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Effect of Perinatal secondhand tobacco smoke exposure on *in vivo* and intrinsic airway structure/function in non-human primates

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Abstract

Infants exposed to second hand smoke (SHS) experience more problems with wheezing. This study was designed to determine if perinatal SHS exposure increases intrinsic and/or *in vivo* airway responsiveness to methacholine and whether potential structural/cellular alterations in the airway might explain the change in responsiveness. Pregnant rhesus monkeys were exposed to filtered air (FA) or SHS (1 mg/m³ total suspended particulates) for 6 hours/day, 5 days/week starting at 50 days gestational age. The mother/infant pairs continued the SHS exposures postnatally. At 3 months of age each infant: 1) had *in vivo* lung function measurements in response to inhaled methacholine, or 2) the right accessory lobe filled with agarose, precision-cut to 600 micron slices, and bathed in increasing concentrations of methacholine. The luminal area of the central airway was determined using videomicroscopy followed by fixation and histology with morphometry. *In vivo* tests showed that perinatal SHS increases baseline respiratory rate and decreases responsiveness to methacholine. Perinatal SHS did not alter intrinsic airway responsiveness in the bronchi. However in respiratory bronchioles, SHS exposure increased airway responsiveness at lower methacholine concentrations but decreased it at higher concentrations. Perinatal SHS did not change eosinophil profiles, epithelial volume, smooth muscle volume, or mucin volume. However it did increase the number of alveolar attachments in bronchi and respiratory bronchioles. In general, as mucin increased, airway responsiveness decreased. We conclude that perinatal SHS exposure alters *in vivo* and intrinsic airway responsiveness, and alveolar attachments.

Keywords

lung slice; videomicroscopy; alveolar attachments; airway responsiveness; smooth muscle; alveolar attachments; eosinophils; mucin; epithelium

Introduction

Perinatal exposure to second hand smoke (SHS) is associated with increased respiratory symptoms in infants and young children. Perinatal SHS exposure increases cough, wheeze, and severity of bronchiolitis (Joad, 2000). The cause is unknown and may involve changes in the nervous (Joad *et al.*, 2007b) or immune (Wang *et al.*, 2007) systems, the structure of the

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Conflict of Interest Statement

The authors have no conflicts of interest.

lung and airways (Pinkerton and Joad, 2006), and/or airway responsiveness (Lodrup Carlsen and Carlsen, 2001).

In this study, we examined the effects of perinatal SHS exposure on *in vivo* and *in vitro* airway responsiveness. In our *in vitro* method, we are able to study the structure/function of bronchi separately from respiratory bronchioles in young monkeys (Kott *et al.*, 2002; Joad *et al.*, 2006; Joad *et al.*, 2007a). This allows for an understanding of how SHS changes different regions of the airway separate from potential effects on the nervous system or circulating cells and factors. With this method, we can study the physiological responsiveness of an airway region to methacholine and then examine the histological features of the same airway. Thus we are able to connect function with structure. We used a monkey model of perinatal SHS because the anatomy of the airways of the monkey are most like humans. Mice and rats do not have respiratory bronchioles, yet humans and monkeys have many generations of respiratory bronchioles which are surprisingly reactive (Joad *et al.*, 2006) to methacholine.

Using videomicroscopy, we have previously shown that exposing infant monkeys to allergen with or without ozone from 1 to 6 months of age increased responsiveness of bronchi and respiratory bronchioles (Joad *et al.*, 2006). In that paper we were able to show that in bronchi but not in respiratory bronchioles, there was a correlation between airway responsiveness and the number of eosinophil profiles and between airway responsiveness and the number of pulmonary neuroendocrine cells (PNECs), an effect that could be blocked by an antagonist to a PNEC mediator, bombesin. We used this same methodology to examine the effects of perinatal SHS. We hypothesized that perinatal SHS exposure would increase responsiveness *in vivo* and in the bronchi and respiratory bronchioles *in vitro*. Perinatal SHS exposure might also increase the number of eosinophil profiles, the thickness of the epithelium, smooth muscle volume, mucin, and decrease alveolar attachments. Finally, there would be a correlation between airway responsiveness and histological variables.

Methods

All protocols were approved by the Institutional Animal Care and Use Committee in compliance with the American Association of Accreditation of Laboratory Animal Care.

