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Sidestream Smoke Effects on Lung Morphology and C-Fibers in Young Guinea Pigs[1]

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Children raised in homes with smokers have more frequent respiratory symptoms, decreased lung function, and increased airway reactivity. This study was designed to evaluate whether chronic exposure of developing guinea pigs to sidestream smoke (SS) would impair lung function and morphology and/or change the activity of a pulmonary defense mechanism, the local bronchopulmonary C-fiber system. Duncan–Hartley guinea pigs (n = 29) were exposed to filtered air (FA) or to SS for 6 h/day, 5 days/week from 8 to about 43 days of life. Their lungs were then studied in an isolated buffer perfused system where increasing doses of capsaicin (a C-fiber stimulant) or substance P (SP, a C-fiber neurotransmitter) were injected into the pulmonary artery. SS exposure significantly increased baseline dynamic compliance (C_{dyn}) by 17% but did not change baseline pulmonary resistance (R_L). SS exposure reduced the capsaicin-induced change in R_L and C_{dyn} but did not change lung responsiveness to SP. SS exposure did not change fixed lung volume, surface area, mean linear intercept length, or elastin deposition. We conclude that SS exposure to developing guinea pigs (1) increased lung compliance without affecting alveolar size or elastin deposition and (2) decreased the airway reactivity of the C-fiber system without changing reactivity to one of its neurotransmitters, SP. If humans are similarly affected, children raised in the homes of smokers may have a diminished pulmonary defense mechanism.

A number of studies have suggested that children living in homes with environmental tobacco smoke (ETS) exposure have increased respiratory problems. Children raised in homes with smokers have more 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). Pulmonary function evaluation of infants and children raised with ETS reveals that they have 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 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, ETS exposure is associated with more severe asthma and greater airway reactivity to histamine (Murray and Morrison, 1992), cold air (O'Connor *et al.*, 1987), and exercise (Frischer *et al.*, 1992).

Exposure to the mother's smoking rather than the father's smoking correlates best with pulmonary problems in children (Martinez *et al.*, 1988; Murray and Morrison, 1992; O'Connor *et al.*, 1987; Tager *et al.*, 1983; Weitzman *et al.*, 1990; Wright *et al.*, 1991). One explanation for this is that maternal smoking damages the lungs of the fetus as it develops *in utero*. Another explanation is that mothers are physically closer to their children while providing care, thus exposing them to a larger dose of ETS. Since mothers who smoke during pregnancy rarely quit after the birth of their child, the relative importance of these explanations is difficult to evaluate epidemiologically. Thus, animal studies are greatly needed which clearly define if *in utero* and/or postnatal exposure to smoke is responsible for the pulmonary changes.

One possible mechanism by which ETS may cause respiratory symptoms is by stimulating C-fiber endings in the lungs and airways. When bronchopulmonary C-fibers are activated, the nerve impulse travels to the CNS resulting in rapid shallow breathing and a cholinergic bronchoconstriction. The nerve impulse also causes a local release of tachykinins: (1) Substance P (SP) which interacts with NK_1 receptors to cause mucus secretion, airway microvascular leak, and (in guinea pigs) bronchoconstriction; and (2) neurokinin A (NKA) which interacts with NK_2 receptors to

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cause bronchoconstriction (Barnes, 1991). This C-fiber system is felt to contribute to the "airway defense response" (Coleridge *et al.*, 1989). C-fibers are known to be stimulated by mainstream cigarette smoke (Delay-Goyet and Lundberg, 1991; Lee *et al.*, 1989) and by components of ETS such as nicotine (Saria *et al.*, 1988), acrolein (Lee *et al.*, 1989), and oxidants (Coleridge *et al.*, 1993).

We hypothesize that if ETS chronically stimulates C-fibers causing cough, airway obstruction, mucus secretion, and airway hyperresponsiveness, the C-fibers may adaptively become downregulated, indirectly adding to the respiratory symptoms by interfering in the airway defense response and leaving the lung more susceptible to lung irritants and infection.

