Chapter 44
Effects of Cigarette Smoke and Chronic Hypoxia on Ventilation in Guinea Pigs. Clinical Significance

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Abstract Ventilatory effects of chronic cigarette smoke (CS) alone or associated to chronic hypoxia (CH), as frequently occurs in chronic obstructive pulmonary disease (COPD), remain unknown. We have addressed this problem using whole-body plethysmography in guinea-pigs, common models to study harmful effects of CS on the respiratory system. Breathing frequencies (Bf) in control (2–5 months old) guinea pigs is 90–100 breaths/min, their tidal volume (TV) increased with age but lagged behind body weight gain and, as consequence, their minute volume (MV)/Kg decreased with age. MV did not change by acutely breathing 10% O_2 but doubled while breathing 5% CO_2 in air. Exposure to chronic sustained hypoxia (15 days, 12% O_2, CH) did not elicit ventilatory acclimatization nor adaptation. These findings confirm the unresponsiveness of the guinea pig CB to hypoxia. Exposure to CS (3 months) increased Bf and MV but association with CH blunted CS effects. We conclude that CS and CH association accelerates CS-induced respiratory system damage leading to a hypoventilation that can worsen the ongoing COPD process.

Keywords Guinea pig • Ventilation • Tobacco • Hypoxia • Carotid body.

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44.1 Introduction

Guinea pigs exposed to cigarette smoke (CS) are valuable models for chronic obstructive pulmonary disease (COPD) to study lung and respiratory pathology (Wright and Churg 2002). To underscore the mechanisms involved in the pathology found in COPD (Churg et al. 2011) most studies focus their interest on the effects of CS on lung parenchyma and more recently on pulmonary circulation (Ferrer et al. 2011). However, a systematic study of the effects of CS on ventilation, including responses to hypoxia and hypercapnia, has not been performed in any laboratory model. In humans, Tobin et al. (1983) found that healthy chronic smokers had resting MV augmented, being the increase due to Bf and TV.

In middle to advanced stages of COPD, CS and chronic hypoxia (CH) coexist in many patients. The study of the interaction between CS and CH results of great interest in order to find if association of CS and CH accelerates the progression of alterations produced by CS alone. The present work has specifically addressed the study in the guinea pig of the ventilatory effects of CH and CS exposure individually and in combination. Our experimental design consisted in exposing the guinea pigs to a smoking protocol of 4 cigarettes/day for 3 months; this smoking protocol does not produce emphysema (Ferrer et al. 2009) nor produces alterations in blood gases (Wright and Churg 1991). Therefore, simultaneous exposure of smoking animals to CH was an absolute requirement to define the combined effects of both stressors, CS and CH, at these early stages of CS induced lung damage.

Prior to the effects of CS and CH we have aimed to settle a basic aspect in the respiratory physiology of guinea pigs, namely, if hypoxia increases their MV as it does in most mammals. Most authors have found that carotid body (CB) mediated ventilation or electrical activity in the carotid sinus nerve in response to hypoxia is very small or totally absent when compared to rat and other laboratory rodents (Blake and Banchero 1985; Curran et al. 1995; Yilmaz et al. 2005; Schwenke et al. 2007). Fernandez et al. (2003) have shown that guinea pigs exhibit a chemosensory drive both in normoxia and hypoxia comparable to that seen in the rat. Blake and Banchero (1985) found that acute hypoxia did not augment ventilation. On the other hand, if the animals were maintained (>3 months) at 4,600 m of altitude they observed a small increase in ventilation (36% above sea level MV; see also Yilmaz et al. 2005). We have performed a basic characterization of the ventilatory pattern of guinea pigs, including the responses to acute hypoxic and hypercapnic tests and the effects of exposure to CH on ventilation.

