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Acute cigarette smoke inhalation blunts lung responsiveness to methacholine and allergen in rabbit: differentiation of central and peripheral effects

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Porra L, Peták F, Strengell S, Neitola K, Janosi TZ, Suhonen H, Suortti P, Sovijärvi AR, Habre W, Bayat S. Acute cigarette smoke inhalation blunts lung responsiveness to methacholine and allergen in rabbit: differentiation of central and peripheral effects. *Am J Physiol Lung Cell Mol Physiol* 299: L242–L251, 2010. First published June 11, 2010; doi:10.1152/ajplung.00033.2010.—Despite the prevalence of active smoking in asthmatics, data on the short-term effect of acute mainstream tobacco smoke exposure on airway responsiveness are very scarce. The aim of this study was to assess the immediate effect of acute exposure to mainstream cigarette smoke on airway reactivity to subsequent nonspecific and allergenic challenges in healthy control ($n = 5$) and ovalbumin-sensitized rabbits ($n = 6$). We combined low-frequency forced oscillations and synchrotron radiation CT imaging to differentiate central airway and peripheral airway and lung parenchymal components of the response to airway provocation. Acute exposure to smoke generated by four successive cigarettes (CS) strongly inhibited the central airway response to subsequent IV methacholine (MCh) challenge. In the sensitized animals, although the response to ovalbumin was also inhibited in the central airways, mainstream CS did not blunt the peripheral airway response in this group. In additional groups of experiments, exposure to HEPA-filtered CS ($n = 6$) similarly inhibited the MCh response, whereas CO (10,000 ppm for 4 min, $n = 6$) or nitric oxide inhalation instead of CS (240 ppm, 4×7 min, $n = 5$) failed to blunt nonspecific airway responsiveness. Pretreatment with α -chymotrypsin to inhibit endogenous VIP before CS exposure had no effect ($n = 4$). Based on these observations, the gas phase of mainstream cigarette smoke may contain one or more short-term inhibitory components acting primarily on central airways and inhibiting the response to both specific and nonspecific airway provocation, but not on the lung periphery where both lung mechanical parameters, and synchrotron-imaging derived parameters, showed large changes in response to allergen challenge in sensitized animals.

asthma; smoking; tomography; X-ray computed; synchrotrons; respiratory mechanics

ACTIVE CIGARETTE SMOKING IS common in asthmatic patients, with prevalence rates similar to those of the general population (34). Asthmatic smokers have more severe disease symptoms and greater need for rescue medication than never smokers (34). Cigarette smoking does not change airway hyperresponsiveness in patients with asthma, despite evidence for neutro-

philic inflammation (6). Although several studies have assessed the effects of acute exposure to environmental or passive smoke with varying results, data on the short-term effect of acute exposure to mainstream tobacco smoke on airway reactivity are very scarce. Chronic smokers with mild-to-moderate airflow limitation demonstrate an increased airway responsiveness to inhaled methacholine (33). However, in asymptomatic nonsmokers, smoking a single cigarette does not increase airway responsiveness to methacholine (15, 32). In patients with asthma, studies of the effect of acute exposure to passive environmental cigarette smoke have produced conflicting results. Wiedemann et al. (36) in nine asthmatic subjects found that bronchial responsiveness to methacholine actually decreased after 1 h of acute smoke exposure. Menon et al. (18), however, showed methacholine hyperresponsiveness in 32% of “smoke-sensitive” subjects with asthma 6 h after acute exposure to environmental tobacco smoke. Direct comparison of data between the human studies is difficult, however, due to differences in the selected patient populations and methodology.

The aim of this study was therefore to assess the short-term effect of acute exposure to mainstream cigarette smoke on airway reactivity to both nonspecific and allergenic challenges in an ovalbumin-sensitized rabbit model. Our working hypothesis was that cigarette smoke, a known potent irritant of the airways, would enhance airway responsiveness in this model. We recently combined low-frequency forced oscillation and synchrotron radiation CT imaging to simultaneously assess functional and structural changes in the lungs following methacholine and allergen challenges (5). In the present study, we used the same approach to differentiate central airway and peripheral lung, including subresolution distal airway and parenchymal components, responses to airway provocation.

METHODS

General

Animal care and experimental procedures were in accordance with the Guidelines for the Care and Use of Animals published by the American Physiological Society and approved by the local institutional authorities. The experiments were performed in male New Zealand rabbits (2.6 ± 0.4 kg). The animals were housed in a specific pathogen-free facility and had constant access to rabbit chow and water ad libitum.

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Ovalbumin Sensitization and Provocation Procedures

Six animals were sensitized over a 1-mo period before the start of the experimental protocol, as detailed previously (23). Briefly, intraperitoneal injections of a solution containing 0.1 mg of ovalbumin (Sigma-Aldrich, St. Quentin Fallavier, France) and 10 mg of aluminum hydroxide as adjuvant (Merck, Darmstadt, Germany) were administered 30 and 17 days before the start of the protocol. The animals were then exposed to daily aerosolized ovalbumin (10 mg/ml, 20 min) on five subsequent days before the experiments using an ultrasonic nebulizer (System LS290, Velleneuve sur Lot, France).

Surgical Preparation

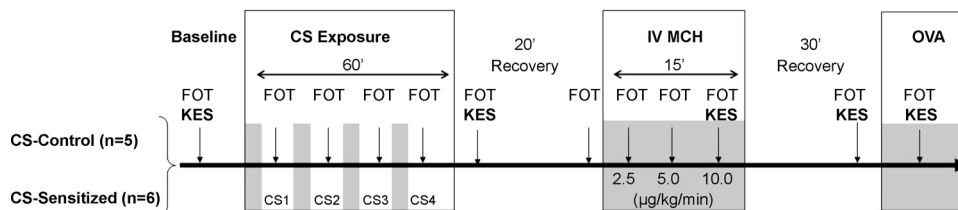
Before forced oscillation lung mechanics measurements and synchrotron imaging, a catheter (22 G) was inserted into the marginal ear vein (Cathlon IV; Ethicon, Rome, Italy) under local anesthesia, using 5% topical lidocaine (Emla, Astra-Zeneca, France). Anesthesia was induced by intravenous injection of thiopental sodium (25 mg/kg iv; Nesdonal, Rhone-Poulenc-Rohrer, Paris, France). The animal was tracheostomized, and an endotracheal tube (no. 3.5; Portex, Berck sur Mer, France) was inserted and secured with a gas-tight seal. A 22-G catheter was inserted into the left carotid artery for blood pressure monitoring and arterial blood sampling for blood gas measurements (Radiometer, ABL77, Copenhagen, Denmark). The left jugular vein was also catheterized for methacholine infusion. The lower extremities of the animal were wrapped in bandage, and the animal was immobilized in the vertical position in a custom-made cylindrical PVC holder. The chest and diaphragm were entirely free, and the lower limbs were securely maintained in the cylindrical holder with the use of foam. Anesthesia was maintained with intravenous midazolam ($0.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; Aguettant, Lyon, France). Paralysis was induced with intravenous atracurium ($1.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; Tracrium, GlaxoSmithKline, Münchenbuchsee, Switzerland). The respiratory gas flow was monitored by using a heated pneumotachometer (Hans Rudolph, Kansas City, MO). The endotracheal pressure was monitored continuously. All monitored signals were amplified, digitized at 400 Hz (Powerlab; ADI Instruments, Oxfordshire, UK), and recorded on a computer.

