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In Utero and Postnatal Effects of Sidestream Cigarette Smoke Exposure on Lung Function, Hyperresponsiveness, and Neuroendocrine Cells in Rats[1]

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We evaluated whether sidestream smoke (SS) exposure *in utero* and/or postnatally causes airway obstruction and hyperresponsiveness, and whether the effect is associated with neuroendocrine cell hyperplasia. Pregnant Sprague–Dawley rats were exposed to filtered air (FA) or to SS (total suspended particulate concentration, 1.00 ± 0.07 mg/m^3; CO, 4.9 ± 0.7 ppm; nicotine, 344 ± 85 μg/m^3; mean ± SD) for 4 hr/day, 7 days/week from Day 3 of gestation until birth and then their female pups were exposed to either FA or SS for 7–10 weeks postnatally. This resulted in four exposure conditions: *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS). The lungs from the pups (n = 6–8 of each exposure combination) were then placed in an isolated buffer-perfused system where transpulmonary pressure, airflow, and pulmonary artery pressure (P_{pa}) were measured while increasing doses of methacholine were injected into the pulmonary artery. Three lungs from each group were then fixed in 1% paraformaldehyde and neuroendocrine cells were identified immunohistochemically using antibodies to neuron-specific enolase. As compared to lungs from FA/FA-exposed rats, lungs from SS/SS-exposed rats exhibited 24% lower C_{dyn} (p = 0.0006, ANOVA), greater reactivity to methacholine (p = 0.0001, repeated measures ANOVA), and more neuroendocrine cells per centimeter basal lamina (p = 0.0006, ANOVA). Lungs from SS/FA- or FA/SS-exposed rats were not different from lungs from FA/FA-exposed rats in any of these parameters. We conclude that exposure to SS both pre- and postnatally (but not only pre- or only postnatally) results in lungs which are less compliant, more reactive to methacholine, and have a greater number of neuroendocrine cells.

Children raised in homes with smokers have more respiratory symptoms including cough (Dodge, 1982; Forastiere *et al.*, 1992; Ekwo *et al.*, 1983), wheeze (Dodge, 1982), sputum production (Dodge, 1982), and respiratory illnesses (Schulte-Hobein *et al.*, 1992; Wright *et al.*, 1991; Forastiere *et al.*, 1992; Ekwo *et al.*, 1983); decreased pulmonary function including decreased FEV_1 (Tager *et al.*, 1983; O'Connor *et al.*, 1987), FEV_1/FVC (Sherrill *et al.*, 1992), FEF_{25-75} (O'Connor *et al.*, 1987), and MMEF (Martinez *et al.*, 1992); and increased airway reactivity (Young *et al.*, 1991; Martinez *et al.*, 1988; Frischer *et al.*, 1992). Children exposed to environmental tobacco smoke (ETS) also have an increased rate of asthma (Martinez *et al.*, 1992; Weitzman *et al.*, 1990), an increased likelihood of using asthma medications, and an earlier (first year of life) onset of asthma (Weitzman *et al.*, 1990). For children with asthma, exposure to environmental tobacco smoke exposure is associated with more severe asthma (Murray and Morrison, 1992); a greater risk for asthma exacerbations (Chilmonczyk *et al.*, 1993); lower FEV_1, FEV_1/FVC, and FEF_{25-75} (Chilmonczyk *et al.*, 1993); and greater airway reactivity to histamine (Murray and Morrison, 1992), cold air (O'Connor *et al.*, 1987), and exercise (Frischer *et al.*, 1992). Since these are epidemiological data, they can only show a relationship between ETS exposure and decreased lung function. Other variables such as early viral infections or exposure to other indoor and outdoor pollutants and antigens may confound these data. Thus, animal models are needed which can evaluate the effects of ETS in a controlled fashion.

Exposure to the mother's smoking rather than the father's smoking correlates best with pulmonary problems in children (Murray and Morrison, 1992; O'Connor *et al.*, 1987; Tager *et al.*, 1983; Weitzman *et al.*, 1990; Wright *et al.*, 1991). This may be due to mothers being physically

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closer to their children while providing care, thus exposing them to a larger dose of ETS, or to the effect of maternal mainstream smoking on the lungs of the fetus as they develop *in utero*. Despite attempts to use epidemiologic methods to separate them (Martinez *et al.*, 1988; Hanrahan *et al.*, 1992; Frischer *et al.*, 1992; Holberg *et al.*, 1993; Tager *et al.*, 1983; Cunningham *et al.*, 1994), the relative importance of pre- vs postnatal exposure to smoke on the eventual lung function of the child has not been established.

Although there is a greater relationship between the mother's rather than the father's smoking and the eventual pulmonary function of the child, several studies have shown that the father's smoking can also be a factor (Martinez *et al.*, 1988; Ekwo *et al.*, 1983; Sherrill *et al.*, 1992). This is usually interpreted as support for the hypothesis that postnatal ETS exposure causes pulmonary problems in children. However, in a household where the father smokes, the mother is also exposed to ETS during her pregnancy, and some of the effects of the father's smoking on the eventual pulmonary function of his child may be due to ETS exposure to the developing fetus. As part of the intensified interest in determining the effects of ETS on human health, it is important to determine whether ETS exposure to the pregnant nonsmoking mother can change pulmonary function in the child, and what role continued exposure to ETS plays.