44.2 Material and Methods

44.2.1 Animals and Experimental Groups

Forty eight male Hartley guinea pigs (7 weeks of age) purchased to Harlam Iberica were housed (3/cage) in the vivarium in a 12 h light–dark cycle at room temperature (20–23 °C). Animals were provided standard chow and water supplemented with vitamin C (1 g/l) *ad libitum*. After 1 week of adaption (i.e., at 2 months of age), animals were distributed in 2 groups of 24 animals and divided in subgroups. Twenty four animals were exposed to cigarette smoke for 3 months: 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 3 months (control animals, Control) and 16 were exposed to hypoxia for the last 2 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).

44.2.2 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.

44.2.3 Exposure to Chronic Hypoxia

The chamber and experimental maneuvers for CH exposure (12% O_2 in N_2; $PO_2 \approx 85$ mmHg; equivalent to ≈4,300 m; 15 days) have been described in prior studies (Caceres et al. 2007).

44.2.4 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 (Bf, breaths/min). The spurious fluctuations due to animal movements were electronically rejected. The TV is provided by the software from the integration of the inspiratory curve. The system was calibrated automatically by software after steady injection into the chamber of 5 ml air. Ventilatory parameters were measured in conscious, freely moving guinea pigs by whole body plethysmography while breathing room air, 10% O_2, and 5% CO_2 in air. The system (Emka Technologies, Paris, France with software IOX version 1.8.9.4) and recording conditions have been described elsewhere (Agapito et al. 2009). Parameters measured or computed include Bf, TV, and MV.

44.2.5 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) with Newman-Keuls and Bonferroni multiple comparison tests for repeated measurements, respectively. P values of 0.05 or less were considered to represent significant differences.

44.3 Results

44.3.1 Animal's General Status, Body Weight and Hematocrit

The general status of the smoking animals was apparently not different from controls. Weight gain in all groups ran parallel up to the 3 months of age or 1 month of exposure to CS. As exposure increased, weight gain in CS and CSCH was less than in control so that at completion of exposure they weighed 19.5% less than control animals.

Hematocrit was measured at the end of the study was significantly increased (by 5–10%) in all experimental groups, being CS and CH nearly additive in eliciting polycythemia.

Table 44.1 Ventilatory parameters in growing guinea pigs maintained in control atmosphere and in animals exposed during 15 days to (12 O_2) (statistical differences in the main text)

Breathing atmosphere	Age				
	Months, 2	Months, 3	Months, 4	Months, 5	Months, 4. 5;0.5, 12% O_2
Room Air					
Bf (breaths/min)	95.6 ± 2.6	101.8 ± 2.6	94.1 ± 2.2	96.0 ± 6.4	83.2 ± 4.4
TV (ml)	2.1 ± 0.1	3.0 ± 0.1	3.4 ± 0. 1	4.1 ± 0.1	4.0 ± 0.1
MV (ml/Kg/min)	600.6 ± 15.9	528.2 ± 17.4	401.7 ± 9.7	446.4 ± 22.8	388.9 ± 2.4
Hypoxia (10% O_2)					
Bf (breaths/min)	95.5 ± 2.8	100.6 ± 2.7	95.5 ± 2.6	89.9 ± 5.0	93.8 ± 4.5
TV (ml)	2.0 ± 0.1	2.9 ± 0.1	3.3 ± 0.1	4.2 ± 0.2	4.5 ± 0.2
MV (ml/Kg/min)	580.2 ± 19.9	511.6 ± 17.4	403.4 ± 12.4	455.7 ± 28.7	506.0 ± 19.2
Hypercapnia (5% CO_2 in air)					
Bf (breaths/min)	116.1 ± 3.4	111.8 ± 2.4	105.1 ± 2.8	110.2 ± 7.1	86.6 ± 3.7
TV (ml)	4.2 ± 0.1	5.4 ± 0.1	5.8 ± 0.1	6.9 ± 0.4	7.7 ± 0.3
MV (ml/Kg/min)	1,542.0 ± 48.4	1,065.8 ± 33.3	772.3 ± 24.0	852.8 ± 58.1	760.0 ± 30.1

44.3.2 Effects of Acute Hypoxia and Hypercapnia on Ventilation. Effects of CH Exposure

Table 44.1 shows ventilatory parameters in Control and CH while animals breathe room air, hypoxia (10% O_2, 10 min), and hypercapnia (10 min, 5% CO_2 in air).