Study Design, Exposure to Mainstream Cigarette Smoke, and Methacholine Airway Challenge

Protocol 1. The study protocol is described in Fig. 1. An initial set of experiments were performed in six sensitized and five control rabbits (Fig. 1, *top*). In these experiments, following baseline acquisitions of respiratory impedance (Z_{rs}) data and synchrotron K-Edge Subtraction (KES) imaging, both control and sensitized animals were exposed to mainstream cigarette smoke. Cigarette smoke was generated using a custom-made Plexiglas chamber connected to the inspiratory branch of the ventilation circuit. Commercial filter cigarettes (Marlboro; Philip Morris International, Lausanne, Switzerland) were used, with an average yield of 0.8 mg of nicotine, 10 mg of tar, and 10 mg of CO, as stated by the manufacturer. The cigarette was lit and placed inside the chamber, connected in parallel to the ventilation circuit. The smoke emitted from the cigarette filter was diluted due to bypass apertures inside the smoke chamber. During exposure, the airflow was directed through the chamber using three-way valves where part of the air stream passed through the cigarette and the rest was used to dilute the cigarette smoke via bypass holes. The animals were exposed to mainstream smoke from each cigarette for 7 min, or down to the butt plus 1–2 mm, followed by 5 min of exposure to 40% FiO_2 , up to a total of four cigarettes. Arterial blood gases were measured before and after cigarette smoke exposure. Following a 20-min recovery phase after the exposure, methacholine was administered by intravenous infusion at 2.5, 5.0, and $10 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ administered stepwise over 15 min. After a 30-min recovery from the effect of methacholine, a new set of imaging and FOT data was collected before ovalbumin provocation (2.0 mg iv), immediately after which data acquisition was repeated. To standardize the volume history, single sighs defined as an inflation to a tracheal pressure of 30 cmH_2O were given at baseline, before methacholine challenge, upon recovery and before ovalbumin administration.

Protocol 2. To study potential mechanisms of the effect of mainstream cigarette smoke on subsequent airway response to methacholine, we performed a series of additional experiments (Fig. 1, *bottom*). The effect of filtering the mainstream smoke was studied in five animals. A High Efficiency Particle Arrest filter (HEPA Capsule 12144; PALL, Port Washington, NY) was used that retains over

Protocol 1



Protocol 2

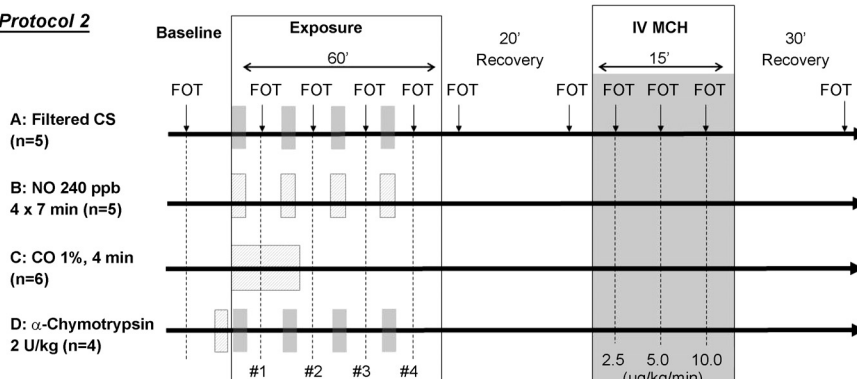


Fig. 1. Summary of the study protocol. *Top:* Protocol 1 experiments performed with combined forced oscillation and K-Edge Subtraction (KES) synchrotron imaging; *bottom:* Protocol 2; supplementary experiments performed without synchrotron imaging. FOT, forced oscillation data acquisition; CS1–4, exposure to smoke from cigarettes 1–4 (gray bars); IV MCH, infused methacholine airway challenge; OVA, ovalbumin. Protocol 2A was exposed to filtered cigarette smoke. Protocol 2B, C, were exposed to nitric oxide and carbon monoxide, respectively, instead of cigarette smoke. Protocol 2D was pretreated with α -chymotrypsin before cigarette smoke exposure.

99.0% of $<0.3\text{-}\mu\text{m}$ particles in unfiltered smoke (8), which contains the nicotine vapor phase (24, 29). These and the following additional experiments were performed in nonsensitized animals, where lung *Zrs* data were obtained, without synchrotron imaging.

To determine the effect of exogenous nitric oxide (NO) contained in mainstream cigarette smoke, NO gas (1,000 ppm in N_2 ; Carbagas, Gümligen, Switzerland) was diluted in air to a final concentration of 240 ppm in five other rabbits. This level was based on measurements of NO concentration in the outlet of the HEPA-filtered mainstream cigarette smoke. NO concentration was monitored during administration using an electrochemical analyzer (Dräger Polytron, Antony, France).

Since the gas phase of mainstream cigarette smoke contains significant amounts of CO, a separate group of six animals was exposed to 1% CO in air (10,000 ppm, 0.01 fractional gas content, Carbagas) for 4 min. This amount of CO exposure produced similar levels of HbCO than that measured after exposure to HEPA-filtered mainstream cigarette smoke (Table 1). To determine whether vasoactive intestinal peptide (VIP) is involved in the CS-induced inhibition of the airway response to methacholine, we administered α -chymotrypsin, which degrades VIP (1, 2), before mainstream cigarette smoke exposure in an additional group of animals.

Synchrotron Lung CT Imaging

The synchrotron CT imaging technique employed in this study or KES imaging, uses dual X-ray beams at two slightly different energies. Simultaneous acquisition and subtraction of two CT images allows the direct quantitative measurement of xenon gas within the airways, and of the regional gas volume (20). Dynamic imaging during xenon wash-in allows the measurement of regional specific ventilation, i.e., the ventilation normalized to voxel volume (25). Visualization and quantitative measurement of the xenon within the airways is based on the property that the attenuation coefficient of xenon increases by a factor of 5.4 when the energy of the incident X-ray beam crosses the energy threshold of 34.56 keV, which is the xenon K-edge (10), but the changes in the attenuation coefficients of cortical bone and lung tissue are negligible. Imaging is performed simultaneously with two X-ray beams at slightly different energies, above and below the xenon K-edge. Subtraction of these two mono-energetic images on a logarithmic scale allows the observation of small anatomic structures carrying the contrast agent, while removing practically all features due to other structures. The intensity scale of the resulting image is directly proportional to the content of the contrast element per image voxel, based on the property that for mono-energetic X-rays the logarithm of the transmitted intensity is proportional to the line integral of the attenuation coefficients. This allows for the direct quantitative measurement of xenon. X-rays from a synchrotron radiation source are required since, as opposed to standard X-ray sources, they allow the selection of monochromatic beams from the full X-ray spectrum while conserving enough intensity for imaging. Images were acquired at mid-thoracic and caudal thoracic levels allowing for measurements of airway cross-sections

down to approximately six generations. A detailed description of the synchrotron imaging method and instrumentation is included in the data supplement (supplemental material for this article is available online at the *AJP-Lung* website).

Image Analysis

Images were processed by using the MatLab programming package (Mathworks) as described previously (5). The total lung area is defined as the area within the mono-energetic images corresponding to lung as determined by image segmentation, with the exclusion of large airways and blood vessels. The density of the lung tissue was calculated within the same presegmented image regions corresponding to lung, using an algorithm based on dual energy subtraction. Gas content defines the fraction of gas contained in the image regions corresponding to lung. Gas content was calculated using the ratio of the mean asymptotic xenon concentration during wash-in, to the xenon concentration in the inhaled gas measured within corresponding central airway lumens. A detailed description of the image processing methods is included in the online data supplement.

