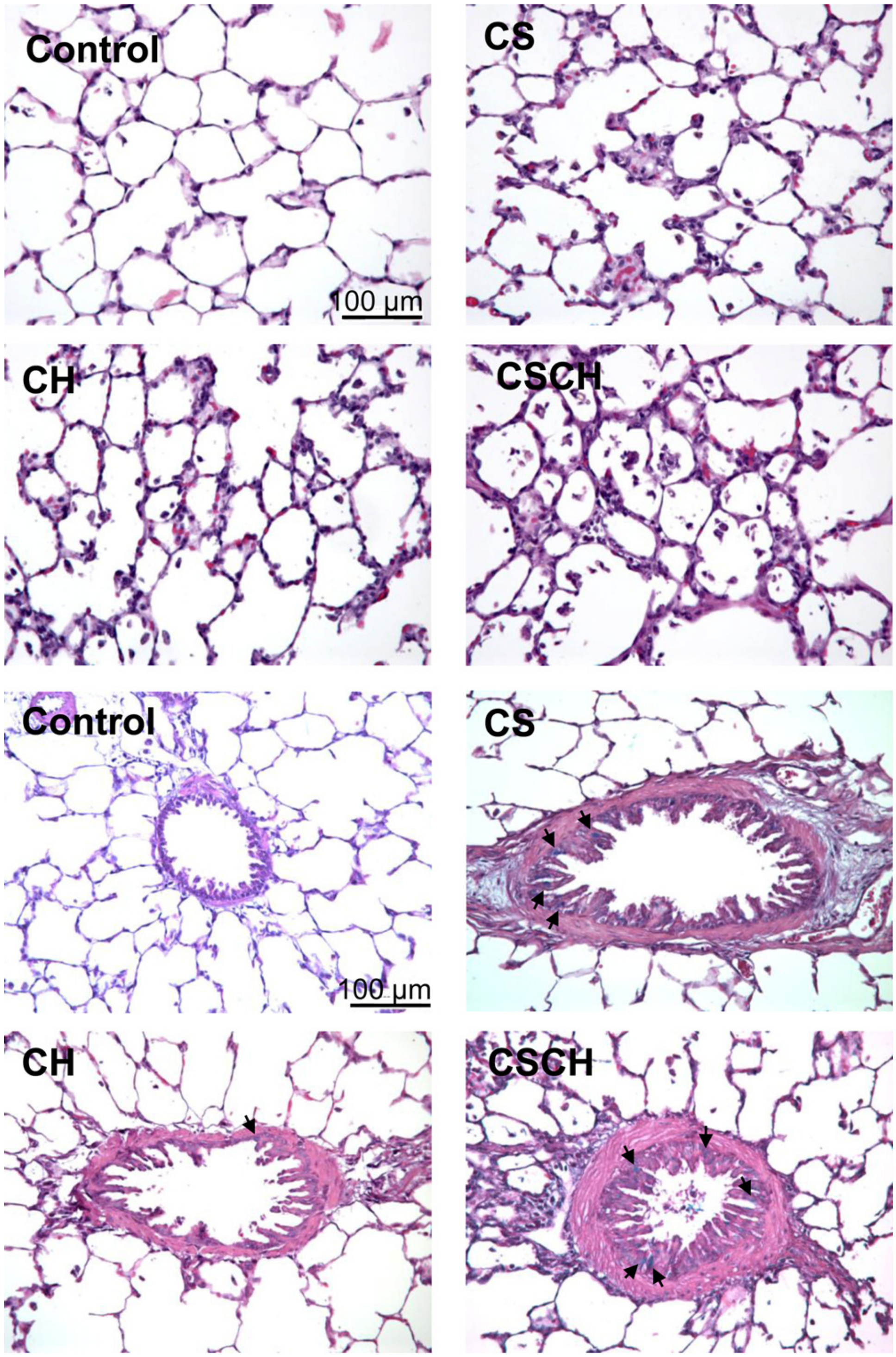

Fig. 6. Parenchymal lung tissue and small bronchi photomicrographs. The top four images are sections of lung parenchyma obtained from control, smoking (CS), chronic hypoxic (CH) and hypoxic smoking (CSCH) animals. Four lower sections corresponding to same experimental groups contain small caliber bronchi to evidence the thickness of bronchial wall and the density of Goblet cells (arrows).

in the three conditions and, association of cigarette smoke and sustained hypoxia (CSCH group) showed an additive effect to increase Penh in the three conditions; (6) thickness of the bronchiolar wall, thickness of the bronchial muscle layer, and number of Goblet cells in bronchi roughly paralleled to the increase in Penh values in all groups.