Because families whose members smoke during a pregnancy rarely stop smoking when the infant is born, the natural experiment which would help determine the relative importance of ETS exposure pre- vs postnatally is difficult to evaluate epidemiologically. Thus, an animal model is particularly needed in which exposure to ETS pre- and postnatally can be separated. We have previously exposed rats to sidestream smoke (SS, a surrogate for ETS which also contains exhaled mainstream smoke) for their first 100 days of postnatal life. This exposure did not change lung function or airway reactivity to methacholine (Joad *et al.*, 1993). Therefore, we postulated that *in utero* exposure to SS with or without subsequent postnatal exposure to SS might cause the development of pulmonary function changes and airway hyperresponsiveness.

One possible developmental mechanism by which SS exposure could result in decreased lung function and airway hyperresponsiveness is by hyperplasia and persistence of neuroendocrine cells. Neuroendocrine cells are granulated cells possessing both endocrine and neural characteristics located in airways either singly or as clumps of cells known as neuroepithelial bodies. In humans, they contain a number of mediators including serotonin, bombesin, calcitonin, chromogranin, and calcitonin gene-related peptide (Polak, 1993) of which at least two, serotonin (Joad *et al.*, 1993) and bombesin (Lach *et al.*, 1993), are bronchoconstrictors, and bombesin is a growth factor (Aguayo, 1994). They are greatest in number during the fetal and newborn period and decrease dramatically thereafter (Cho *et al.*, 1989). Neuroendocrine cell hyperplasia occurs in airway diseases associated with hyperresponsiveness including cystic fibrosis, asthma, and bronchopulmonary dysplasia (Aguayo, 1993). Mainstream smoke exposure to humans (Aguayo, 1993) and adult hamsters (Tabassian *et al.*, 1989) causes neuroendocrine cell hyperplasia. Nicotine, a component of both SS and mainstream smoke stimulates the growth of neuroendocrine cells in culture, increases the concentration of immunoreactive calcitonin in the lungs of the developing fetus *in vivo* (Nylen *et al.*, 1993), and increases the number of neuroepithelial bodies in neonatal mice when given perinatally to their pregnant and lactating mothers (Wang *et al.*, 1984). The effect of *in utero* and/or postnatal SS exposure on neuroendocrine cells and neuroepithelial bodies has not been studied.

This study was designed to test the hypothesis that SS exposure *in utero* with or without SS exposure postnatally results in lungs with impaired function, hyperresponsiveness, and neuroendocrine cell hyperplasia.

METHODS

General protocol. The study was designed to provide four exposure combinations: *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS). Pregnant Sprague–Dawley rats (Zivic Miller, Zelienople, PA) were concurrently exposed to either FA or to SS for 4 hr/day, 7 days/week from Day 3 of gestation until delivery. At birth the female pups were randomly assigned to litters of 13–14 pups/dam which were exposed to either FA or SS postnatally. At 21 days of life the pups were weaned and 6–8 under each exposure condition were continued on their same exposure regimen. At 7–10 weeks of life, their lungs were removed and placed in an isolated buffer-perfused system where measurements were made of dynamic compliance (C_{dyn}), pulmonary resistance (R_L), pulmonary artery pressure (P_{pa}), and reactivity to methacholine. Three of the lungs in each group were then evaluated histologically for neuroendocrine cells. Since pulmonary function and reactivity to methacholine change with age in rats (Joad *et al.*, 1993), we studied the lungs in a randomized order so that age at the time of evaluation would not differ by exposure regimen.

Generation of SS exposure atmosphere. SS, the smoke which comes off the burning tip of the cigarette, was used as a surrogate for ETS which also contains a small amount of exhaled mainstream smoke. The exposure system and monitoring methods have been previously described (Teague *et al.*, 1994). Briefly, SS was generated by a modified ADL/II smoke exposure system (Oakridge National Laboratory) using conditioned 1R4F cigarettes from the Tobacco and Health Research Institute of the University of Kentucky. Two cigarettes at a time were smoked under Federal Trade Commission conditions in a staggered fashion at a rate of 1 puff (35 ml, 2 sec duration) per minute. The SS was diluted with filtered air in a mixing chamber and then passed into the stainless steel and glass Hinners-type exposure chamber 0.44 m^3 in size. Relative humidity, temperature, total suspended particulates (TSP), and nicotine concentrations were measured with probes 15–20 cm inside the back wall of the exposure chamber right next to the cages. Carbon monoxide was sampled from the back wall of the chamber. Carbon monoxide, temperature, and humidity were sampled continuously. Nicotine was sampled for 15 min twice during each 6 hr

exposure period. TSP (using the peizobalance technique) was sampled for 30 min of every hour. The exposure chamber was characterized by a relative humidity of 41.8 ± 7.2%, a temperature of 24.4 ± 0.8°C, a TSP of 1.00 ± 0.07 mg/m^3, a carbon monoxide concentration of 4.9 ± 0.7 ppm, and a nicotine concentration of 344 ± 85 μg/m^3 (mean ± SD of measurements from all exposure days).

Isolated perfused lung system. The lungs were studied in an isolated buffer-perfused system to separate them from the effects of circulating blood components and central neural control. Each rat was anesthetized with 150 mg/kg pentobarbital ip. The trachea was cannulated and the rat ventilated with room air at a rate of 60 breaths/min and a V_T of 3 ml. The chest was opened and 100 units of heparin was injected into the right ventricle. The inspired gas was then changed to 5% CO_2 mixed with room air, the right ventricle was incised, and a canula was placed into the main pulmonary artery. The left ventricle was incised, a canula was placed in the left atrium, and the lungs were washed free of blood with a warmed (37°C) Krebs–Ringer bicarbonate buffer (NaCl 119 mM, $CaCl_2$ 3.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 21.0 mM, KH_2PO_4 1.2 mM, albumin 4.5%, glucose 0.1%, pH 7.30–7.40). The lung was then dissected free and hung by the trachea in a heated (37°C) water-saturated chamber.