Measurement of Respiratory Mechanics

The experimental setup used to measure the mechanical properties of the respiratory system by the low frequency, i.e.: the forcing signal containing the breathing frequency-forced oscillation technique was identical to that described previously (5). Three to five respiratory impedance (*Zrs*) spectra were ensemble-averaged under the baseline conditions, following cigarette smoke exposures, and during steady-state methacholine infusions. Because of the absence of a steady response after the intravenous ovalbumin challenge, parameters obtained from the *Zrs* data collected 2 min after the ovalbumin injections were included in the analysis (11). Airway resistance (*R_{aw}*), inertance (*I_{aw}*), tissue damping (*G*), and elastance (*H*) were estimated from a model fit to the averaged *Zrs* data (10). The respiratory tissue hysteresivity (η) was calculated as G/H (9). A detailed description of the method is included in the online data supplement.

Statistical Analysis

The scatters in the parameters were expressed by the SD. Two-way repeated measures ANOVA was used to evaluate the differences in the mechanical, functional imaging parameters and blood gases among groups and compared with baseline. Identical tests were applied to assess the effects of ovalbumin on the lung functional changes. Pairwise comparisons were performed by using Student-Newman-Keuls multiple comparison procedures. Statistical tests were carried out with the significance level of $P < 0.05$.

RESULTS

Responses in Lung Mechanics

Baseline lung mechanics parameters *R_{aw}* (12.0 ± 4.3 vs. 12.9 ± 3.2 cmH₂O/s/l) and *G* (135.1 ± 34.7 vs. 129.4 ± 16.8

Table 1. Arterial blood gas data

	pH		PO ₂ , mmHg		PCO ₂ , mmHg		HCO ₃ ⁻ , mmol/l		HbCO, %	
	Baseline	Exposure	Baseline	Exposure	Baseline	Exposure	Baseline	Exposure	Baseline	Exposure
CS control	7.43 $\pm$ 0.07	7.17 $\pm$ 0.07*	98.6 $\pm$ 4.1	85.5 $\pm$ 25.4*	36.0 $\pm$ 2.9	40.4 $\pm$ 13.7	24.7 $\pm$ 3.5	14.0 $\pm$ 2.8*		
CS sensitized	7.53 $\pm$ 0.07	7.29 $\pm$ 0.08*§	100.5 $\pm$ 5.8	81.3 $\pm$ 22.3*	27.0 $\pm$ 3.9§	37.3 $\pm$ 16.3	25.3 $\pm$ 2.3	17.2 $\pm$ 2.4*		
FS	7.49 $\pm$ 0.06	7.44 $\pm$ 0.04§	81.2 $\pm$ 10.0	71.4 $\pm$ 6.7	29.9 $\pm$ 4.4§	28.0 $\pm$ 2.6	22.9 $\pm$ 4.6	19.0 $\pm$ 3.0*	2.8 $\pm$ 0.3	34.7 $\pm$ 3.2*
NO	7.52 $\pm$ 0.07	7.52 $\pm$ 0.06*§	88.6 $\pm$ 4.9	85.3 $\pm$ 4.4	27.9 $\pm$ 1.4§	23.6 $\pm$ 1.9	23.1 $\pm$ 4.4	19.5 $\pm$ 2.9*	3.1 $\pm$ 0.3	2.9 $\pm$ 0.3
CO	7.52 $\pm$ 0.07	7.49 $\pm$ 0.04§	90.6 $\pm$ 4.9	102.7 $\pm$ 7.0*	22.8 $\pm$ 3.0§	23.0 $\pm$ 3.2	18.5 $\pm$ 2.3§	17.6 $\pm$ 2.6	3.1 $\pm$ 0.4	36.7 $\pm$ 9.5*
α -Chemotrypsin	7.55 $\pm$ 0.03	7.40 $\pm$ 0.04*§	83.0 $\pm$ 7.1	59.6 $\pm$ 15.3*	24.7 $\pm$ 4.6§	23.3 $\pm$ 0.9	21.4 $\pm$ 3.1	14.4 $\pm$ 1.7*	2.9 $\pm$ 0.3	33.4 $\pm$ 2.8*

CS, cigarette smoke exposure; FS, filtered smoke; NO, nitric oxide; CO, carbon monoxide. * $P < 0.05$ vs. baseline, § $P < 0.05$ vs. the CS control group by 2-way repeated measures ANOVA.

cmH₂O·s/l) were not different between the control and sensitized groups, respectively. Although H tended to be greater in sensitized than in control rabbits (527.5 ± 72.5 vs. 455.5 ± 24.7 cmH₂O/l), this difference did not reach statistical significance ($P = 0.068$). The effect of acute exposure to cigarette smoke on lung mechanics parameters is shown in Fig. 2. The significant interactions in the changes in R_{aw} ($P = 0.009$), G ($P < 0.001$), H ($P = 0.002$), and η ($P < 0.001$) revealed by the ANOVA analyses demonstrate that the smoke exposures differently affected the central airway and peripheral responses to airway provocation. Airway resistance slightly but significantly decreased following exposure to smoke produced by the first cigarette both in control and sensitized animals, and did not further decrease after each of the three following exposures. Subsequent airway response to IV methacholine was almost completely inhibited in both groups. Intravenous ovalbumin provocation, however, still produced a significant rise in R_{aw} , although this response was smaller than that observed in sensitized animals not exposed to cigarette smoke in a previous study (5), shown here for comparison. Peripheral lung changes in response to methacholine provocation were similarly inhibited by acute cigarette smoke exposure (Fig. 2). Neither tissue damping G , tissue elastance H , nor hysteresivity η showed a statistically significant increase with the exposure to smoke from the successive cigarettes. The rise in G and η in response to increasing doses of methacholine was smaller than that seen previously in nonexposed sensitized animals. Despite the inhibition of the peripheral airway response to methacholine, ovalbumin provocation still produced a large rise in G , H , and η , which was not significantly different than that observed in non CS-exposed sensitized animals from our previous study.

Responses in Functional Lung Images

Examples of specific ventilation images in representative control and sensitized animals in each experimental condition are shown in Fig. 3. Following acute cigarette smoke exposure, the mean total central airway luminal cross-section area increased in both ovalbumin-sensitized and control animals (Fig. 4). This increase was larger and statistically significant in controls. Mean central airway cross-section remained higher than baseline even after the highest dose of methacholine and was significantly larger than that of the sensitized animals upon recovery from methacholine. On the other hand, the total area of the lung fields for the combined middle and caudal slices significantly decreased in sensitized but not in control animals (Fig. 5, top). The mean total lung area contained within the images remained low in these animals, whereas it did not change significantly in the controls. The fractional gas content in the ventilated regions in each image slice (Fig. 5, middle) was significantly lower in the sensitized animals at baseline, but did not change significantly after acute exposure to cigarette smoke or following methacholine. The gas content did show a small but significant drop after ovalbumin provocation in the sensitized animals. The density of the lung tissue was consistently higher in sensitized animals compared with controls, even at baseline (Fig. 5, bottom). The data were not significantly different in the middle and caudal image slices; therefore, the data from both image levels were combined. On the other hand, the ventilated alveolar area or the percentage of well-ventilated lung zones was not significantly affected by the