Overall protocol

Pregnant rhesus monkeys (*macaca mulatta*) from the California National Primate Research Center (Davis, CA) were selected. Fetuses/infants began exposures to: 1) filtered air (FA) from 40 days gestation (FA, $n=5$ *in vivo* studies, $n=5$ *in vitro* studies) or 2) SHS from 50 days gestation (perinatal SHS, $n=4$ *in vivo* studies, $n=5$ *in vitro* studies). At 3 months of age, monkeys were given pulmonary function tests in response to methacholine (*in vivo* studies) or were necropsied and their bronchi and respiratory bronchioles in lung slices were studied for reactivity to methacholine then fixed for histology (*in vitro* studies).

SHS Exposures

Aged and diluted sidestream smoke was used as a surrogate for SHS and produced in a smoking apparatus built in our laboratory, as previously described (Teague *et al.*, 1994). Research cigarettes (2R4F) were obtained from the Tobacco Research Institute at the University of Kentucky. Exposure to SHS occurred for 6 h/d, 5 d/wk at a total suspended particulate target concentration of 1 mg/m³.

In vivo Studies

On the day of the pulmonary function test, the monkeys were anesthetized with Ketamine (0.2cc i.m.) and then propofol (initially 2 mg/kg i.v., then titrated to effect) then intubated and

ventilated. Utilizing a pneumotachograph and esophageal catheter, baseline respiratory rate (f), tidal volume (V_t), pulmonary resistance (RL) and dynamic compliance (C_{dyn}) were measured (Modular Instruments, Malvern, PA). Following baseline measurements, increasing concentrations of aerosolized methacholine (0.0625 – 0.5 mg/ml) was administered for 15 breaths on the ventilator. This was followed by measurement of the pulmonary function parameters for the next 3 minutes. Methacholine was aerosolized using a microstat ultrasonic nebulizer (Caire, Littleton, CO) connected in series with the inspiratory line from the ventilator so that during each ventilatory cycle air was drawn through the nebulizer.

In Vitro Studies

At necropsy, the right accessory lobe was filled with warm agarose, cooled and cut into 600 μ m slices perpendicular to the main central airway. Each slice was secured with surgical glue to a cover-slip near the pleural edges, then bathed in increasing concentrations of methacholine (-10 to -4 log M) with images captured using a light microscope with water-immersion lenses connected to a computer running NIH Image software via a video camera (Kott *et al.*, 2002). The provocative concentration of methacholine that decreased luminal size to 50% of that at 10^{-4} M (PC_{50}) was calculated using linear interpolation. Slices were also challenged with increasing concentrations methacholine in the presence of the bombesin antagonist ([Leu¹³-(ψ - CH₂NH)-Leu¹⁴-]-Bombesin, 100 nM, Sigma chemical, St Louis, Mo).

Histology

Following videomicroscopy measurements, each lung slice was fixed in 1% paraformaldehyde and embedded in paraffin (Paraplas-20, Oxford labware, St. Louis, MO). Airways were classified as a bronchus by the presence of cartilage in the wall, a bronchiole by the absence of cartilage in the wall or a respiratory bronchiole by occasional alveolar outpocketings along the airway wall. Due to the paucity of bronchioles in the lungs of monkeys (only 1–2 generations in total), they were excluded from the analysis. Histological measurements included: eosinophil profiles, epithelial volume, smooth muscle volume, mucin volume, number of alveolar attachments, outermost circumference of the airway, and basal lamina length.

Eosinophils were identified using a specific stain (CEM, American Master Tech Scientific, Inc., Lodi, CA) which identifies eosinophils and mast cells. Eosinophil profiles in the submucosa of each airway were counted and expressed as profile number per unit length of airway basal lamina.

For *epithelial volume*, the sections were stained with hematoxylin and eosin and the volume of epithelium was measured and expressed as volume epithelium per basal lamina surface area (E_s/BL_s).

Smooth muscle was identified with Masson's Trichrome Stain (American Master Tech Scientific, Inc., Lodi CA) to distinguish smooth muscle from fibroblasts, collagen and elastin. Smooth muscle surrounding the entire airway was measured and expressed as the volume of smooth muscle per basal lamina surface area (SM_s/BL_s) where SM_s is the areal measurement of smooth muscle and BL_s is the linear measurement of the basal lamina.

Mucin was identified by Alcian Blue and Periodic Acid Schiff's (AB/PAS, American Master Tech Scientific, Inc., Lodi, CA). Four quadrants of each airway were captured and the area of AB/PAS- positive intracellular material was measured and expressed as the volume of mucosubstances per basal lamina surface area using the morphometric formula: V_s/BL_s where V_s is the areal measurement of mucosubstances and BL_s is the linear measurement of the basal lamina.