Normoxia. Bf in normoxia was nearly identical at all ages: around 100 breaths/min. TV doubled from 2 to 5 months of age, from 2.06 ± 0.06 ml to 4.15 ± 0.14 ml ($p < 0.001$ vs. 2 months). This increase runs behind body weight gain so that MV/Kg of body weight decreased with age from 601 ± 16 at 2 months down to 446 ± 23 ml/min/Kg at 5 months ($p < 0.001$ vs. 2 months).

Hypoxia. Acute hypoxia did not alter the Bf, TV nor MV/Kg encountered in normoxia indicating that guinea pigs do not hyperventilate in response to acute hypoxic hypoxia.

Hypercapnia. Bf in hypercapnia was statistically identical at all ages. Hypercapnia caused a moderate increase (vs. normoxia) in the Bf at all ages but 5 months ($p < 0.001$ and $p < 0.05$). TV 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 TV in hypercapnic atmosphere was nearly double than in air ($p < 0.001$). Consequently MV/Kg in hypercapnia was nearly double normoxic value ($p < 0.001$; i.e., hypercapnia nearly doubled ventilation), but also declined with age being maximum at 2 months and minimum at 5 months ($p < 0.001$ at all ages vs. 2 months).

Last column of Table 44.1 shows the effects of CH: neither Bf, TV nor MV/Kg were significantly modified in any conditions. Alternatively stated, CH did not elicit any adaptive response.

44.3.3 Ventilatory Effects of CS Exposure and its Association with CH

Exposure to CS changed the respiratory pattern observed in Control animals (Table 44.2, compare with Table 44.1). In CS animals Bf increased with age and duration of the smoke exposure in normoxia, hypoxia, and hypercapnia ($p < 0.01$ and $p < 0.001$ at all times vs. 0 exposure or 2 months old animals). Bf in all atmospheres was statistically higher than in their age-matched controls ($p < 0.001$). Close inspection of the data suggests some positive interaction between CS and acute hypoxia as Bf tended

Table 44.2 Ventilatory parameters in guinea pigs of different ages and different times of cigarette smoking (CS) exposure maintained in normal atmosphere or exposed to chronic hypoxia (12% O_2) for 15 days (statistical differences are given in the main text)

	Age, CS				
	Months	Months	Months	Months	Months
Breathing	Age, 2;	Age, 3;	Age, 4;	Age, 5;	4.5;
Atmosphere	CS, 0	CS, 1	CS, 2	CS, 3	CS 2,5; 0.5, CS + 12% O_2
Room air					
Bf (breaths/min)	81.8 ± 2.3	116.6 ± 8.0	117.7 ± 7.9	155.6 ± 22.7	102.37 ± 5.24
TV (ml)	2.1 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	4.1 ± 0.3	3.9 ± 0.3
MV (ml/Kg/min)	600.6 ± 15.9	676.4 ± 50.9	548.9 ± 64.7	901.4 ± 185.6	571.36 ± 50.47
Hypoxia (10% O_2)					
Bf (breaths/min)	86.0 ± 2.6	125.6 ± 9.7	141.3 ± 11.9	168.9 ± 19.3	141.5 ± 9.3
TV (ml)	2.0 ± 0.1	3.2 ± 0.1	3.6 ± 0.2	4.8 ± 0.4	4.2 ± 0.2
MV (ml/Kg/min)	580.2 ± 19.9	695.8 ± 42.1	749.5 ± 82.5	1086.6 ± 152	813.9 ± 49.7
Hypercapnia (5% CO_2 in air)					
Bf (breaths/min)	101.1 ± 2.4	137.0 ± 9.0	131.6 ± 25.0	188.4 ± 25.0	120.3 ± 7.0
TV (ml)	4.5 ± 0.1	5.0 ± 0.2	5.3 ± 0.3	6.61 ± 0.6	5.5 ± 0.3
MV (ml/Kg/min)	1,542.0 ± 48.4	1,180.5 ± 47.0	995.3 ± 73.1	1,649.0 ± 207	931.5 ± 44.5