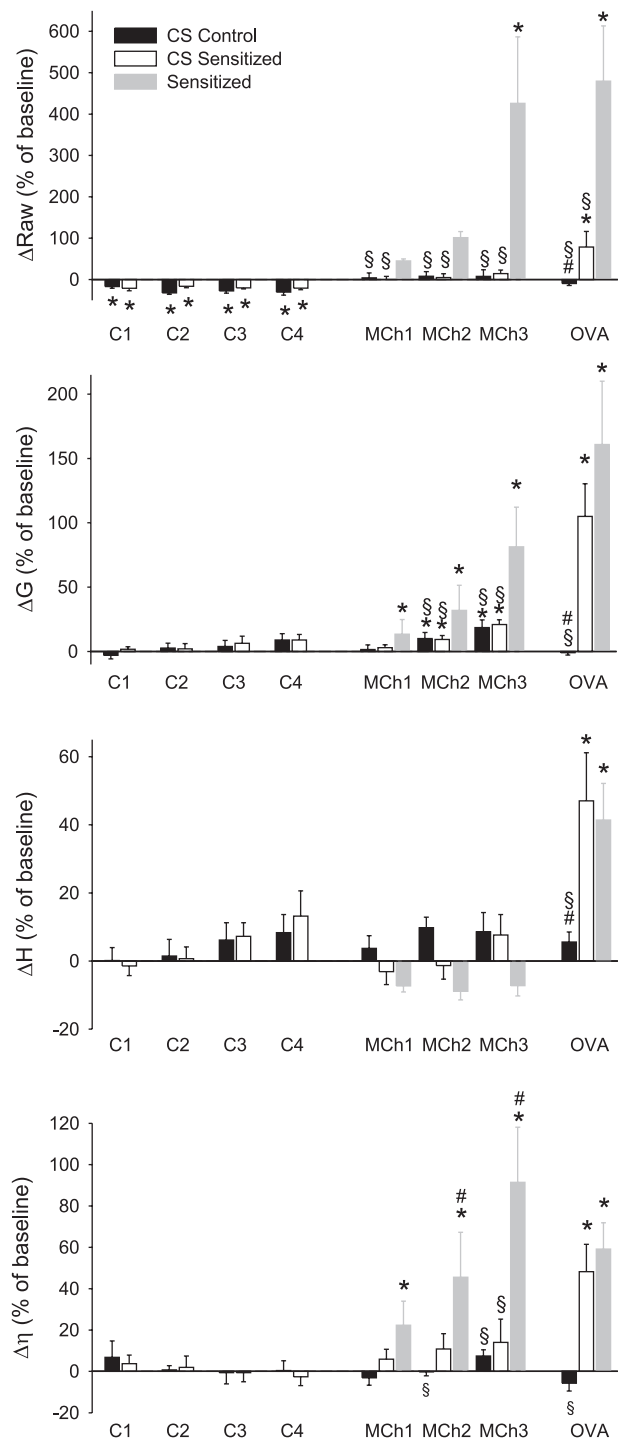


Fig. 2. Relative changes in airway resistance (ΔR_{aw}), tissue damping (ΔG), tissue elastance (ΔH), and tissue hysteresivity (η) following exposure to smoke from 4 subsequent cigarettes (C1–C4), infusion of 2.5, 5.0, and 10 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ of methacholine (M10), and after IV ovalbumin challenge in control (black bars) and sensitized animals (white bars). A separate group of previously studied sensitized animals nonexposed to cigarette smoke was included for comparison (gray bars); * $P < 0.05$ vs. baseline, # $P < 0.05$ vs. the CS-exposed control group, and \$ $P < 0.05$ vs. nonexposed sensitized animals from a previous study shown for comparison by 2-way repeated measures ANOVA.

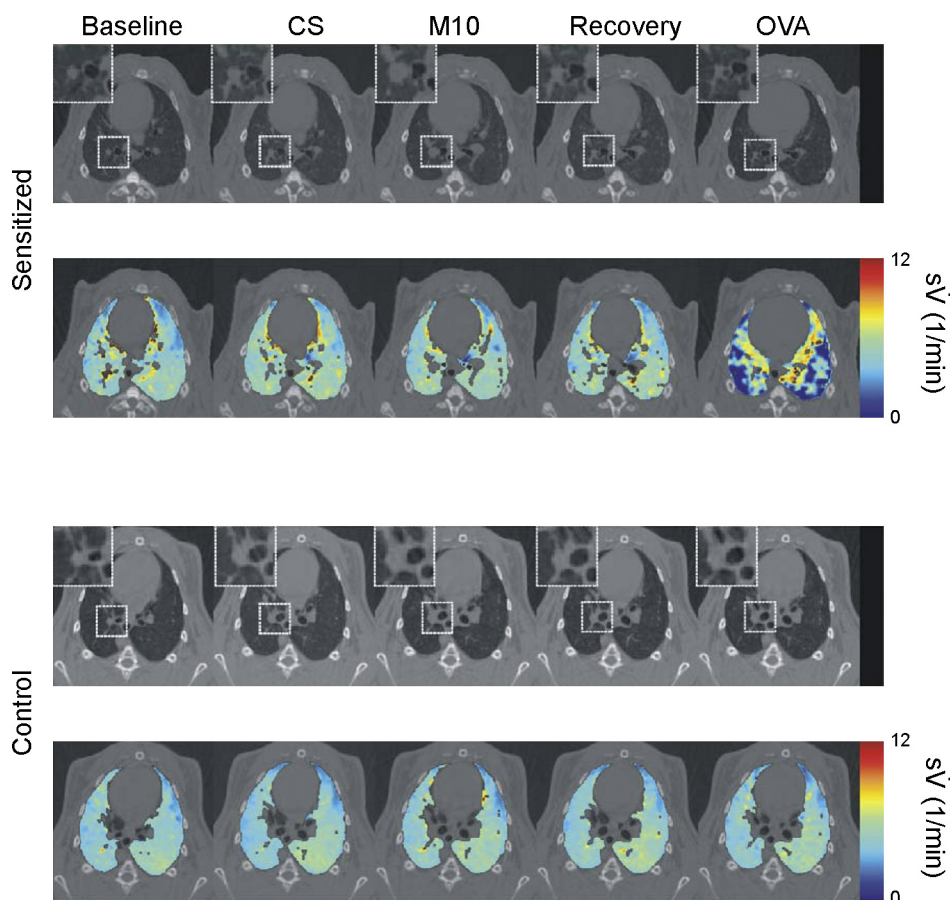


Fig. 3. Sensitized: mono-energetic CT images (*top row*) showing the central airway cross-section with 1 airway magnified (box) in the mid-thoracic image slice and specific ventilation images (*bottom row*) in 1 representative sensitized rabbit at baseline, following exposure to smoke from the 4th cigarette (CS4), during methacholine infusion of $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$, upon recovery and after ovalbumin provocation (OVA). Control: similar images in a representative control animal. CS did not cause significant heterogeneity in the peripheral lung ventilation, but significantly blunted central airway constriction in response to methacholine infusion in both control and sensitized animals. Intravenous ovalbumin provocation, however, did produce both a significant patchiness and a drop in the area of adequately ventilated lung regions in CS-exposed sensitized subjects.

acute exposure to cigarette smoke and decreased significantly in sensitized animals, only after ovalbumin challenge (Fig. 6, *top*). Similarly, ventilation heterogeneity, estimated by the CV of $s\dot{V}$ was not different between the sensitized and control groups and significantly increased only after ovalbumin challenge in the sensitized animals (Fig. 6, *bottom*).

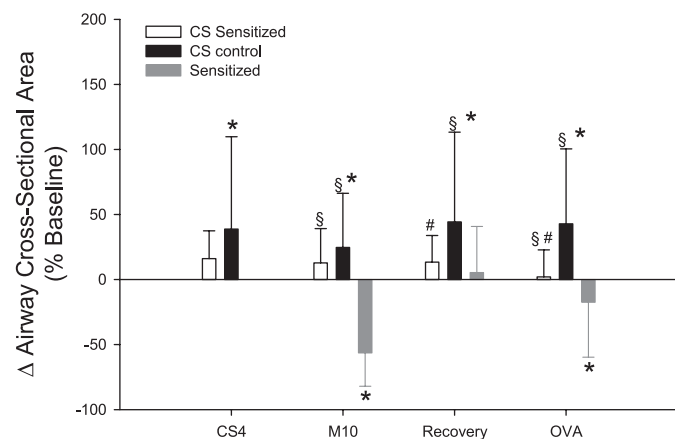


Fig. 4. Changes in central airway cross-sectional areas measured in mono-energetic synchrotron images following exposure to smoke from the 4th cigarette (CS4), during methacholine infusion of $10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (M10), upon recovery, and after ovalbumin provocation (OVA); $*P < 0.05$ vs. baseline, $\#P < 0.05$ vs. the CS-exposed controls; $\$P < 0.05$ vs. nonexposed control animals from a previous study shown for comparison by 2-way repeated measures ANOVA. Cigarette smoke inhalation caused central airway dilatation that was significant in control, but not in sensitized animals.