For evaluation of *alveolar attachments*, sections were stained with hematoxylin and eosin and the outer boundary of each airway wall (P) where the airway adventitia directly attached to alveoli was measured. The number of alveolar septal attachments (A) to the corresponding outer perimeter (P) of the airway was counted. The number of attachments per length of outer airway wall was calculated as A/P (Joad *et al.*, 2007a).

Statistical analysis

Repeated measures analysis of variance was used to compare the concentration response curves. When appropriate, *post hoc* analyses consisted of a series of Scheffe contrast tests among the treatment groups (SAS/Stat, SAS Institute, Cary, NC). The effects of SHS exposure on baseline pulmonary function parameters and on histological variables within airway regions were compared using a t-test if normally distributed or a rank sum test if not. Linear regression was used to determine the relationship of each histological variable (across both exposures) with airway responsiveness as measured by PC₅₀ (SigmaStat 3.1; Systat Software Inc Richmond, CA). Data are presented as mean $\pm$ SEM unless otherwise noted.

RESULTS

SHS exposure characteristics

The exposure conditions for the monkeys used for *in vivo* studies were (mean $\pm$ SD) : total suspended particulates 0.97 ± 0.13 mg/m³, nicotine 259 ± 62 μ g/m³, carbon monoxide 5.3 ± 0.8 ppm, temperature 77.0 ± 4 °F, relative humidity $39 \pm 10\%$. Exposure conditions for monkeys used in *in vitro* studies were (mean $\pm$ SD): total suspended particulates 0.99 ± 0.10 mg/m³, nicotine 160 ± 57 μ g/m³, carbon monoxide 4.9 ± 0.5 ppm, temperature 74.0 ± 1.3 °F, relative humidity $38.6 \pm 6.5\%$.

In vivo Studies

There was no significant effect of SHS exposure on tidal volume, lung resistance or dynamic compliance, but SHS exposure significantly ($P < 0.05$) increased respiratory rate approximately 20% (Table 1). The methacholine-induced changes in respiratory rate (Fig. 1), pulmonary resistance (Fig. 2) and tidal volume (data not shown) were not significantly affected by perinatal SHS exposure. Methacholine challenge induced a significantly smaller reduction in dynamic lung compliance in monkeys exposed to SHS as compared to those exposed to perinatal FA ($P = 0.049$; Fig. 3).

Airway slices

For the bronchial region, there were 1 to 4 slices per animal with a median of 2 slices per animal. For the respiratory bronchiole region, there were 1 to 4 slices per animal with a median of 2 slices per animal. There was no statistical difference in baseline luminal area between exposure groups in bronchi (113 ± 16.8 FA, 123 ± 18.4 SHS) or respiratory bronchioles (54.2 ± 9.03 FA, 34.3 ± 6.81 SHS).

In Vitro Studies

Perinatal exposure to SHS did not change airway responsiveness to methacholine in bronchi, nor were any pairwise comparisons significantly different (Fig. 4A). Repeated measures analysis of respiratory bronchiole responsiveness, showed a significant interaction between the SHS and FA exposed airways. Perinatal exposure to SHS increased responsiveness at lower methacholine concentrations (-9 log M, but decreased it at higher concentrations (-6.5 to -4 log M) where the majority of the dose response relationship occurred. When bronchi and respiratory bronchioles from both exposure groups were challenged in the presence of the

bombesin antagonist, no significant difference was seen in responsiveness to methacholine (data not shown).

Eosinophil profiles, epithelial volume, smooth muscle volume, mucin volume

There were no perinatal SHS exposure effects on the number of eosinophil profiles, epithelial volume, smooth muscle volume, or mucin volume in bronchi or respiratory bronchioles (Table 2). There were also no correlations between PC₅₀ and either eosinophil profiles, epithelial volume, or smooth muscle volume. There was a correlation between mucin volume and PC₅₀; as the mucin volume increased, airway responsiveness decreased ($P=0.007$, $n = 15$, $r^2 = 0.44$; Fig. 5).

Alveolar Attachments

Perinatal SHS exposure significantly increased the number alveolar attachments per perimeter length in bronchi ($P = 0.01$) but not in respiratory bronchioles ($P = 0.10$; Fig. 6). In respiratory bronchioles, there was a trend for a correlation between the number of alveolar attachments and PC₅₀; as the number of attachments increased the airway responsiveness decreased ($P = 0.08$, $r^2 = 0.17$, Fig. 7).