to be higher in hypoxia than in normoxia, while in controls Bf in hypoxia was nearly identical to that seen in normoxia. In fact, at 4 months of age (2 of CS exposure) Bf was higher in hypoxia than in normoxia ($p < 0.05$).

In CS animals, TV increased in normoxia, hypoxia, and hypercapnia with the duration of tobacco exposure (and age) following a trend comparable to control animals (i.e., there were significant increases in TV at 3, 4, and 5 months of age vs. 2 months; $p < 0.05$; $p < 0.01$, and $p < 0.001$). However, there was a trend for CS animals to have slightly higher hypoxic TV than age-matched Controls. Additionally, CS animals have lower hypercapnic TV than their age-matched Controls, at 1 and 2 months of CS exposure ($p < 0.05$; $p < 0.001$).

While in Control group MV/Kg decreases with age in all atmospheres (Table 44.1), in CS animals the age-dependent decrease in MV is nearly abolished in all atmospheres, implying that compared to age-matched controls, CS hyperventilate in all atmospheres ($p < 0.05$; $p < 0.01$, and $p < 0.001$). Even further, in normoxia and in hypoxia animals have MV that augment with age and the duration of the exposure to CS ($p < 0.05$; $p < 0.001$ vs. 2 month old, 0 time exposure). CS animals tend to breathe more in hypoxic than in normoxic atmosphere, appearing that CS time-dependently sensitized CB-mediated hypoxic ventilation. Alternatively, CS might have triggered a centrally mediated hypoxic ventilatory response. As stated above CS animals exposed to hypercapnia had higher MV/Kg than their age-matched controls: the marked age-dependent decrease in MV/Kg seen in control animals was lessened and after 3 months of exposure it recovered levels encountered at 2 months of age.

Assuming that dead space in guinea pigs is 1/3 of TV (Crosfill and Widdicombe 1961) it is possible to estimate alveolar ventilation (ml/min/Kg). It was found that alveolar ventilation in Control animals breathing air ranged from 260 to 350 ml/min/Kg at different ages. Following this simple reasoning CS animals should have alveolar ventilations 125% (1 month of exposure), 134% (2 months of exposure), and 192% (3 months of exposure) higher than age matched Control animals. However these alveolar ventilations in CS group are in all likelihood underestimations as in these CS animals TV would have increased without a parallel increase in dead space. Although we have not measured functional residual capacity (FRC) nor residual volume (RV) we can exclude significant alterations of these parameters, as Ferrer et al. (2009) have shown that the smoking protocol here employed does not cause emphysema after 3 months.

In CSCH group (last column in Table 44.2) Bf were intermediate between CS and CH groups in the three conditions studied ($p < 0.05$ and $p < 0.001$ vs. CH group and $p < 0.01$ vs. CS). TV in CSCH