The mean specific ventilation ($s\dot{V}_m$) was higher in the sensitized animals compared with controls (Fig. 7). The $s\dot{V}_m$ was not significantly affected by the acute exposure to cigarette smoke or methacholine provocation in either group, but decreased after ovalbumin challenge in the sensitized animals.

Effect of Filtered Cigarette Smoke, CO, NO, and α -Chymotrypsin Pretreatment on Subsequent Airway Response to Methacholine Challenge

Supplemental experiments (*protocol 2*) were performed to explore possible mechanisms through which acute exposure to cigarette smoke inhibited subsequent airway response to methacholine, based on the changes in R_{aw} measured by the forced oscillation technique in non-sensitized animals. Results of these experiments performed without KES imaging are summarized in Fig. 8. Exposure to the gas phase of cigarette smoke by filtering caused a similar inhibition of the airway response to methacholine as that observed with unfiltered smoke. Neither exposure to NO with a similar concentration as that measured in the filtered smoke, nor to CO with concentrations producing similar levels of HbCO as with the filtered smoke, inhibited the airway response to a subsequent methacholine challenge. Also, pretreatment with α -chymotrypsin, a peptidase administered to prevent the effect of the inhibitory non-adrenergic noncholinergic system, before exposure to unfiltered cigarette smoke, did not produce a significant difference in the methacholine airway response inhibition.

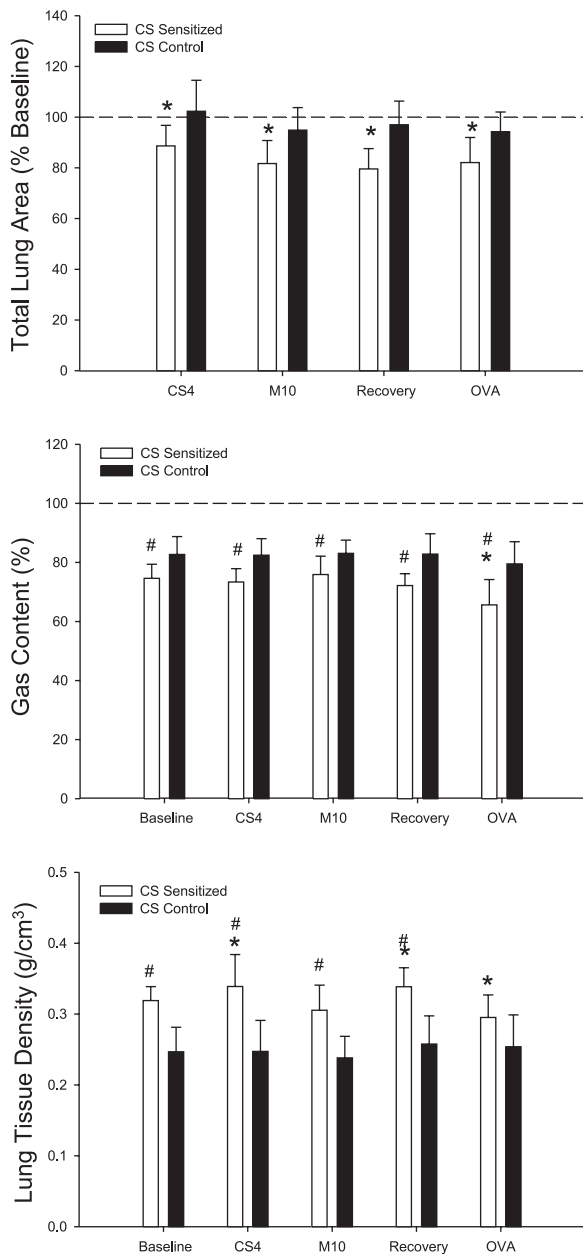


Fig. 5. *Top*: changes in the total area of image regions corresponding to lung; *middle*: fractional gas content in lung tissue; *bottom*: lung density. * $P < 0.05$ vs. baseline, # $P < 0.05$ vs. the CS-exposed sensitized group by 2-way repeated measures ANOVA.

Arterial Blood Gases

Arterial blood gas data are summarized in Table 1. Cigarette smoke exposure caused metabolic acidosis in the control, sensitized, and α -chymotrypsin pretreatment groups, but not in the animals exposed to filtered smoke, CO, or NO. A lower average PaO_2 was observed in the α -chymotrypsin-pretreated animals following cigarette smoke exposure, although this difference was not statistically significant. The HbCO level was measured in protocols 2A to 2D, and was similar in animals exposed to filtered mainstream smoke, those pretreated with α -chymotrypsin, and those exposed to CO alone.

DISCUSSION

The objective of this study was to describe the effect of acute exposure to cigarette smoke on airway reactivity to subsequent intravenous methacholine challenge. Given the known deleterious effects of chronic exposure to both mainstream and environmental cigarette smoke on lung function and airway reactivity, our hypothesis was that acute exposure to cigarette smoke causes bronchoconstriction and enhanced airway response to methacholine. We used two different approaches to differentiate the effect of such an exposure on central conducting airways and on the lung periphery including subresolution distal airway and parenchymal components: measurement of forced oscillatory lung mechanics parameters and K-edge subtraction lung imaging. The main contribution of K-edge subtraction imaging is the ability to quantify the changes in peripheral ventilation and its heterogeneity.

Unexpectedly, our data demonstrate that acute exposure to mainstream smoke from four successive cigarettes largely inhibited the airway response to a subsequent methacholine challenge in both control and ovalbumin-sensitized rabbits. This effect persisted even when exposure was limited to the gas

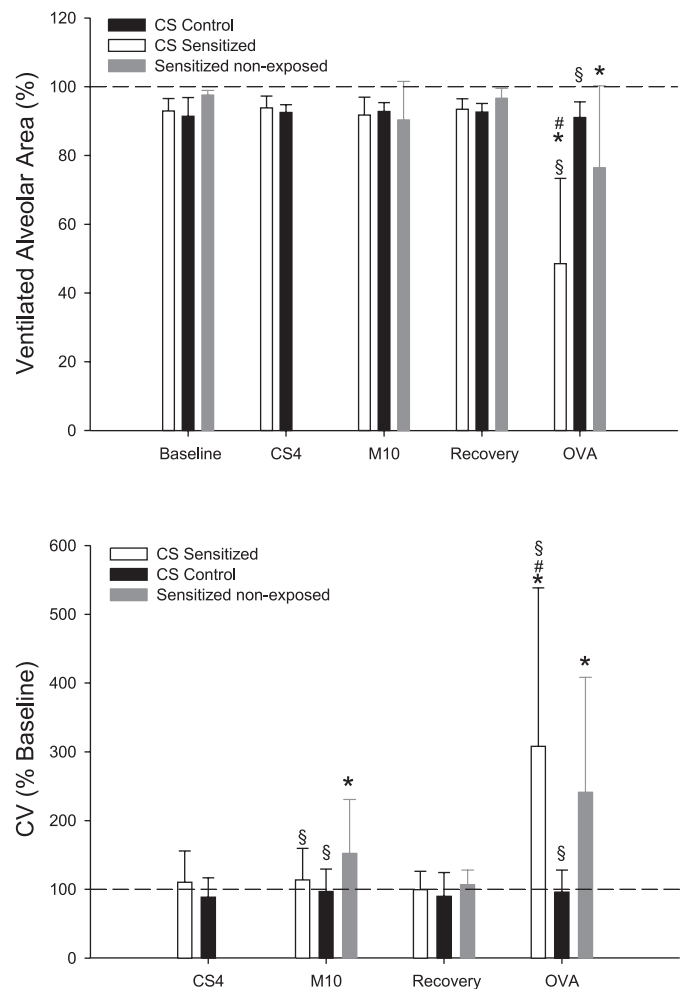
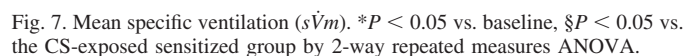