This study was designed to evaluate whether chronic postnatal sidestream smoke (SS) exposure to young guinea pigs obstructs airways, alters lung morphology, or downregulates the local bronchopulmonary C-fiber axon reflex. SS was used as a practical surrogate for ETS, which also contains exhaled mainstream smoke. The guinea pig was chosen since it has an active C-fiber system regulating airway tone. The isolated buffer-perfused lung was chosen to study lung mechanics and the C-fiber axon reflex because it allows for study of the lung C-fiber axon reflex without influences from the CNS, circulating cells, and systemic effects of C-fiber stimulants or tachykinins. The activity of the local C-fiber system was tested by measuring pulmonary resistance (R_L) and dynamic compliance (C_{dyn}) while administering increasing doses of capsaicin, the pungent ingredient in hot pepper, which is known to stimulate C-fibers. The potential location(s) of the alteration in the C-fiber system was tested by administering increasing doses of SP, one of the C-fiber neurotransmitters.

METHODS

General protocol. Male Duncan-Hartley guinea pigs (n = 29) were exposed to (1) filtered air (FA) or (2) SS for 6 hr/day, 5 days/week from age 8 days to age 37–48 days of life. They were fed guinea pig chow (#5025, Purina Mills, Inc., St. Louis, MO) and water *ad lib* including during the exposures. Guinea pigs were housed in polycarbonate cages (69 × 69-cm cross sectional area) with a wire lid and autoclaved wood carvings for bedding. They were housed 2 per cage until they weighed 350 g, and then they were housed individually. The cages were placed inside the exposure chamber around the clock. At the end of the exposure period, their isolated lungs were studied for baseline pulmonary mechanics (C_{dyn}, R_L, n = 14–15 each group). Increasing doses of either capsaicin (n = 6 FA, n = 8 SS) or SP (n = 8 FA, n = 7 SS) were then injected into the pulmonary artery. Some of the lungs which received capsaicin (n = 4 FA and SS) were then fixed for morphologic examination. The age at evaluation did not differ by exposure condition (42.9 ± 0.6 days old for the FA group and 43.1 ± 0.6 days old for the SS group, mean ± SEM, p = 0.85, t test).

All methods and procedures used complied with the Animal Welfare Act and the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. All experiments were approved by the UC Davis Committee on Animal Use and Care.

Generation of SS exposure atmosphere. The exposure system and monitoring methods have been previously described (Teague *et al.*, 1994). Briefly, dilute 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 one puff (35 ml, 2-sec duration) per minute. The mainstream smoke was collected on a filter and discarded. 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. The exposure chamber was characterized by a relative humidity of 38.2 ± 10.5%, temperature of 24.2 ± 1.8°C, respirable suspended particulate (RSP) concentration of 1.01 ± 0.02 mg/m^3, carbon monoxide concentration of 5.6 ± 0.7 ppm, and nicotine concentration of 586 ± 106 μg/m^3 (mean ± SD). Relative humidity, temperature, RSP, 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 was sampled continuously. Nicotine was sampled for 15 min twice during each 6-hr exposure period. RSP (using the peizobalance technique) was sampled for 30 min out of every hour.

Isolated perfused lung system. As we have done previously, we studied the lungs in an isolated perfused system to separate them from the effects of circulating blood components and central neural control (Pino *et al.*, 1992; Joad *et al.*, 1993). Guinea pigs were anesthetized with 65 mg/kg pentobarbital IP. The trachea was canulated and the guinea pig was ventilated with room air at a rate of 60 breaths/min and a tidal volume (V_T) of 3 ml. The chest was opened and 100 units of heparin and 400 μg/kg isoproterenol were 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 cannula was placed into the main pulmonary artery. The left ventricle was incised and the lungs were washed free of blood with a warmed (37°C) Krebs-Henseleit bicarbonate buffer (119 mM NaCl, 4.7 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 21 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.5% albumin, 0.1% glucose, pH 7.35–7.40). After the left atrium was cannulated, the heart, lung, and trachea were dissected from the animal and suspended by the trachea in a water-saturated chamber.

