ACUTE CIGARETTE SMOKE INHALATION BLUNTS LUNG RESPONSIVENESS TO METHACHOLINE AND ALLERGEN IN RABBIT: DIFFERENTIATION OF CENTRAL AND PERIPHERAL EFFECTS

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Running head: Acute cigarette smoke inhalation blunts lung responsiveness.

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ABSTRACT

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 non-specific 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 in order to differentiate central airway and peripheral airway and lung parenchymal components of the response to airway provocation. Acute exposure to smoke generated by 4 successive cigarettes (CS) strongly inhibited the central airway response to subsequent IV methacholine (MCh) challenge. In the sensitized animals, although the response to ovalbumin (OVA) 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 (10000 ppm for 4 min, n=6) or NO inhalation instead of CS (240 ppm, 4 x 7 min, n=5), failed to blunt non-specific 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 non specific 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.

Keywords: Asthma; Smoking; Tomography, X-Ray Computed; Synchrotrons; Respiratory Mechanics.
INTRODUCTION

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 neutrophilic 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 non smokers, 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. in 9 asthmatic subjects, found that bronchial responsiveness to methacholine actually decreased after one hour of acute smoke exposure (36). Menon et al. however (18), showed methacholine hyper responsiveness in 32% of "smoke-sensitive" subjects with asthma, 6 hours 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 non specific 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 in order 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.

Ovalbumin Sensitization and Provocation procedures. Six animals were sensitized over a one-month 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 5 subsequent days prior the experiments using an ultrasonic nebulizer (Systam LS290, Velleneuve sur Lot, France).

Surgical preparation. Prior to 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 mg/kg/h, Aguettant, Lyon, France). Paralysis was induced with intravenous Atracurium (1.0 mg/kg/h, Tracrium, Glaxo-SmithKline, Münchenbuchsee, Switzerland). The respiratory gas flow was monitored by using a heated pneumotachometer (Hans Rudolph, Kansas City MO, USA). 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 Figure 1. An initial set of experiments were performed in 6 sensitized and 5 control rabbits (Figure 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 in parallel 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 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 3-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 minutes, or down to the butt plus 1 to 2 mm,
followed by 5 minutes of exposure to 40 % FiO₂, up to a total of four cigarettes. Arterial 
blood gases were measured prior to 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 μg/kg/min administered stepwise over 15 minutes. After a 30-min recovery 
from the effect of methacholine, a new set of imaging and FOT data was collected prior to 
ovalbumin provocation (2.0 mg IV), immediately after which data acquisition was repeated. 
In order to standardize the volume history, single sighs defined as an inflation to a tracheal 
pressure of 30 cmH₂O were given at baseline, prior to methacholine challenge, upon recovery 
and before ovalbumin administration.

Protocol 2. In order to study potential mechanisms of the effect of mainstream cigarette 
smoke on subsequent airway response to methacholine, we performed a series of additional 
experiments (Figure 1; bottom). The effect of filtering the mainstream smoke was studied in 5 
animals (group 2A). A High Efficiency Particle Arrest filter (HEPA Capsule 12144, PALL, 
Port Washington, New York) was used that retains over 99.0% of <0.3 μm particles in 
unfiltered smoke (8) which contains the nicotine vapour phase (24),(29). These and the 
following additional experiments were performed in non-sensitized animals, where lung Zrs 
data were obtained, without synchrotron imaging.

In order to determine the effect of exogenous nitric oxide (NO) contained in mainstream 
cigarette smoke, NO gas (1000 ppm in N₂, Carbagas AG, Gümligen, Switzerland) was diluted 
in air to a final concentration of 240 ppm in 5 other rabbits (group 2B). 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 6 animals were exposed to 1% CO in air (10,000 ppm, 0.01 fractional gas content, Carbagas AG, Gümligen, Switzerland) for 4 minutes (group 2C). This amount of CO exposure produced similar levels of HbCO than that measured after exposure to HEPA-filtered mainstream cigarette smoke (Table 1). In order 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), prior to mainstream cigarette smoke exposure in an additional group of animals (group 2D).

**Synchrotron lung CT imaging.** The synchrotron CT imaging technique employed in this study is K-edge subtraction 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 6 generations (Bayat, 2006 #357). A detailed description of the synchrotron imaging method and instrumentation is included in the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site.

Image analysis. Images were processed by using the MatLab programming package (Mathworks Inc., MI, USA) 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 contained 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 $Z_{rs}$ data (10). The respiratory tissue hysteresivity ($\eta$) 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 analysis of variances (ANOVA), was used to evaluate the differences in the mechanical, functional imaging parameters and blood gases among groups and as compared to 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 Raw (12.0±4.3 vs. 12.9±3.2 cmH₂O⋅s/l) and G (135.1±34.7 vs. 129.4±16.8 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 Figure 2. The significant interactions in the changes in Raw (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 Raw, 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 (Figure 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 non-exposed 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 Figure 3. Following acute cigarette smoke exposure, the mean total central airway luminal cross-section area increased in both ovalbumin-sensitized and control animals (Figure 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 (Figure 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 (Figure 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 to controls, even at baseline (Figure 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 acute exposure to cigarette smoke and decreased significantly in sensitized animals, only after ovalbumin challenge (Figure 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 (Figure 6; bottom).