Fig. 6. *Top*: changes in the area of adequately ventilated lung regions (see text for definition); *bottom*: heterogeneity of regional ventilation measured as the CV of $\dot{V}_A$. * $P < 0.05$ vs. baseline; # $P < 0.05$ vs. the CS-exposed control group; § $P < 0.05$ vs. nonexposed sensitized animals from a previous study shown here for comparison by 2-way repeated measures ANOVA.

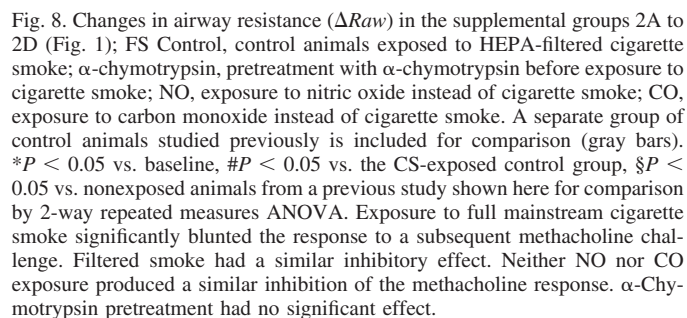


Effect of Acute Cigarette Smoke Exposure on Lung Mechanics

rabbit model is more relevant than guinea pig with regard to the role of sensory neural activity in the airways. On the other hand, Alving et al. (3) found that inhalation of mainstream smoke from a single cigarette caused significant bronchodilation in pig that was reduced but persisted after filtering. The authors suggested that exogenous NO contained in the gas phase of cigarette smoke may have mediated this effect. In the present study, however, NO administered at a concentration similar to that measured in the gas phase of cigarette smoke did not cause a significant bronchodilation. Exogenous carbon monoxide (CO) has also been suggested to exert a bronchodilatory effect in ovalbumin-sensitized and -challenged mice but not in control mice despite HbCO levels of 38% (4). Since the gaseous phase of cigarette smoke contains CO, we tested the effect of exogenous CO exposure producing an HbCO level similar to that measured following the filtered cigarette smoke exposure. Exogenous CO, however, failed to produce a significant decrease in *Raw* in either control or ovalbumin-sensitized rabbit. Together, our findings and evidence in the literature suggest that a bronchodilatory effect of one or more components of the gas phase of mainstream cigarette smoke cannot be excluded in the present experimental conditions and under general anesthesia.

Effect of Acute Mainstream Tobacco Smoke Exposure on Subsequent Airway Reactivity to Methacholine and Ovalbumin

In the present study, we found that acute exposure to smoke generated by four successive cigarettes considerably blunts the central airway response to a subsequent intravenous methacholine challenge. We have previously shown that the effect of



methacholine infusion challenge predominates in the central airways in both healthy and sensitized smoke-naïve rabbits, whereas in sensitized animals, the larger part of the airway response to allergen occurs in the lung periphery, causing significant heterogeneity in the peripheral lung ventilation along with elevations in G and H (5). In the present study, although the response to ovalbumin challenge was also inhibited in the central airways of sensitized animals, mainstream cigarette smoke exposure did not inhibit the allergen-induced peripheral lung response in this group (Figs. 2 and 3). The observed rises in G and H following ovalbumin challenge in sensitized animals may have resulted from tissue distortions induced by conducting airway constriction due to airway/tissue interdependence forces, the mechanical load on the parenchyma increasing as parenchymal airways constrict (16, 19). In this study, the measurement of central conducting airway cross-sections based on mono-energetic CT images did not show any significant constriction following ovalbumin challenge (Fig. 4). However, the constriction of conducting airways distal to those measured in the images cannot be ruled out. Furthermore, peripheral airway closures may increase G and H by reducing the ventilated volume. The latter mechanism may also be involved in the rise in ventilation heterogeneity and the decrease in the ventilated alveolar area (Fig. 6). Moreover, allergen challenge can cause changes in lung tissue mechanics through other mechanisms, namely, inflammatory cell infiltration, septal thickening, interstitial edema, and vascular congestion (26).

Few animal studies have been carried out to assess the short-term effects of acute mainstream cigarette smoke on airway response to methacholine. Roehrs et al. (28) found that reactivity to inhaled methacholine was reduced in chronically smoking baboons. In the same species, Wallis et al. (35) found that nicotine inhalation had no effect on lung function but decreased bronchial reactivity to inhaled methacholine. The authors suggested that nicotine might inhibit bronchial reactivity through both ganglionic stimulation and release of catecholamines, and postganglionic blockade of parasympathetic pathways. In the present study, smoke filtration, which removes >99% of the particulate phase (8) that contains nicotine, did not significantly change the inhibitory effect of smoke inhalation on airway response to methacholine, suggesting that other components of the gas phase may have played an inhibitory role. In a separate study by Melgert et al. (17), 3 wk of exposure to mainstream cigarette smoke in ovalbumin-sensitized and -challenged mice significantly decreased airway reactivity to both ovalbumin and methacholine. No significant inhibition of airway reactivity was observed, however, in the nonsensitized animals. This effect was accompanied by a significant decrease in BAL and tissue eosinophilia. The authors suggested that induction of protective enzymes such as inducible nitric oxide synthase and heme oxygenase-1, producing endogenous CO, or the exogenous CO contained in mainstream smoke may have contributed to the observed blunting in the methacholine response. Similarly, Robbins et al. (27) found that mainstream cigarette smoke exposure significantly decreased both airway response to methacholine and lung eosinophilic allergic inflammation, in ovalbumin-sensitized mouse. Conversely, Nishikawa et al. (21) showed that acute exposure to 10 and 20 puffs of mainstream cigarette smoke increased airway reactivity to methacholine in guinea pig, and that this

effect persisted 5 h after exposure. In that study, the amount of cigarette smoke administered was relatively smaller than in our study. In another study, Emms and Rogers (7) found that inhalation of 50 tidal volumes of cigarette smoke had no significant effect on pulmonary insufflation pressure, and that it reduced the magnitude of subsequent bronchoconstriction induced by neurokinin A. A potential reason for the observed inhibitory effect of acute mainstream cigarette smoke exposure on airway reactivity to methacholine may be the prechallenge airway caliber. An equivalent amount of airway narrowing will produce a much smaller rise in Raw if the challenge is performed in dilated airways. We previously showed that Raw is largely determined by the central airway calibers in the experimental model used in this study (5), and that infused methacholine produces very significant reductions in central airway cross-section, which is strongly correlated to Raw . However, the magnitude of central airway dilatation following cigarette smoke exposure in this study was small and significant only in the control subjects. Therefore, the latter phenomenon alone may not fully explain the considerable inhibition of the response to methacholine, particularly at the highest dose (Fig. 2).