As previously reported (Ardite et al., 2006), prolonged smoke exposure curtails weight gain in guinea pigs. Coherent with findings in smokers humans (Yanbaeva et al., 2007), CS group showed an increase in the hematocrit, that probably results from a high carboxyhemoglobinemia (Wright and Churg, 1991). The increased hematocrit in CH group is also expected as an adaptive response to hypoxia mediated by HIF-1α (Semenza et al., 2006). In CSCH group both, hypoxic hypoxia and anaemic hypoxia (carboxyhemoglobinemia) would generate a higher compensatory-adaptive hematopoietic response.

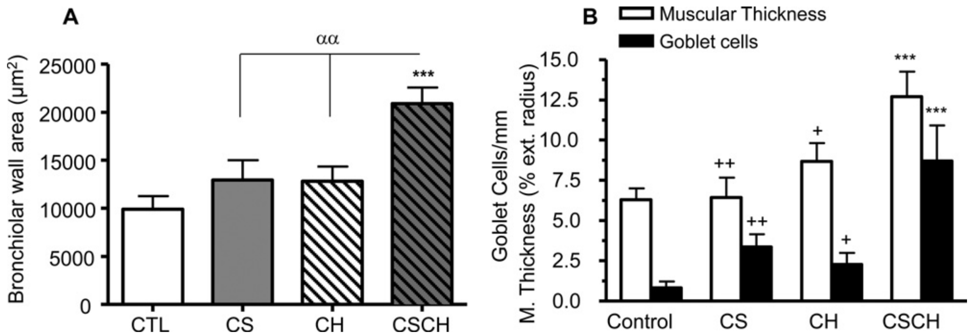

Fig. 7. (A) Bronchiolar wall area determined as the difference between total and internal areas of the bronchioles. $^{***}p < 0.001$ vs. control; $^{\alpha\alpha}p < 0.01$ vs. CS and CH (one way ANOVA). (B) Goblet cell number and thickness of bronchial muscular layer in the four experimental groups. Statistical significance: $^{***}p < 0.001$ CSCH vs. Control; Crosses differences among CS and CH vs. CSCH groups as labeled (independent one way ANOVA for each parameter). In every case, sample sizes as depicted in Fig. 1.

The age-dependent variation in breathing pattern in the age window studied, 2–5 months, is characterized by a constant f_{resp} and a progressive V_T increase; yet, V_T increased less than body weight causing an age dependent decrease in $\dot{V}_E$/kg. Absolute values of f_{resp}, V_T and $\dot{V}_E$ are comparable to those found by other authors (Yilmaz et al., 2005; Wiester et al., 2005). This age-dependent variation in ventilatory pattern is common in all mammals (Mortola, 2001) and parallels the mass specific metabolic rate. It should be noted, however, that maintenance of f_{resp} and increase in V_T tends to increase alveolar ventilation, and therefore, O_2 uptake/kg of body weight would decrease less than the measured $\dot{V}_E$/kg of body weight. Consistent with most of the published studies (Blake and Banchero, 1985; Curran et al., 1995; Yilmaz et al., 2005; Schwenke et al., 2007; but see Fernández et al., 2003), our data indicate a lack of hypoxia driven CB chemoreflex in adult guinea pigs. Admittedly, the intensity of the hypoxic test was moderate ($F_{I_{O_2}}$ 0.10), but the same stimulus applied in the same conditions augmented f_{resp}, V_T and $\dot{V}_E$/kg in rats by a factor of 1.6 (Agapito et al., 2009). Schwenke et al. (2007) in their experiments in anaesthetized guinea pigs used 8% O_2 and found that no recorded CSN responded to this hypoxic level and additionally that ventilation under 8% O_2 was not affected by CSN denervation. Contrary to the situation with hypoxia, in present experiments we observe that ventilatory response to hypercapnia is comparable to that obtained in the rat in comparable experimental conditions (Agapito et al., 2009) suggesting that in guinea pigs, as it is the case in the rat, CO_2-triggered hyperventilation is mediated by CB and central chemoreceptors (Gonzalez et al., 1994), and implying that the lack of CB chemoreflex in guinea pigs refers exclusively to the hypoxia driven reflex. Consistent with our interpretation, Schwenke et al. (2007) observed in their anaesthetized guinea pigs that denervation of the CB in guinea pigs caused a ca. 28% decrease in the hyperventilation produced by 8% CO_2 breathing (Schwenke et al., 2007), a percentage which is comparable to that observed in most mammals, i.e., in guinea pigs, as in most mammals, the CB would contribute by around 30% to the CO_2 triggered hyperventilation, with the remaining response being of central origin (Gonzalez et al., 1994). This in turn would indicate that the CB and its central projections to brainstem respiratory controllers are functional implying that the oxygen-sensing machinery (Gonzalez et al., 1994, 2007) could be absent in the CB of this species.