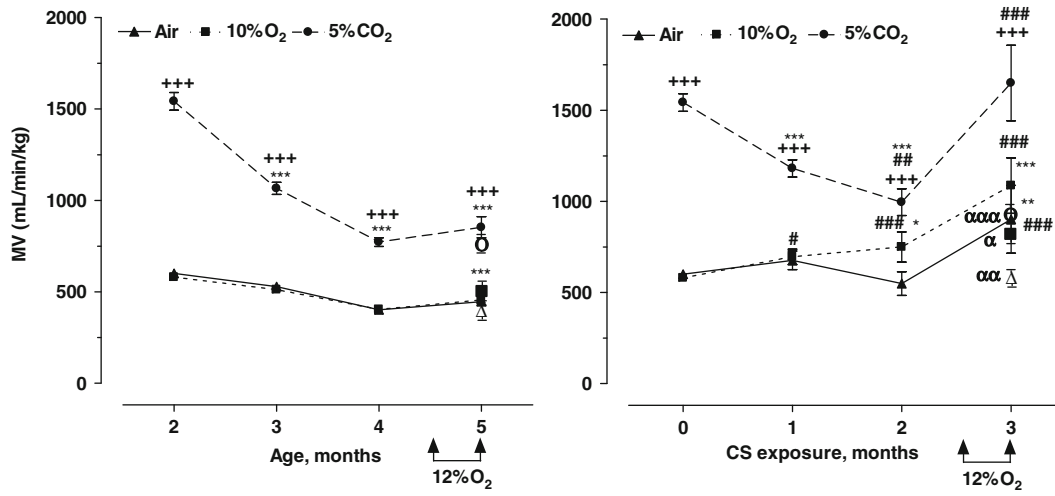

Fig. 44.1 Age-dependent variation in MV/Kg body weight in control (C, left) and cigarette smoke exposed guinea pigs (CS, right) while breathing in the atmospheres shown in the Figure labels. Empty symbols correspond to animals that have been exposed to chronic hypoxia during 2 weeks to conform groups CH and CSCH. Statistics: *,**,*** $p<0.05$, 0.01, and 0.001, respectively vs. 2 months of age. +,++,+++$p<0.05$, 0.01, and 0.001, respectively vs. air breathing. # and ### $p<0.05$ and 0.001, respectively, CS vs. C; α, αα, and ααα $p<0.05$, 0.01, and 0.001, respectively, CSCH vs.CS

group was slightly smaller than Control, CS, and CH groups (at 5 months of age) in the three atmospheres studied. Again, the interaction between cigarette smoke and chronic hypoxia in CSCH group resulted in an intermediate MV/Kg between Control and CS groups in the three conditions ($p<0.05$, $p<0.01$, and $p<0.001$ vs. CS; $p<0.001$ vs. CH in FiO_2 0.10), implying that CH partially blunts the stimulatory action of CS on ventilation.

For the sake of clarity Fig. 44.1 depicts the age dependent variation in MV/Kg in Control and CS animals in the three atmospheres as well as the effects of CH exposure on those relationships.

44.4 Discussion

Our findings include: (1) CS exposure causes a moderate time-dependent decrease in the body weight and an increase in haematocrit. (2) CH increases haematocrit acting in a synergistic manner with CS to increase the haematocrit. (3) Ventilatory pattern of control guinea pigs in the 2–5 months window is: a constant Bf, an increase in TV with age, and an age-dependent decrease in MV/Kg. (4) Acute hypoxia does not alter the ventilatory pattern at any age, while acute hypercapnia causes a marked TV-dependent increase in MV at all ages. (5) CS exposure increases Bf proportionally to exposure duration in air, 10% O_2, and 5% CO_2 atmospheres, preventing the age-dependent decrease in MV/Kg seen in Control. (6) Exposure to CH does not increase Bf, TV, and MV/Kg when data are compared with age matched controls. (7) Exposure of CS animals to CH, CSCH group, eliminates the Bf increase produced by CS causing a very significant decrease in MV/Kg when compared with CS group.

Consistent with findings in smokers humans (Yanbaeva et al. 2007), CS group showed an increased hematocrit, probably resulting from a high carboxyhemoglobinemia. The increased hematocrit in CH group should be considered a HIF-1α mediated adaptive response to hypoxia. In CSCH group both, hypoxic hypoxia and anaemic hypoxia (carboxyhemoglo- binemia), would generate a higher adaptive hematopoietic response.