The lung was ventilated at 60 breaths/min with warmed (37°C) humidified gas (95% air and 5% CO_2) at an initial tidal volume which was adjusted to provide a transpulmonary pressure fluctuating between −2.5 and −10 cm H_2O. During the initial 30-min stabilization period, the lung was hyperinflated with 20 cm H_2O for 10 sec at 10-min intervals to prevent and reverse atelectasis. A differential pressure transducer (Valindyne, Northridge, CA) measured transpulmonary pressure and a Fleisch 0000 pneumotachograph (OEM, Richmond, VA) via a second pressure transducer measured airflow. All voltages were passed through carrier demodulators (Valindyne, Northridge, CA) into a Modular Instruments Data Acquisition System (Malvern, PA), where R_L and C_{dyn} (method of Amdur and Mead (1958)) were calculated. The average value over a 5-sec period was used except for dose–response curves, where the maximum value was used for R_L, and the minimum value was used for C_{dyn}.

The lungs were perfused with the warmed (37°C) Krebs–Ringer bicarbonate buffer in a recirculating fashion via a peristaltic pump at a rate of 0.04 ml/g body wt/min. pH of the perfusate was maintained between 7.30 and 7.40 by the addition of $NaHCO_3$ if needed. P_{pa} was measured with a pressure transducer (Gould; Cupertino, CA) with voltage passed into the Modular Instruments System.

After the stabilization period, R_L, C_{dyn}, and P_{pa} were measured. Then, increasing doses of methacholine ($10^{-9.75}$ to 10^{-7} mol) were injected in 100-μl bolus volumes every 1 min into a port in the pulmonary artery catheter and carried to the pulmonary artery with the perfusate which continued to flow at 0.04 ml/g body wt/min. The maximum change in R_L and minimum change in C_{dyn} were recorded.

At the end of the experiments, three lungs in each group were perfused with 1% paraformaldehyde for histologic examination. After the final dose of methacholine was administered, the transpulmonary pressure was held at 20 cm H_2O for 10 sec to reverse atelectasis. Lungs were removed from the chamber and approximately 4 ml cold (4°C) 1% paraformaldehyde was instilled into the lung via the trachea. The lung with the syringe still attached was then covered with moist gauze and suspended in cold 1% paraformaldehyde for 15 min. The syringe was then removed without ligation of the trachea and the lung (which remained inflated) was stored at 4°C in 1% paraformaldehyde.

Methods for immunohistochemistry of neuroendocrine cells. Neuroendocrine cells were identified immunohistochemically using an antibody to neuron-specific enolase (Polak, 1993). The validity of the procedure was confirmed by identifying the same or nearby cells with antibodies to serotonin (Polak, 1993). All lung tissues were embedded in paraffin. An avidin–biotin peroxidase method as outlined by Hsu *et al.* (1981) was used (kit purchased from Vector Laboratories, Burlingame, CA). Briefly, the sections were deparaffinized in three changes of xylene for 5 min each and hydrated in decreasing concentrations of ethanol. Sections were then treated with 3% hydrogen peroxide in double-distilled water to eliminate endogenous peroxidase and to unmask the antigenic sites. Bovine serum albumin (10%) in phosphate-buffered saline (PBS) containing 2% rat serum was then added to block nonspecific binding sites. The tissue was incubated with serotonin antibody overnight at 4°C or with neuron-specific enolase antibody for 20 min at room temperature. The neuron-specific enolase polyclonal antibody is raised in rabbits against neuron-specific enolase γ/γ homodimer (approximately 90 kDa) isolated from bovine brain (anti-neuron-specific enolase: Biogenex Laboratories, San Ramon, CA). Serotonin monoclonal antibody was made in rat against synthetic serotonin (Fitzgerald Industries International, Inc., Concord, MA). The appropriate dilution for both antibodies was 1:100. As a control, primary antibody was substituted with PBS or normal serum in each run to examine the tissue for nonspecific reaction.

Antibodies to neuron-specific enolase were used to identify neuroendocrine cells in tissue sections used for morphometry. At least two neuron-specific enolase-stained sections from each animal were examined by light microscopy (20×) and the number of positively stained airway epithelial cells and the number of positively stained cell aggregate sites (neuroepithelial bodies) from all airways were recorded. Nine to 36 airways were examined from each animal. Among these airways, at least two airways in each animal were central or axial pathways. To measure the basement membrane length, an image (10×) was captured onto a computer screen via a videocamera using the Image 1.47 program (National Institutes of Health). Data were collected by digitizing the entire length of basal lamina of the airways. Then, the number of positive cells/sites per centimeter basement membrane was calculated.

Statistical evaluation. All data except the methacholine dose–response curves were compared using a one-way analysis of variance (ANOVA) with a post hoc Fisher PLSD test if appropriate (Statview 512+ statistical computer package, BrainPower, Calabasas, CA). The methacholine dose–response data were log-transformed to equalize variance and analyzed with a repeated measures ANOVA with exposure group as a between-subject effect, and dose level as a within subject effect. Post hoc analyses consisted of a polynomial decomposition of the dose effect, and a series of Scheffe contrast tests among the treatment groups (SAS/STAT, SAS Institute). Significance is claimed whenever $p < 0.05$.