Discussion

We had hypothesized that perinatal SHS exposure would increase airway responsiveness but instead saw a decrease in responsiveness to methacholine challenge with perinatal SHS exposure in both the *in vivo* and *in vitro* studies. In our *in vivo* study perinatal SHS exposure resulted in a trend toward less of an increase in pulmonary resistance (Fig. 2) and a significant effect of less of a decrease in dynamic compliance (Fig. 3) in response to methacholine. Similarly, in our *in vitro* study perinatal SHS exposure decreased airway reactivity to methacholine during most of the concentration response curve in respiratory bronchioles (Fig. 4B).

Our hypothesis that SHS exposure would induce hyperreactive airways was based on animal studies (Joad *et al.*, 1993; Joad *et al.*, 1999) and human studies that show maternal smoking during pregnancy is associated with increased prevalence of asthma and history of wheezing (Tager *et al.*, 1995; Gilliland *et al.*, 2001; Lannero *et al.*, 2006). It has also been shown that older children with a history of perinatal cigarette smoke exposure have decreased lung function parameters (Moshhammer *et al.*, 2006) and that maternal smoking during pregnancy is associated with bronchial hyperresponsiveness by the age of 2 years and asthma by the age 17 to 20 years (Goksor *et al.*, 2007). The evidence is clear that perinatal cigarette smoke exposure is associated with pathological effects on pulmonary function in young children; but whether cigarette smoke exposure is associated with airway hyperresponsiveness in very young infants is not clear (Stocks and Dezateux, 2003). The changes we saw in pulmonary function and reduced methacholine responsiveness in respiratory bronchioles, may be related to the immunosuppressive effect of cigarette smoke (Selgrade, 2007). There is evidence that in the rat model, nicotine suppresses inflammatory/allergic responses (Mishra *et al.*, 2008). In OVA challenged rats, mainstream cigarette smoke increased cytokine production, but reduced much of the airway immune inflammatory response and furthermore significantly reduced the airway hyperresponsiveness induced by the allergen (Robbins *et al.*, 2005).

We thought that a number of histological features might be altered by perinatal SHS exposure and might correlate with airway responsiveness. We did not see any exposure effects on eosinophils, epithelial volume, smooth muscle volume, or mucin volume. Despite the lack of a SHS effect on mucin, there was a negative correlation between the volume of mucin and bronchi responsiveness i.e., PC₅₀ (Fig. 5). This may have been due to increased mucin volume

causing an increased diffusion barrier, thus limiting methacholine access to smooth muscle receptors. Perinatal SHS exposure did induce a significant increase in alveolar attachments in bronchi as well as in the respiratory bronchioles although not to statistical significance. Alveolar attachments are the main tether for transferring the preload of parenchyma onto smooth muscle and change in alveolar attachment number can have a profound effect on airway responsiveness (Macklem, 1996). Increased numbers of alveolar attachments found at the level of the bronchi in infant monkeys might explain in part the observed airway hypo-responsiveness of these airways in precision tissue slices, due to facilitating counterforces against airway smooth muscle contraction (during methacholine challenge) to maintain a more open airway lumen.

Our results demonstrating a decreased responsiveness to methacholine both *in vivo* and *in vitro* seem to be in contradiction with the preponderance of evidence that perinatal SHS is associated with asthma, history of wheezing and bronchial hyperresponsiveness in children. This apparent disparity could be due to the fact that we are testing SHS and not mainstream smoke exposure (i.e. the mothers in this study were exposed to SHS, not smoking directly). To our knowledge this is the first time that the effects of perinatal SHS exposure have been tested on primates, from gestational day 50 to 3 months of age in a controlled setting. The monkeys in the present study were exposed only to cigarette smoke, whereas subjects in clinical or epidemiological studies are much more likely to have other co-exposures (e.g. allergens, particulate matter). The three-month old primate lung is also in a rapidly developing phase, with a changing combination of anatomy and physiology, which could account for decrease in airway responsiveness in the young infant and increases later in life. We postulate that perinatal exposure to SHS decreases airway responsiveness in the very young infant but may increase it later in life.

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Figure 1. Methacholine induced changes in respiratory rate

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS). On the day of pulmonary function tests, they were anesthetized, intubated and ventilated. Increasing concentrations of aerosolized methacholine were administered. Perinatal SHS exposure did not affect the change in respiratory rate induced by increasing concentrations of methacholine (ANOVA, $P > 0.05$, $n = 5$ monkeys per group).