Exogenous CO has been shown to reduce airway responsiveness to methacholine in ovalbumin-sensitized mice (4). In the present study, however, exogenous CO exposure mimicking that contained in mainstream cigarette smoke did not blunt the response to methacholine. On the other hand, the gaseous phase of mainstream cigarette smoke contains significant amounts of NO, which has been shown to produce bronchodilation in pig (3). In this study we did not find any evidence that exogenous NO is involved in the inhibition of airway response to methacholine. Alternatively, endogenous VIP has been suggested to inhibit nonadrenergic noncholinergic neurogenic bronchoconstriction (14). In the absence of a selective VIP receptor antagonist, we administered α -chymotrypsin, which degrades VIP (1, 2) before mainstream cigarette smoke exposure. However, α -chymotrypsin pretreatment did not modify the blunting of the subsequent response to methacholine either. Based on these observations, a plausible hypothesis is that the gas phase of mainstream cigarette smoke contains one or more short-term inhibitory components. The effect of acute exposure to these components acts primarily on central airways inhibiting the response to both specific and nonspecific airway provocation, but not on the lung periphery where both lung mechanical parameters G and H , and synchrotron-imaging derived parameters showed large variations in response to allergen provocation in the sensitized animals. However, mainstream cigarette smoke is a complex mixture of several hundred components, and further study is needed to identify potential substances.

During cigarette smoke exposure, metabolic acidosis was found in both control and sensitized animals. However, this was not the case in the animals exposed to filtered smoke, excluding the latter as the inhibitory mechanism of the airway response to methacholine.

Baseline Lung Mechanics and Image-Derived Parameters in Control and Sensitized Rabbits

In this study, we did not observe significant differences in Raw and G between sensitized and control animals. Although H tended to be greater in the sensitized vs. control rabbits (517

vs. 451, respectively), this difference did not reach statistical significance. Synchrotron imaging revealed a significantly higher lung tissue density and lower fractional gas content as estimated from Xe concentrations within the KES images (Fig. 5) in sensitized compared with control animals. Conversely, specific ventilation was significantly higher in the sensitized animals. A potential cause could be higher overall minute ventilation in the sensitized animals. However, although the sensitized animals demanded a higher minute ventilation to maintain similar arterial Po_2 levels (887 ± 184 vs. 792 ± 51), this difference was not statistically significant ($P = 0.085$). Alternatively, a smaller alveolar gas compartment in the sensitized animals could lead to a shorter Xe wash-in time constant and a larger $s\dot{V}$ for the same minute ventilation. The underlying reason for the baseline differences in lung tissue density and gas fraction between sensitized and control animals is not clear. Inflammatory cell infiltration, septal thickening, interstitial edema and vascular congestion, and constriction of contractile elements within the parenchymal interstitium could potentially increase the estimated lung tissue densities. Histological data in an ovalbumin-sensitized and -challenged rabbit lung model similar to our study demonstrated the presence of allergic lung inflammation features including alveolar septal thickening and patchy cellular infiltration by lymphocytes, eosinophils, mononuclear cells, and macrophages (26). The presence of allergic inflammation could therefore be a likely explanation for these findings.

Summary and Conclusions

In this study, we combined low-frequency forced oscillations and synchrotron CT imaging to assess the short-term effect of acute exposure to mainstream cigarette smoke on airway reactivity. This approach allowed us to differentiate central and peripheral airway components of the responses to both nonspecific and allergenic airway challenges. Mainstream exposure to CS in anesthetized and mechanically ventilated rabbit profoundly inhibited central and peripheral airway responsiveness to methacholine. Although the response to ovalbumin challenge was also inhibited in the central airways of sensitized animals, mainstream cigarette smoke exposure did not inhibit the allergen-induced peripheral lung response in this group, as evidenced by both specific ventilation images and respiratory mechanics data. Our findings suggest that the gas phase of mainstream cigarette smoke contains one or more short-term inhibitory components other than exogenous NO or CO, acute exposure to which acts primarily on central airways inhibiting the response to both specific and nonspecific airway provocation. Identification of such component(s) is of potentially important clinical implications and may contribute to the development of new therapeutic pathways in asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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On-line supplement for: Acute cigarette smoke inhalation blunts lung responsiveness to methacholine and allergen in rabbit: differentiation of central and peripheral effects

By Porra L et al.

Detailed Description of Methods

Synchrotron lung CT imaging. Imaging experiments were performed at the Biomedical Beamline of the European Synchrotron Radiation Facility (ESRF, Grenoble, France). The synchrotron CT imaging technique employed in this study or K-edge subtraction imaging, uses dual X-ray beams at two slightly different energies. Simultaneous acquisition and subtraction of two CT images, as explained further under *image analysis*, allows the direct quantitative measurement of xenon gas within the airways, and of the regional gas volume (9). Dynamic imaging during xenon wash-in allows the measurement of regional specific ventilation, i.e. the ventilation normalized to voxel volume (11). Visualization and quantitative measurement of the xenon within the airways is based on the property that the attenuation coefficient of xenon increases by a factor of 5.4 when the energy of the incident X-ray beam crosses the energy threshold of 34.56 keV, i.e.: the xenon K-edge (5), but the changes in the attenuation coefficients of tissues such as cortical bone and lung are negligible.

The mono-energetic beams were produced from the continuous synchrotron radiation spectrum by a bent silicon crystal monochromator and the energy difference between the beams was 250 eV. The beams focused and crossed at the animal position, beyond which they diverged and were recorded by a liquid nitrogen-cooled high-purity germanium dual-line detector (Eurisy Measure, Lingolsheim, France). The horizontal pixel size of the detector was 0.35 mm, and the beam vertical width was 0.7 mm. Since the X-ray beam is stationary, the animal was moved for imaging. For projection imaging, the animal was moved vertically

through the beams and for CT imaging the animal was rotated around an axis perpendicular to the beams (6.56° off-vertical). The detector was read out synchronously with the animal motion to provide 0.35 mm vertical pixel size in the projection images, and 0.5° angular resolution in the CT images. The rotation speed in CT imaging was $180^\circ/\text{s}$ and images were recorded from 720 angular projections per full 360° rotation. Images were acquired at mid-thoracic and caudal thoracic levels. CT reconstruction was performed using the filtered-back-projection algorithm, with the Interactive Data Language (IDL, RSI, Boulogne-Billancourt, France).

The experimental set-up for animal positioning and mechanical ventilation was essentially identical to that used in previous experiments (2). An additional electromagnetic valve was included, which allowed the switching of the ventilation circuit from the ventilator to the forced oscillatory measurement system. Details of the ventilation system were published previously (2). The set-up consisted of a custom-made apparatus, including a T-tube and two electromagnetic valves, used to synchronize mechanical ventilation with the image acquisition. The input gas flow was maintained constant at baseline. The tidal volume was approximately 7.0 ml/kg, with the respiratory rate set to achieve an arterial PCO_2 close to 40 Torr. Ventilation and the inhaled gas mixture were remotely controlled with electromagnetic valves. Gas flows were measured continuously, recorded with mass flow meters (Aalborg, Orangeburg, New York) and adjusted prior to data acquisition. During the resting period the animal breathed air, and during imaging it breathed a mixture of xenon (70%) and oxygen (30%). Ventilation was paused in expiration at atmospheric pressure for image acquisition.