RESULTS

Evaluation of the isolated lungs revealed that SS/SS exposure in contrast to FA/FA exposure resulted in a 24% lower C_{dyn} ($p = 0.0006$, ANOVA, Fig. 1). On the other hand, SS exposure either only prenatally (SS/FA) or only postnatally (FA/SS) did not change C_{dyn}. SS/SS exposure decreased R_L an insignificant 6% (Fig. 2). P_{pa} was not changed by SS exposure (15.7 ± 1.0 mmHg in rats FA/FA-exposed, 15.8 ± 0.4 mmHg in rats FA/SS-exposed, 15.6 ± 0.9 mmHg in rats SS/FA-exposed, and 16.1 ± 0.8 mmHg in rats SS/SS-exposed, mean ± SEM, $p = 0.96$, ANOVA).

Lungs from SS/SS-exposed animals were also hyperresponsive to methacholine (Figs. 3 and 4). The increase in R_L at the four highest doses of methacholine was more than twofold greater in the SS/SS-exposed lungs than those in the FA/FA-exposed lungs (Fig. 3). In contrast, lungs from rats exposed to SS only prenatally or only postnatally were not hyperresponsive to methacholine (Fig. 3). The effect of SS exposure on methacholine-induced changes in C_{dyn} was

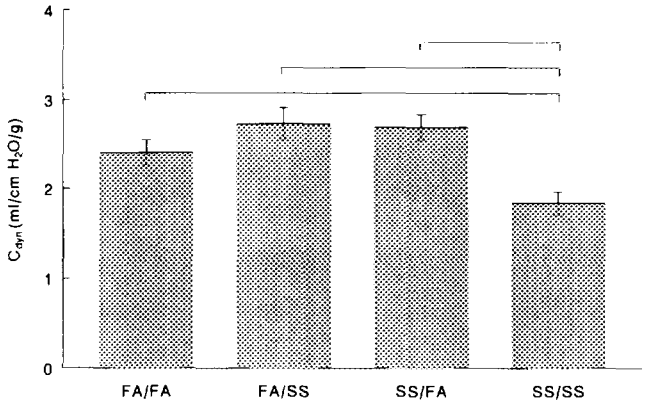

FIG. 1. Dynamic compliance/body weight in rat lungs exposed to *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS). Lungs from SS/SS-exposed rats had a lower C_{dyn}/body weight than lungs from any other exposure condition ($p = 0.0006$, ANOVA, brackets indicate Fisher PLSD <0.05, $n = 6$–8 each group).

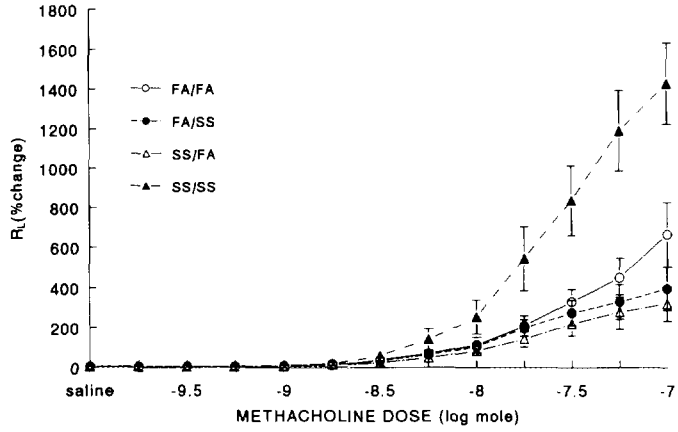

FIG. 3. Methacholine-induced changes in R_L in isolated lungs from rats exposed to *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS, $n = 6$–8 each group). SS/SS exposure resulted in hyperresponsiveness to methacholine ($p = 0.0001$, repeated measures ANOVA on the log-transformed data, with SS/SS different from all other exposure groups by post hoc polynomial decomposition of the dose effect, and a series of Scheffe contrast tests among the treatment groups (SAS/STAT, SAS Institute)).

similar to that with R_L but the differences were not as great (Fig. 4).

Neuroendocrine cells present in airway epithelium were identified by antibodies to neuron-specific enolase and serotonin (Figs. 5A and 5B). As expected, the antibody to neuron-specific enolase labeled both neuroendocrine cells and nerves (Fig. 6), while the serotonin antibody labeled both neuroendocrine cells and mast cells (Fig. 7). The negative controls with the primary antibody deleted confirmed that the antibodies were specific (Fig. 5C). Airway epithelial cells stained with neuron-specific enolase were found in all generations of airways, but more often in proximal airways. They were predominantly found to stain as clusters of cells.