The experimental conditions used in present experiments to measure ventilatory response to hypoxia might not be ideal because ambient temperature is lower than neutral temperature in guinea pigs [32 °C according to Hill (1959)]. In young adult guinea pigs comparable in weights to those used in our present experiments, Hill observed that at temperature below neutral, hypoxia (10% O_2 as in our experiments) causes a rapid hypometabolism assessed as a nearly 50% decrease in minute O_2 consumption (this response with a higher or lower intensity also occurs in other mammals; Frappell et al., 1992; see also Mortola and Frappell, 2000). In the rat, this decrease in oxygen consumption is mediated by mechanisms centered in the posterior hypothalamus (and probably additional brain areas) and produces a decrease in ventilation (Hinrichsen et al., 1998). Additionally, hypoxia as a result of the hypometabolism causes a slow and progressive decrease in body temperature (Hill, 1959; Mortola and Frappell, 2000), which via CB chemoreceptors (see Zapata, 1997) would also tend to lessen any potential hypoxia-triggered CB-mediated hyperventilation. In sum, these two factors, hypometabolism and hypothermia linked depression of ventilation, could be distorting our findings and masking any potential hypoxia-triggered CB-mediated hyperventilation. Although there are not specific data quantifying these effects in guinea pigs, it appears that in our experimental conditions they would be quantitatively very small by the following reasons: (1) the depression of ventilation by hypometabolism is variable from species to species (Frappell et al., 1992) and it is best evidenced at neutral or near neutral temperature in newborn animals with poorly developed CB chemoreflex (Mortola et al., 1989), (2) the hypothermic depression of CB function would be nil due to the short duration of the hypoxic exposure (10 min) and to the fact that the temperature of the plethysmography chamber was 22–24 °C (in Hill's experiments (1959) working at 20 °C, body temperature dropped ≤0.5 °C in 10 min), and (3) the above-mentioned study of Schwenke et al. (2007) showing a lack of CB response to hypoxia carried out in adult guinea pigs was performed at constant body temperature of 39 °C and the study by Curran et al. (1995) also showing the lack of a hypoxic ventilatory response in young 10–14 days old guinea pigs was performed at 33 °C in the plethysmographic chamber, an ambient temperature that according to Hill (1959) would not change O_2 consumption or body temperature. In sum, although guinea pigs as all mammals in hypoxia appear to have an increased ventilation to metabolism ratio (i.e., hyperventilate in relation to metabolic needs), it would appear that in our experimental conditions (temperature and duration of hypoxia) such hyperventilation would be minimal, but if any, it would be generated exclusively by a decrease in O_2 consumption due to the unresponsiveness of their CB to hypoxia. This abnormal response of guinea pigs, in comparison to other mammals, prevents us from reporting the impact of CS and CSCH on chemosensitivity.