The mean specific ventilation ($s\dot{V}m$), was higher in the sensitized animals compared to controls (Figure 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 a-chymotrypsin pre-treatment on subsequent airway response to Methacholine challenge.** Supplemental experiments (protocol 2) were performed in order to explore possible mechanisms through which acute exposure to cigarette smoke inhibited subsequent airway response to methacholine, based on the changes in $Raw$ measured by the forced oscillation technique in non-sensitized animals. Results of these experiments performed without KES imaging are summarized in Figure 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. Conversely, pre-treatment with $\alpha$-chymotrypsin - a peptidase administered in order to prevent the effect of the inhibitory non-adrenergic non-cholinergic system – prior to exposure to unfiltered cigarette smoke did not produce a significant difference in the methacholine airway response inhibition.

**Arterial blood gases.** Arterial blood gas data are summarized in table 1. Cigarette smoke exposure caused metabolic acidosis in the Control, Sensitized and $\alpha$-chemotrypsine pre-treatment groups, but not in the animals exposed to filtered smoke, CO or NO. A lower average PaO2 was observed in the $\alpha$-chemotrypsine 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 $\alpha$-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 in order 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 phase of cigarette smoke obtained by absolute HEPA filtering that removes > 99% of the fine particulate phase (8) containing nicotine (24).

Effect of acute cigarette smoke exposure on lung mechanics. In the present study, acute CS exposure produced a slight but significant decrease in Raw. At the same time the cross-sections of large-caliber central airways increased, and did not show any decrease even after the highest dose of methacholine despite a significant rise in Raw. This suggests that acute exposure to mainstream cigarette smoke blunted the airway tone in the proximal airways. This result was unexpected since cigarette smoke is a known irritant that has been shown to trigger a number of protective neural reflexes and to
provoke cough, mucus secretion, and bronchoconstriction. The neural pathways involved in
these immediate responses include central nervous system stimulation, activation of
autonomic ganglia (22) and stimulation of C-fiber endings, causing the endogenous release of
tachykinins acting on NK1 and NK2 receptors (12), and rapidly adapting pulmonary receptors
(RAR’s) eliciting cholinergic reflex bronchoconstriction (13). Sellick et al. showed that the
activity of the latter receptors significantly increased in rabbit following 2 minutes of
exposure to mainstream cigarette smoke (30). Despite this, no significant change in
pulmonary mechanics was found. This may have been due to the fact that in their study, the
animals were bilaterally vagotomized, which may have suppressed the vagally-mediated
reflex due to the stimulation of RAR’s. Similarly, in the present study, we did not observe
reflex bronchoconstriction despite the fact the animals were vagally intact. Suppression of the
central nervous system by anesthetics may have depressed the reflex responses mediated by
the activation of RAR’s (13). The relative contribution of C-fiber afferents to that of RAR’s in
the acute irritant-induced bronchoconstriction is not very well known, however, Spina et al.
(31) found significantly higher concentrations of sensory neuropeptides in guinea pig
compared to rabbit or human airways in isolated bronchial preparations. The authors
demonstrated that high concentrations of capsaicin, which stimulates sensory C-fiber activity,
elicited a relaxation response in rabbit and human airways, but not in guinea pig. The authors
suggested that the 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. found that inhalation of
mainstream smoke from a single cigarette caused significant bronchodilatation in pig, that
was reduced but persisted after filtering (3). 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 bronchodilatation. 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. Taken 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 anaesthesia.

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 4 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-naive 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 (Figures 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 (Figure 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 (Figure 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. Roers et al. found that reactivity to inhaled methacholine was reduced in chronically smoking baboons (28). 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 post-ganglionic 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 weeks 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 non sensitized 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. found that mainstream cigarette smoke exposure significantly decreased both airway response to methacholine and lung eosinophilic allergic inflammation, in ovalbumin sensitized mouse (27). Conversely,
Nishikawa et al. 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 hours after exposure (21). In that study, the amount of cigarette smoke administered was relatively smaller than in our study. In another study, Emms et al. (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 calibre. 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 calibres 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 (Figure 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 bronchodilatation 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 vasoactive intestinal peptide (VIP) has been suggested to inhibit non-adrenergic non-cholinergic neurogenic
bronchoconstriction (14). In the absence of a selective VIP receptor antagonist, we administered $\alpha$-chymotrypsin which degrades VIP (2),(1), prior to mainstream cigarette smoke exposure. However, $\alpha$-chymotrypsin pre-treatment 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 non specific 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 (Figure 5) in sensitized compared to 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 non specific 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 non specific 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 are declared by the authors.
FIGURE LEGENDS

Figure 1. Summary of the study protocol. *Above dotted line: Protocol 1* experiments performed with combined forced oscillation and k-edge subtraction synchrotron imaging; *below dotted line: Protocol 2;* supplementary experiments performed without synchrotron imaging. FOT: Forced oscillation data acquisition; KES: K-edge subtraction imaging; CS1-4: exposure to smoke from cigarettes 1 to 4 (solid grey bars); IV MCH: infused methacholine airway challenge; OVA: ovalbumin. Group 2A was exposed to filtered cigarette smoke. Groups 2B and 2C were exposed to nitric oxide and carbon monoxide respectively, instead of cigarette smoke. Group 2D was pretreated with α-chemotrypsin prior to cigarette smoke exposure.