The average number of cells within a cluster was five and did not differ by exposure regimen. Based on our morphometric analysis, there were significantly more neuroendocrine cells in the lungs of SS/SS-exposed rats than in the lungs from rats which had never been exposed to SS (Fig. 8). In contrast, although there was a trend for lungs from

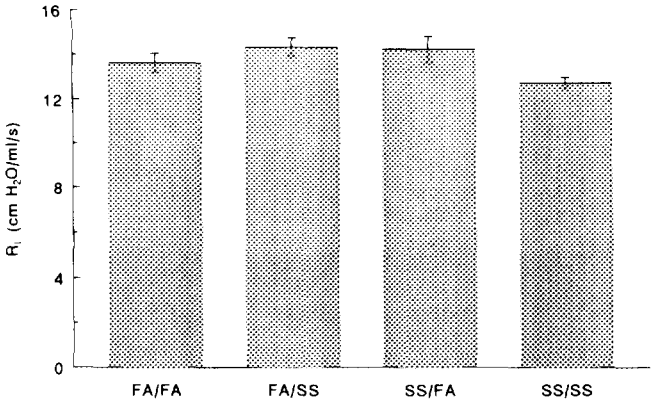

FIG. 2. Resistance of the lung in rat lungs exposed to *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS). There was no significant effect of exposure regimen on R_L ($p = 0.06$, ANOVA, $n = 6$–8 each group).

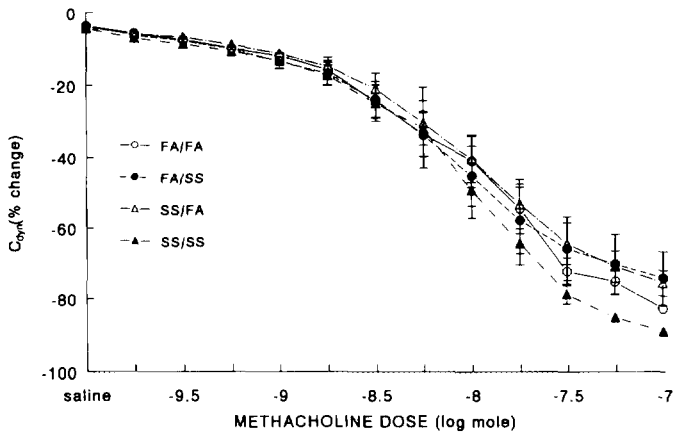

FIG. 4. Methacholine-induced changes in C_{dyn} in isolated lungs from rats exposed to *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS, $n = 6$–8 each group). SS/SS exposure resulted in hyperresponsiveness to methacholine ($p = 0.005$, repeated measures ANOVA on the log-transformed data, with SS/SS different from all other exposure groups by post hoc polynomial decomposition of the dose effect, and a series of Scheffe contrast tests among the treatment groups (SAS/STAT, SAS Institute)).

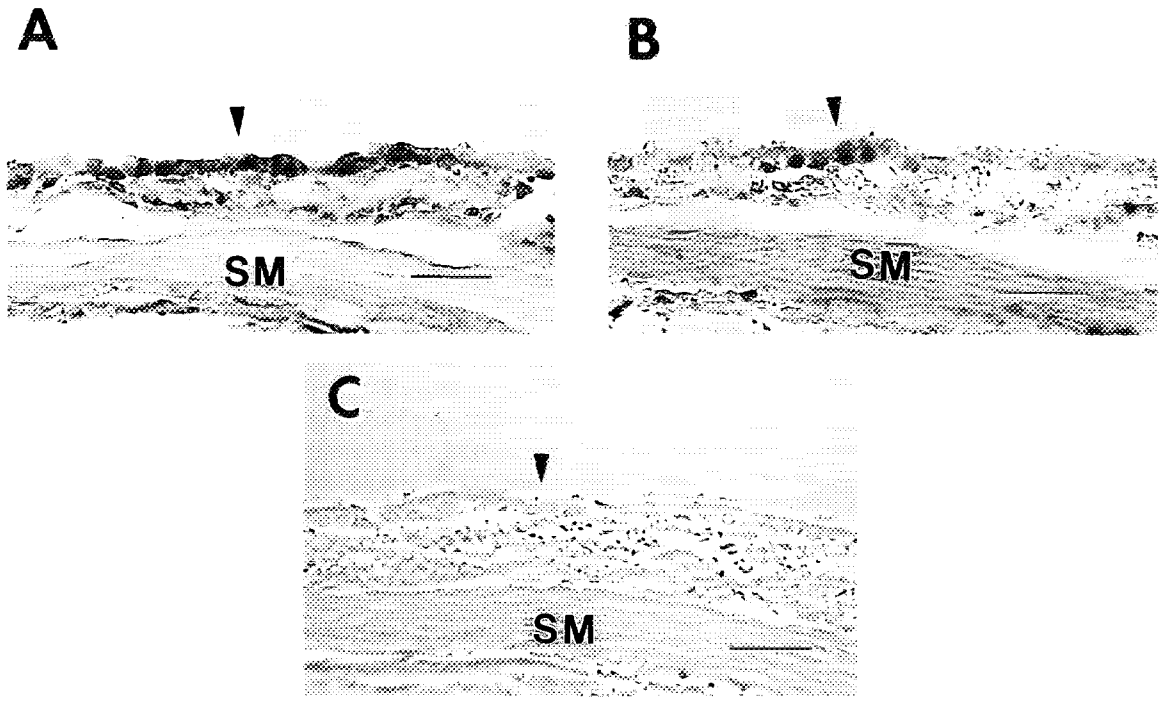

FIG. 5. Serial step sections from the same region of a central airway from the lung of a rat exposed to sidestream smoke both *in utero* and postnatally (SS/SS) which has been immunostained for serotonin (A) or neuron-specific enolase (B). A negative control where the primary antibody has been deleted is shown in (C). These sections show that epithelial cells in the same region are immunoreactive to both serotonin and neuron-specific enolase (arrowheads). SM, smooth muscle. Bar = 25 μm.

rats exposed to SS only prenatally or only postnatally to have an increased number of neuroendocrine cells, the difference was not statistically significant. Similar results were found for the number of neuroepithelial bodies (Fig. 9). An example of a section from an SS/SS-exposed rat lung showing multiple neuroendocrine cells in the airway epithelium is shown in Fig. 10.