Absolute values of Bf, TV, and MV are comparable to those found by other authors (Yilmaz et al. 2005; Wiester et al. 2005). The age-dependent variation observed in ventilatory pattern is common in all mammals (Mortola 2001) and parallels the mass specific metabolic rate. Yet, maintenance of Bf and increase in TV would increase alveolar ventilation, i.e., O_2 uptake/Kg decreases less than the observed MV/Kg.

Our data indicate a lack of hypoxia driven CB chemoreflex in adult guinea pigs and a minimal or absent acclimatization to CH which is known to be CB mediated (Gonzalez et al. 1994). In other words, guinea pigs, contrary most mammals, lack hypoxic hypoxia driven CB chemoreflex. However, since acute hypoxia in guinea pigs at the ambient temperature used in present experiments nearly halves O_2 consumption (Hill 1959), our data would imply that in hypoxia guinea pigs hyperventilate for their metabolic needs (i.e., MV/min O_2 consumption would nearly double). Ventilatory response to hypercapnia is comparable to that observed in the rat (Agapito et al. 2009) suggesting it is mediated by CB (≈30%) and central (≈70%) chemoreceptors (Gonzalez et al. 1994). Consistent with that CSN denervation in guinea pigs caused a *ca.* 28% decrease in the hyperventilation produced by 8% CO_2 breathing (Schwenke et al. 2007). As a whole data suggest that brainstem integration of CB signals is appropriate and that guinea pigs lack the oxygen-sensing machinery (Gonzalez et al. 2007, 2010) in their CB.

Our findings constitute the first set of data on the effects of CS on ventilation in animal models. CS MV/Kg mostly via an increase in Bf while in humans, both Bf and TV contributed to it (Tobin et al. 1983). CS in guinea pigs generated some signs of the activation of the CB chemoreflex evidenced by the discrete increase in MV/Kg produced by acute hypoxia in CS. This last finding would suggest that the origin of changes produced by chronic cigarette smoke probably is multifactorial with central and peripheral structures and mechanisms involved.

As stated before CH exposure alter minimally control breathing parameters in non-smoking animals, but associated to CS very significantly reversed the facilitating effects of tobacco smoke on ventilation. Thus, it would appear that hypoxia associated with nicotine and/or with lung damage, as it occurs in many COPD patients, would precipitate a decrease in ventilation with the subsequent worsening of oxygenation and CO_2 wash out. Yet, we must recall the singularity guinea pigs as they do not hyperventilate in response to hypoxia. This would limit, *a priori*, the extrapolation of our findings to other mammals including humans. Although we shall also note that severe COPD patients exhibit depressed ventilatory responses to hypoxia and hypercapnia (Gorini et al. 1996). Within limitations of extrapolation, our findings would indicate is that even limited lung and lung airways damage (Ferrer et al. 2009; Olea et al. 2011), as found in our animals, association of hypoxia (i.e., ascension to high altitude or mild pneumonia) can precipitate hypoventilation and a worsening of blood oxygenation. These changes would accelerate the appearance or deterioration of the cardiovascular components of COPD (e.g., increased pulmonary hypertension and risk of right and left heart dysfunction or even failure). This pathophysiological picture constitutes a feed-forward process that would negatively affect survival rate in COPD patients and would advise prompt oxygen therapy as soon as hypoxemia appears aiming to break the vicious circle (Coleta et al. 2008). The mechanisms of the negative nicotine-CH interation on ventilation are unknown. Yet, it is well founded that in the sudden infant death syndrome such association (smoking mothers or smoke ambient 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 dead (Kinney and Thach 2009).

In summary, guinea pigs lack functional O_2-driven ventilatory chemoreflex. Exposure of guinea pigs to CS during 3 months causes an increase in ventilation. Co-exposure to CS and CH blunts the ventilation increase induced by tobacco. From the clinical standpoint we consider 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.

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