Figure 2. *In vivo* methacholine induced changes in pulmonary resistance

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS). On the day of pulmonary function tests, they were anesthetized, intubated and ventilated. Increasing concentrations of aerosolized methacholine were administered. Although there did appear to be a trend of perinatal SHS exposure decreasing pulmonary resistance induced by increasing concentrations of methacholine, this affect was not statistically significant (ANOVA, $P > 0.05$, $n = 5$ monkeys per group).

Figure 3. Methacholine induced changes in dynamic compliance

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS). On the day of pulmonary function tests, they were anesthetized, intubated and ventilated. Increasing concentrations of aerosolized methacholine were administered. The decreases in dynamic compliance induced by increasing concentrations of methacholine were significantly reduced in monkeys perinatally exposed to SHS (ANOVA, $P < 0.05$, $n = 5$ monkeys per group).

Figure 4. In vitro Airway responsiveness to methacholine

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS) then increasing concentrations of methacholine were applied to the central airway of lung slices and the luminal area determined. **A.** Bronchi. Perinatal SHS exposure did not change responsiveness ($P > 0.05$, $n = 5-12$ slices per group). **B.** Respiratory bronchioles. Perinatal SHS exposure increased responsiveness in the lower methacholine concentrations but decreased it at the higher concentrations ($P = 0.0005$ interaction, ANOVA, $n = 8-11$ slices per group.) Asterisks indicate a significant difference in responsiveness to specific methacholine concentrations between those exposed to SHS or FA (pairwise contrasts). SHS exposed respiratory bronchioles as compared to those exposed to FA were significantly more

responsive to log -9 M methacholine, but were significantly less responsive to methacholine starting at log -6.5 M methacholine concentration.

Figure 5. Correlation between mucin and airway reactivity in bronchi

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS). After measuring airway responsiveness to methacholine of the central airway of lung slices, the slices were fixed and mucin volume measured. Linear regression analysis of all slices from both exposures showed that in bronchi, as the mucin volume increased, airway responsiveness, as indicated by PC₅₀, decreased ($P = 0.007$, $r^2 = 0.44$).

Figure 6. Alveolar attachments

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS). After measuring airway responsiveness to methacholine of the central airway of lung slices, the slices were fixed and the number of alveolar attachments per length of perimeter of the airway measured. Perinatal exposure to SHS increased the number of alveolar attachments/perimeter length in bronchi ($P = 0.013$, $n = 16$ – 20 slices per group) and but not in respiratory bronchioles ($P = 0.104$, $n = 18$ – 24 slices per group).

Figure 7. Correlation between alveolar attachments and airway reactivity in respiratory bronchioles

Monkeys were exposed perinatally to filtered air (FA) or second hand smoke (SHS). After measuring airway responsiveness to methacholine of the central airway of lung slices, the slices were fixed and the number of alveolar attachments/perimeter length measured. Linear regression analysis of all slices from both exposures showed that in respiratory bronchioles, as the number of alveolar attachments increased, airway responsiveness tended to decrease ($P = 0.08$, $r^2 = 0.17$, $n = 19$ slices).

Table 1

Baseline respiratory rate and pulmonary function tests.

	FA	SHS	P
<i>f</i>	47.7 ± 2.9	57.7 ± 4.1	<0.05
Vt (ml)	6.79 ± 0.40	8.06 ± 1.3	ns
RL (cmH ₂ O/ml/s)	0.063 ± 0.008	0.048 ± 0.016	ns
Cdyn (ml/cmH ₂ O)	1.81 ± 0.23	3.16 ± 0.81	ns

f = respiratory rate Vt = tidal volume RL = pulmonary resistance Cdyn = dynamic compliance

Table 2

Histology

	Bronchi		Respiratory Bronchioles	
	FA	SHS	FA	SHS
Eosinophil profiles/basal lamina length (# $\text{mm}^{-1} \times 10^{-3}$)	482 ± 231	846 ± 396	0 ± 0	0 ± 0
Epithelium/basal lamina ($\mu\text{m}^3/\mu\text{m}^2$)	23.0 ± 1.0	24.7 ± 0.7	5.11 ± 0.78	3.31 ± 0.91
Smooth muscle/basal lamina ($\mu\text{m}^3/\mu\text{m}^2$)	9.22 ± 0.83	13.3 ± 1.8	5.91 ± 0.67	3.94 ± 0.67
Mucin/basal lamina ($\mu\text{m}^3/\mu\text{m}^2$)	5.21 ± 0.86	5.37 ± 0.79	0.302 ± 0.216	0 ± 0

No statistically significant differences. 5–24 slices per group, median = 16.5 group.