DISCUSSION

We conclude that prenatal followed by postnatal SS exposure reduced lung compliance, increased lung respon-

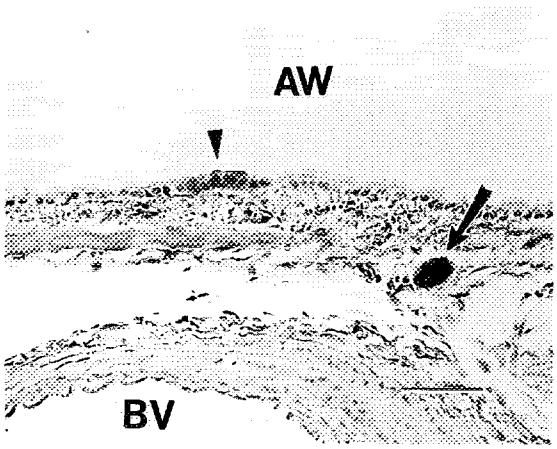

FIG. 6. Proximal airway from a rat lung exposed to sidestream smoke both *in utero* and postnatally (SS/SS) which has been immunostained for neuron-specific enolase. Both a cluster of airway epithelial cells (arrowhead) and a nerve bundle (arrow) within the airway wall are labeled. AW, airway; BV, blood vessel. Bar = 50 μm.

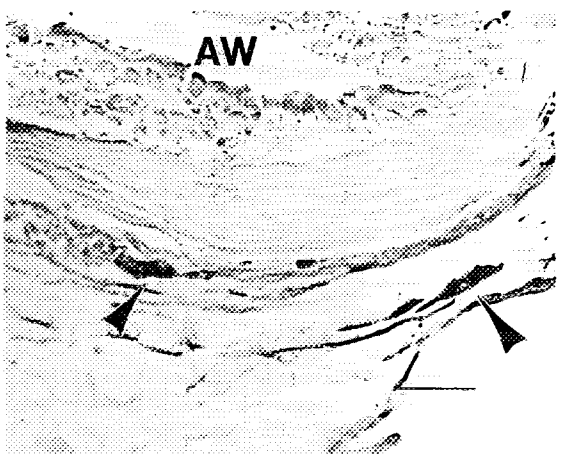

FIG. 7. Proximal airway from a rat lung exposed to sidestream smoke both *in utero* and postnatally (SS/SS) which has been immunostained for serotonin. Note the mast cells (arrowheads) present in the submucosa of the airway wall. AW, airway. Bar = 25 μm.

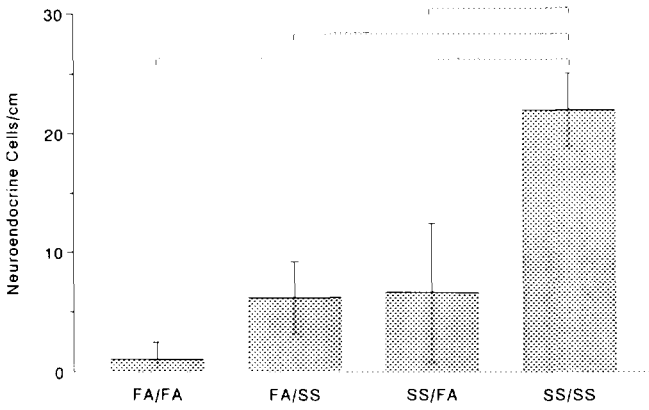

FIG. 8. Number of neuroendocrine cells (identified by neuron-specific enolase staining) per centimeter basal lamina in lungs from rats exposed to *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS). SS/SS resulted in more neuroendocrine cells per centimeter basal lamina ($p = 0.0025$ ANOVA, SS/SS differs from all other groups as indicated by the brackets, $p < 0.01$ Fisher PLSD; values are means ± SEM).

siveness to methacholine, and increased the number of pulmonary neuroendocrine cells and neuroepithelial bodies. These changes did not occur in rats exposed to SS either only prenatally or only postnatally.

Since children raised in homes of smokers have decreased lung function and increased airway reactivity including clinical asthma, we previously tried to reproduce these effects in rats exposed to relevant concentrations of SS during their first 100 days of life. We were surprised, however, to find that chronic SS exposure did not change lung function or reactivity to methacholine (Joad *et al.*, 1993). The present study confirms these findings. Although these results could be explained by species differences, it is possible that in humans and in rats postnatal SS exposure alone may not be sufficient to change lung function, but either *in utero* exposure alone or the combination of *in utero* exposure and postnatal exposure may be required. Furthermore, we wondered whether SS exposure rather than mainstream smoke exposure to the pregnant mother would be sufficient to cause these changes.

Although human studies have not addressed the issue of ETS exposure to the pregnant nonsmoking mother on the eventual lung function of her children, various investigators have tried to separate the relative importance of *in utero* mainstream smoke exposure from postnatal ETS exposure on lung function and airway reactivity in children.