Figure 2. Relative changes in airway resistance ($\Delta$Raw; top), tissue damping ($\Delta$G; middle), tissue elastance ($\Delta$H; bottom) and tissue hysteresivity ($\eta$) following exposure to smoke from 4 subsequent cigarettes (C1 to C4), infusion of 2.5, 5.0, and 10 µg/kg/min of methacholine (M10), and after IV ovalbumin challenge (OVA), in control (black bars) and sensitized animals (white bars). A separate group of previously studied sensitized animals non-exposed to cigarette smoke was included for comparison (grey bars); *: p < 0.05 vs. baseline, #: p < 0.05 vs. the CS-exposed control group and §: p < 0.05 vs. non-exposed sensitized animals from a previous study shown for comparison, by 2-way repeated measures ANOVA.

Figure 3. *Upper panel:* mono-energetic CT images (upper row) showing the central airway cross-section with one airway magnified (box) in the mid-thoracic image slice, and specific ventilation images (lower row) in one representative sensitized rabbit at baseline, following exposure to smoke from the 4th cigarette (CS4), during methacholine infusion of 10 µg/kg/min (Mch), upon recovery and after ovalbumin provocation (OVA); *lower panel:*
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.

**Figure 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 µg/kg/min (M10), upon recovery, and after ovalbumin provocation (OVA); *: p < 0.0.5 vs. baseline, #: p < 0.0.5 vs. the CS-exposed controls; §: p < 0.0.5 vs. non-exposed 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.

**Figure 5.** Top: Changes in the total area of image regions corresponding to lung; **Middle:** fractional gas content in lung tissue; **Bottom:** Lung density; *: p < 0.0.5 vs. baseline, #: p < 0.0.5 vs. the CS-exposed sensitized group, by 2-way repeated measures ANOVA.

**Figure 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 $s\dot{V}$; *: p < 0.0.5 vs. baseline ; #: p < 0.0.5 vs. the CS-exposed control group, §: p < 0.0.5 vs. non-exposed sensitized animals from a previous study shown here for comparison, by 2-way repeated measures ANOVA.

**Figure 7.** Mean specific ventilation ($s\dot{V} m$); *: p < 0.0.5 vs. baseline, #: p < 0.0.5 vs. the CS-exposed sensitized group, by 2-way repeated measures ANOVA.
Figure 8. Changes in airway resistance (ΔRaw) in the supplemental groups A to D; FS Control: control animals exposed to HEPA-filtered cigarette smoke; α-chymotrypsine: pre-treatment with α-chymotrypsine prior to exposure to cigarette smoke; NO: exposure to nitric oxide instead of cigarette smoke; CO: exposure to carbon monoxide instead of cigarette smoke. A separate group of control animals studied previously is included for comparison (light grey bars); *: p < 0.0.5 vs. baseline, #: p < 0.0.5 vs. the CS-exposed control group, §: p < 0.0.5 vs. non-exposed animals from a previous study shown here for comparison, by 2-way repeated measures ANOVA. Exposure to full mainstream cigarette smoke significantly blunted the response to a subsequent methacholine challenge. Filtered smoke had a similar inhibitory effect. Neither NO nor CO exposure produced a similar inhibition of the methacholine response. Alpha-chymotrypsine pre-treatment had no significant effect.

Table 1. Arterial blood gas data. CS: cigarette smoke exposure; FS: filtered smoke; NO: nitric oxide; CO: carbon monoxide. *: p < 0.0.5 vs. baseline, §: p < 0.0.5 vs. the CS Control group, by 2-way repeated measures ANOVA.
REFERENCES


Figure 1.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
<table>
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<tr>
<th></th>
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<th>pCO2 (mmHg)</th>
<th>HCO3 (mmol/l)</th>
<th>HbCO (%)</th>
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<td>Baseline</td>
<td>Exposure</td>
<td>Baseline</td>
<td>Exposure</td>
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<td><strong>CS Control</strong></td>
<td>7.43 ± 0.07</td>
<td>7.17 ± 0.07*</td>
<td>98.6 ± 4.1</td>
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<td>7.29 ± 0.08*§</td>
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<td>81.3 ± 22.3*</td>
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<td><strong>FS</strong></td>
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<td><strong>α-Chemotrypsine</strong></td>
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<td>83.0 ± 7.1</td>
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Table 1.