Image analysis. Lung tissue was selected within the mono-energetic CT images, by segmentation based on tissue density thresholds. In the single-compartment model, the xenon concentration for a given region of interest in the K-edge subtraction images increases in the wash-in sequence following mono-exponential dynamics (1). The time constant (τ) of xenon wash-in was calculated from a mono-exponential fit by using:

$$d(t) = d_A(1 - \exp[-(t-t_0)/\tau])$$

where d_A is the asymptotic xenon concentration and τ is the time constant. The arrival time t_0 includes the transfer time from the gas valve to the trachea, and the transfer time from the trachea to the entrance of the acini. The time scale is directly proportional to the number of breaths, because the apnea pauses during imaging were subtracted. The inverse of τ , equal to the local specific ventilation ($s\dot{V}$) or ventilation normalized to the voxel volume, was calculated for every point of the CT image to produce regional ventilation maps (13). The exponential fit was performed by using multidimensional unconstrained non-linear minimization (Nelder-Mead), and the goodness of fit was assessed by examination of the normalized summed squared residuals (SSRs). Images were smoothed with a 5 by 5 pixel moving average window to produce 1.75 mm x 1.75 mm resolution smoothed $s\dot{V}$ maps, where the effects of small differences (up to two pixels) in the animal position between successive images are eliminated. The heterogeneity of ventilation was calculated with a coefficient of variation (CoV) of $s\dot{V}$. Average regional ventilation $s\dot{V}$ and CoV were calculated separately from each $s\dot{V}$ map.

To determine well-ventilated lung zones, a histogram of $s\dot{V}$ was calculated. At baseline, the distributions were uni-modal. The statistical distribution of the regional ventilation at baseline is best described by a log-normal function due to the fractal structure of the bronchial tree (8). This functional form was used to fit the histograms of regional

ventilation, using the Nelder-Mead minimization. The probability density function of the log-normal distribution for a random variable x is given by the equation (7):

$$f(x; \sigma, \mu) = \exp[-(\ln x - \mu)^2 / 2\sigma^2] / [x\sigma(2\pi)^{1/2}]$$

Since the well-ventilated lung is supplied by fractally distributed flow, even after airway challenge, these were estimated from the total distribution of $s\dot{V}$ by fitting the above function, weighted to the larger values of $s\dot{V}$:

$$SSR = \sum_{i=0}^n w_i (y_i - f(x_i))^2$$

$$w_i = \begin{cases} 1 & \text{if } f(x_i) < \mu \\ 2 & \text{if } f(x_i) \geq \mu \end{cases}$$

Where SSR is the sum of square residuals, and w_i the weighting factor. The goodness of fit was assessed based on the SSRs. The area of well-ventilated zones (VA) was defined as the area where:

$$s\dot{V} > (\mu - 2\sigma)$$

The total lung area is defined as the area within the mono-energetic images corresponding to lung determined by segmentation, with the exclusion of large airways and blood vessels. The lung tissue density was calculated as follows: the two mono-energetic images obtained at the energies E_- and E_+ , below and above the xenon K-edge, respectively. For mono-energetic X-rays the logarithm of the transmitted intensity I is proportional to the line integral of the attenuation coefficient. CT reconstruction produces linear attenuation coefficient maps, which can be used to calculate xenon density maps. For each pixel of the tomographic image, the linear attenuation coefficients (μ_- and μ_+) can be written as a linear combination of the two elements, tissue and xenon:

$$\mu = \mu_T + \mu_{Xe} = \left(\frac{\mu}{\rho} \right)_T \cdot \rho_T + \left(\frac{\mu}{\rho} \right)_{Xe} \cdot \rho_{Xe} \quad (1)$$

Because the changes in the mass-attenuation coefficients (μ/ρ) are known for xenon and tissue

(3, 12), based on equation (6) :

$$\left\{ \begin{array}{l} \rho_T = \frac{\mu_- - \left(\frac{\mu_-}{\rho} \right)_{Xe} \cdot \rho_{Xe}}{\left(\frac{\mu_-}{\rho} \right)_T} = \frac{\mu_+ - \left(\frac{\mu_+}{\rho} \right)_{Xe} \cdot \rho_{Xe}}{\left(\frac{\mu_+}{\rho} \right)_T} \Rightarrow \rho_{Xe} = \frac{\left(\frac{\mu_+}{\rho} \right)_T \cdot \mu_- - \left(\frac{\mu_-}{\rho} \right)_T \cdot \mu_+}{\left(\frac{\mu_+}{\rho} \right)_{Xe} \left(\frac{\mu_-}{\rho} \right)_T - \left(\frac{\mu_-}{\rho} \right)_{Xe} \left(\frac{\mu_+}{\rho} \right)_T} \\ \rho_{Xe} = \frac{\mu_- - \left(\frac{\mu_-}{\rho} \right)_T \cdot \rho_T}{\left(\frac{\mu_-}{\rho} \right)_{Xe}} = \frac{\mu_+ - \left(\frac{\mu_+}{\rho} \right)_T \cdot \rho_T}{\left(\frac{\mu_+}{\rho} \right)_{Xe}} \Rightarrow \rho_T = \frac{\left(\frac{\mu_-}{\rho} \right)_{Xe} \cdot \mu_+ - \left(\frac{\mu_+}{\rho} \right)_{Xe} \cdot \mu_-}{\left(\frac{\mu_+}{\rho} \right)_{Xe} \left(\frac{\mu_-}{\rho} \right)_T - \left(\frac{\mu_-}{\rho} \right)_{Xe} \left(\frac{\mu_+}{\rho} \right)_T} \end{array} \right. \quad (2)$$

Using equation 2, the density due to tissue; ρ_T and to xenon; ρ_{Xe} were calculated separately for each voxel in the image (3).

Gas content was defined as the fraction of gas contained in the image regions corresponding to lung. Gas content was calculated using the ratio of the mean asymptotic xenon concentration based on equation (1), to the xenon concentration in the inhaled gas measured within corresponding central airway lumens, assuming negligible diffusion of xenon out of the airspaces during the short imaging time. Since the calculation of the mean asymptotic xenon concentration is subject to error in poorly ventilated zones, these zones were excluded from the calculation of gas content.

Measurement of respiratory mechanics. The mechanical ventilation was paused at end-expiration, a loudspeaker-in-box system and the tracheal tube were connected through a polyethylene tube (100 cm length, 0.375 cm ID) and a small-amplitude (1 cmH₂O peak to peak) forcing signal was delivered into the trachea. The loudspeaker was driven by a

computer-generated pseudorandom signal ranging from 0.5 to 21 Hz. Lateral pressures were measured at the loudspeaker end (P_1) and the distal end (P_2) of the wave-tube with miniature sidearm transducers (ICS 33NA00D). These pressure signals were low-pass filtered (<25 Hz) and digitized at a sampling frequency of 128 Hz. The pressure transfer function (P_1/P_2) was created by fast Fourier transformation from the 8-s recording. The input impedance of the respiratory system (Z_{rs}) was computed from the pressure transfer function as the load impedance of the wave-tube (10) by using the transmission line theory (14):

$$Z_{rs} = Z_0 \cdot sh(\gamma L) / ([P_1/P_2 - ch(\gamma L)])$$

where Z_0 is the characteristic impedance of the wave-tube and γ is the complex propagation wavenumber; these were determined from the geometrical parameters of the wave-tube, and the material constants of the tube and the gas in it.

Three to five Z_{rs} spectra were ensemble-averaged under the baseline conditions, following cigarette smoke exposures, and during steady-state methacholine infusions. Because of the absence of a plateau response after the intravenous ovalbumin challenge, parameters obtained from the Z_{rs} data collected 2 min after the ovalbumin injections were included in the analysis (6).

A model including an airway resistance (R_{aw}) and inertance (I_{aw}) in series with a constant-phase tissue compartment (6) incorporating tissue damping (G) and elastance (H) was fitted to the averaged Z_{rs} data. The respiratory tissue hysteresivity (η) was calculated as G/H (4). The data at frequencies coinciding with the heart rate and its harmonics were often corrupted, as evidenced by poor coherence and a high SD, and they were omitted from the model fitting. The minimization of the squared sum of weighted differences between the measured and the modeled impedance data was accomplished by a global optimization algorithm (6).

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