Three studies suggest *in utero* exposure to mainstream smoke is more important than postnatal exposure to ETS. Martinez *et al.* (1988) showed that airway hyperreactivity was present in 70% of children whose mothers smoked during pregnancy compared to only 29% of children whose mothers did not smoke during pregnancy. However, they stated that the effect of the mother's current smoking status could not be statistically separated from the prenatal effects. Hanrahan *et al.* (1992) showed that expiratory flow at FRC was 74.3 ml/sec in 4-week-old infants born of continuous smokers vs 150 ml/sec in infants born of nonsmokers. Using multiple regression models, they found that postnatal ETS exposure effects were not related to the pulmonary function of these infants. However, their definition of ETS exposure involved such a small exposure (exposure to smokers at least 2 hr twice a week) that a postnatal ETS

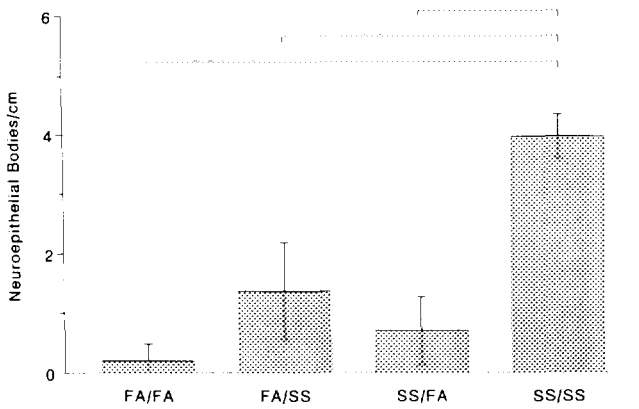

FIG. 9. Number of neuroendocrine cell sites (neuroepithelial bodies, identified by neuron-specific enolase staining) per centimeter basal lamina in lungs from rats exposed to *in utero* FA followed by postnatal FA (FA/FA), *in utero* FA followed by postnatal SS (FA/SS), *in utero* SS followed by postnatal FA (SS/FA), and *in utero* SS followed by postnatal SS (SS/SS). SS/SS resulted in more neuroepithelial bodies per centimeter basal lamina ($p = 0.0006$ ANOVA, SS/SS differs from all other groups as indicated by the brackets, $p < 0.01$ Fisher PLSD; values are means ± SEM).

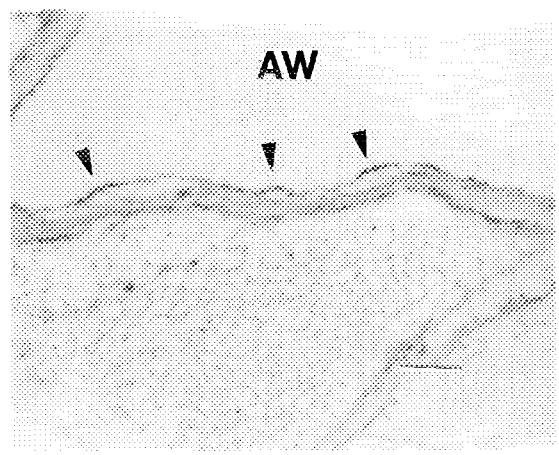

FIG. 10. Proximal airway from a rat lung exposed to sidestream smoke both *in utero* and postnatally (SS/SS) which has been immunostained for serotonin. Several clusters of serotonin-positive cells probably representing neuroendocrine cells (arrowheads) can be seen in the airway epithelium. AW, airway. Bar = 100 μm.

effect may have been missed. Recently, Cunningham *et al.* (1994) studied 8863 children and found that their lung function at 8–12 years of age was lower if their mother smoked during pregnancy. After adjusting for smoking during pregnancy, they found that current maternal smoking was not associated with lower lung function. The 75 children of mothers who smoked during pregnancy but not after their birth were found to have an 11% lower FEF_{25-75} than the never-exposed children. Children whose mothers did not smoke during pregnancy but who smoked in their first 2 years of life had a 2.8% lower FEF_{25-75}. These data suggest that lung function is most compromised by *in utero* exposure to smoke, although early postnatal exposure may play a role.

Three other studies provide the best evidence that postnatal exposure to ETS is more important than prenatal exposure. Frischer *et al.* (1992) showed that airway hyperresponsiveness to exercise in first graders was associated with smoke exposure in the first year of life, but not with ETS exposure during pregnancy or at the time of study. Holberg *et al.* (1993) showed that there was a threefold increase in wheezing and lower respiratory tract illnesses in 3 year olds exposed to ETS by care givers other than the parent. Tager *et al.* (1983) showed that the annual increase in FEV_1 was reduced by 7–11% in children of smoking mothers.

This study suggests that, at least in rats, it is the combination of *in utero* and postnatal smoke exposure and not either one alone which causes a change in lung function and responsiveness. Furthermore, the *in utero* smoke exposure needs not to be from the mother herself smoking, but rather can be from SS exposure to the nonsmoking mother.

The baseline function of lungs from rats exposed to SS/SS in our study had a statistically significant 24% decrease in C_{dyn}/body wt, with a trend ($p = 0.06$, ANOVA) toward a small (6%) decrease in R_L. Human studies have similarly shown that children raised in the homes of smokers have decreased baseline function with a decreased FEV_1 (Tager *et al.*, 1983; O'Connor *et al.*, 1987), FEV_1/FVC (Sherrill *et al.*, 1992), FEF_{25-75} (O'Connor *et al.*, 1987; Cunningham *et al.*, 1994), and MMEF (Martinez *et al.*, 1992).