Literature on the value of Penh as a measure of airways resistance is divided, with some authors been attached to it (Bergren, 2001; Lomask, 2006) and some others neglecting its value (Flandre et al., 2003; for a recent discussion on the significance of Penh please see Vargas et al., 2010). Yet, in present study there are several findings that predicting an increase in airways resistance did indeed cause an increase in Penh values, and vice versa. (1) In Control group Penh values were double in 5% CO_2 than in air or 10% O_2. Taking into account that airways tone is reflexly controlled, being arterial and central chemoreceptors (among other) afferent arms of the reflex, and taking also into account that in guinea pig hypercapnia, but not hypoxia activates chemoreceptors, it should be expected, as indeed it is the case, that only hypercapnia increases Penh (Fitzgerald and Shirahata, 1997; Pérez Fontán et al., 1998; Canning, 2006). In other words, CO_2 that activates peripheral and central chemoreceptors increases Penh, while hypoxia that in guinea pigs does not activate chemoreceptors does not modify Penh. (2) CS animals have increased Penh values in all atmospheres. As mentioned in the introduction CS sensitizes C-fibers in airways by a neurokinin mediated mechanism leading to bronchial hyper reactivity (Joad et al., 2004), an in addition the increased number of Goblet cells would also contribute to generate hyperactive bronchiolar afferents, being well documented that mucous secretion activates airways afferents triggering reflex airways constriction (Canning, 2006). (3) In CH Penh values are higher than in controls. This increase in Penh value would result from the thickening of the bronchial wall and bronchial muscle layer (probably mediated by HIF-1α; Semenza et al., 2006) and concurrent tendency of Goblet cells to increase. (4) Finally, the highest Penh value in CSCH animals is consistent with markedly increased Goblet cell number and thickening of bronchiolar wall and its muscular layer. In this context, we should emphasize the similarities of airways reactivity in guinea pigs and humans (Canning, 2006). As a whole these findings indicate that tobacco smoke produces morphological and functional alterations in airways that would contribute to the obstruction encountered in COPD patients. The association of tobacco smoke and hypoxia thicken bronchial wall, increase Goblet cell number, and augment Penh values, more than each stressor individually, and therefore they could further contribute to airways obstruction. In addition it might be suggested that these structural alterations modify the pattern of stimulation of mechanoreceptors in airways (Yu, 2009) and thereby could alter breathing pattern.

In sum, exposure of guinea pigs to cigarette smoke during three months causes an increase in airways resistance. Co-exposure to cigarette smoke and chronic hypoxia causes a further increase in airways resistance. In addition, pulmonary hypertension and signs of vascular remodeling are exacerbated (Ferrer et al., 2011) on associating CS and hypoxia. These observations might have potentially relevant clinical correlates: it might be suggested that the appearance of hypoxemia in COPD patients would represent a sign that should be promptly corrected to avoid the triggering of feed-forward mechanisms that would endanger the survival of patients. In fact it is well recognized that hypoxemia in COPD patients augments the risk of right and left heart dysfunction or even failure (Nizet et al., 2005; Le Jemtel et al., 2007) as well as the frequency and severity of nocturnal desaturations and complicating lactacidosis (Toraldo et al., 2005). Even further, casual acute or sub acute hypoxic episodes (as for example sojourning at high altitude or a seasonal respiratory illness) might uncover unrecognized COPD patients or aggravate the clinical picture in patients with mild COPD. This could be so because well before the picture of emphysema is evident there is an increase in pulmonary circulation resistance-pulmonary hypertension that would be exaggerated by the hypoxic episodes due to pulmonary hypoxic vasoconstriction (Ferrer et al., 2011). Similarly, to the hyper reactivity of the bronchial wall caused by CS, which also appears before emphysema is apparent, it would add the high reflex bronco constrictor effect mediated by carotid body chemoreceptors and triggered by hypoxia (Gonzalez et al., 1994; Fitzgerald and Shirahata, 1997); the net result would be an exaggerated increase in airways resistance. Finally, our findings (additive effect between CS and hypoxia to increase Goblet cells, thickness of bronchiolar wall and muscle layer, and airways resistance) would provide a tentative explanation to the epidemiologically established observation that association of smoking (e.g., smoking mothers or smoke house) and hypoxia (frequent respiratory infections or sleep related apneas) constitutes a precipitating factor for the respiratory misregulation leading to loss of the arousal reaction to hypoxia and sudden infant dead (Kinney and Thach, 2009). Consistent with our interpretation Mutoh et al. (2000) have observed that the sensitization of the bronchopulmonary C-fiber endings is associated with a prolonged reflexively evoked expiratory apnea.

Conflict of interest

The authors declare no conflict of interest with the study or preparation of the manuscript.

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