The increase in lung reactivity to methacholine in the rats in this study was similar to the increase in airway reactivity (Young *et al.*, 1991; Martinez *et al.*, 1988; Frischer *et al.*, 1992) shown in children raised in the homes of smokers. Since airway reactivity to methacholine is often used as an index of severity of asthma, it is significant that the increase in R_L was up to twofold greater in the animals exposed to SS/SS. These findings suggest that children who are predisposed to asthma may have more severe disease if exposed to ETS both *in utero* and postnatally. This is consistent with the findings of Weitzman *et al.* (1990) who reviewed health surveys of 4331 children 1–5 years old and found that maternal smoking of at least 0.5 packs of cigarettes per day was associated with a twofold risk for asthma and an earlier onset of asthma. Similarly, Chilmoczyk *et al.* (1993) found that children with asthma exposed to high concentrations of ETS had a 1.8-fold greater risk of an asthma exacerbation as well as lower FEV_1, FEF_{25-75}, and FEV_1/FVC.

Since neither prenatal nor postnatal SS exposure alone changed airway function or responsiveness in our rats but rather both exposures were required, we searched for a mechanism which would produce apparent "priming" by *in utero* SS exposure followed by "maintenance" with postnatal ETS exposure. Wang *et al.* (1984) had shown that feeding mother mice nicotine during their pregnancies increased neuroepithelial bodies in the babies, and that although the number of neuroepithelial bodies decreased as expected with postnatal age, the decline was slowed by feeding the mothers nicotine during lactation. We postulated that the nicotine from SS could cause similar effects in the developing rat. Consistent with the work of Wang *et al.* (1984), we found that by 7–10 weeks of age rats exposed to SS both pre- and postnatally had a dramatic 22-fold greater number of pulmonary neuroendocrine cells compared to the rats exposed only to FA, while rats exposed to SS only pre- or only postnatally had no statistically significant increase in neuroendocrine cells. Consistent with this finding, Nylen *et al.* (1993) showed that nicotine stimulates growth of neuroendocrine cells in culture and Chen *et al.* (1987) suggested that human neonates of smoking mothers also appear to have larger and more distorted neuroepithelial bodies, although they were not able to establish this statistically.

We confirmed our identification of neuroendocrine cells by labeling cells in the same region of the airway epithelium with antibodies to serotonin, a component of the granules, and to neuron-specific enolase, a component of the cytoplasm (Fig. 5). However, we chose neuron-specific enolase as the marker for neuroendocrine cells in our morphometric analysis because neuron-specific enolase along with another cytoplasmic protein, protein gene product 9.5, are considered pan-neuroendocrine cell markers (Lauweryns and Seldeslagh, 1993; Gosney, 1993; McDowell *et al.*, 1994; Bousbaa *et al.*, 1994). As such, they are preferable to markers which reflect the state of granulation of neuroendocrine cells, such as chromogranin, serotonin, and calcitonin gene-related peptide which have been shown to change with ontogeny (McDowell *et al.*, 1994) and allergic sensitization (Bousbaa *et al.*, 1994). Future studies, however, should use markers for granular components to further explore the changes in neuroendocrine cells which may occur with SS exposure.

We were not able to address whether there was a causal relationship between the increase in neuroendocrine cells and the change in lung responsiveness in rats perinatally

exposed to SS. In humans, neuroendocrine cells contain at least two bronchoconstrictors, serotonin (Joad *et al.*, 1993) and bombesin (Lach *et al.*, 1993). Although bombesin is not a component of neuroendocrine cells in rats (Wang and Cutz, 1993), serotonin may have increased methacholine reactivity by acting as a functional agonist and by activating C-fibers (Coleridge *et al.*, 1989) which increases airway hyperresponsiveness to muscarinic agents (Hsiue *et al.*, 1992). In addition to increasing the number of neuroendocrine cells present, it is likely that SS enhanced the release of mediators from them. Nylen *et al.* (1993) showed that nicotinic agonists induced the release of immunoreactive calcitonin from cultured neuroendocrine cells. Tabassian *et al.* (1993) showed that hamsters exposed to mainstream cigarette smoke lost monomeric immunoreactive calcitonin from their lungs concomitant with an increase in monomeric immunoreactive calcitonin in their serum. Finally, Agauyao (1993) showed that a subset of mainstream smokers exhibits markedly increased bombesin-like peptides in their bronchoalveolar lavage and urine. Further studies are needed to fully understand the relationship between perinatal SS exposure, neuroendocrine cells, and lung function.

The concentration of SS used in this study (1 mg/m^3 total suspended particulates) was in the high range of that to which humans are exposed (U.S. Department of Health and Human Services, 1986). However, since the concentration of smoke increases inversely with the square of the distance from the source, infants and young children may be exposed to much higher concentrations than those usually reported for homes. Indeed, for the same number of cigarettes smoked per day, mothers' smoking results in higher urinary cotinine concentrations in her children than does fathers' smoking (Cook *et al.*, 1994), and the urinary cotinine levels in bottle-fed infants of smoking mothers is greater than that of adults exposed to ETS (Schulte-Hobein *et al.*, 1992).

In summary, prenatal followed by postnatal exposure to sidestream smoke reduced lung compliance, increased lung responsiveness to methacholine, and increased the number of pulmonary neuroendocrine cells and neuroepithelial bodies. These changes did not occur in rats exposed to sidestream smoke either only prenatally or only postnatally. Further studies are needed to determine if the increase in neuroendocrine cells is causally related to the changes in pulmonary function.

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