Warmed (37°C), humidified gas (95% air and 5% CO_2) was administered in 3 ml V_T breaths at a rate of 60 breaths/min. The lung was hyperinflated with air at 26 cm H_2O hydrostatic pressure for 10 sec at 15-min intervals during the 60-min stabilization period to prevent and reverse atelectasis. A differential pressure transducer (Validyne, 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 (Validyne) into a Modular Instruments Data Acquisition System (Malvern, PA) where R_L and C_{DYN} (method of Amdur and Mead (1958)) and V_T 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 Krebs-Henseleit 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 7.20 and 7.40 by the addition of $NaHCO_3$ if needed.

Lung reactivity to capsaicin and SP. Lung reactivity to capsaicin and SP was measured by administering increasing doses of drug in 100-μl bolus volumes every 45 sec into a port in the pulmonary artery catheter about 70 cm from the heart and measuring the peak change in R_L and C_{dyn}. The doses of capsaicin ranged from $10^{-9.5}$ to $10^{-8.125}$ mol. The doses of SP ranged from $10^{-11.5}$ to $10^{-8.5}$ mol.

Morphology. Lungs examined for histology were from the group of lungs which received capsaicin (four from the FA- and four from the SS-exposed groups). After the final dose of capsaicin was administered, the transpulmonary pressure was held at 30 cm H_2O for 10 sec to reverse atelectasis and then decreased to 12 cm H_2O. The perfusate was changed to 440 mOsm Karnovsky's fixative at room temperature and the perfusion was

continued for 15 min. After the perfusion, the trachea was tied off to maintain pressure of about 12 cm H_2O and the lung was placed in 440 mOsm Karnovsky's fixitive, covered with moist gauze, and stored at 4°C.

The fixed lung volume for each animal was determined by volume displacement (Scherle, 1970). The left lung lobe was cut into serial 2-mm-thick transverse slices. Using the Cavalieri method (Bolender *et al.*, 1993), two slices were selected, one cranial to and one caudal to the hilar level of the left lung, to ensure uniform sampling of parenchymal tissues. These slices were cut into 3 × 6 × 2-mm blocks. From these tissue blocks, four were randomly selected for embedment. The blocks were dehydrated in a graded series of ethanol and embedded in glycolmethacrylate (GMA). Sections 1.5 μm thick were cut using a JB4 Microtome from each of the four tissue blocks per animal and stained with toluidine blue. The distribution of collagen and elastin within sites sampled in the left lung was examined using GMA sections stained with suprasirius blue (Castro, 1988) and Miller's elastin (Castro, 1989), respectively.

From the sections taken from the four tissue blocks, total alveolar surface area and mean linear intercept length of the alveolar airspace were determined. From each section, five nonoverlapping fields were viewed with an optical microscope using a 10× objective and a 21-line test lattice graticule in the eyepiece ocular. Selection of these fields was done by using a random stratified selection scheme (Pinkerton and Crapo, 1985). The number of intercepts of the test lines with the air–tissue interface was counted for each field. A minimum of 1000 intercepts were counted per animal. The alveolar surface density (S_v) was calculated using the formula

$$S_v = 2I_L,$$

where I_L is the number of intersections per test line length. The total alveolar surface area was the product of S_v, fixed lung volume, and the volume fraction of the lung parenchyma. The parenchymal volume fraction used was 0.81 based on the studies of Forrest and Weibel (1975) and Gehr *et al.* (1981). Mean linear intercept length of the alveolar airspace is a derivation of the above equation in which the total test line length is divided by the number of intercepts made with the air–tissue interface of the alveoli (Weibel, 1979).

Statistical evaluation. The effects of SS on C_{dyn}, R_L, fixed lung volume, total alveolar surface area, specific alveolar surface area, and mean linear intercept were evaluated using a two-tailed t test. The effect of SS on the dose–response curves to capsaicin and SP were evaluated with a one-way multivariate repeated measures ANOVA (SAS/STAT, SAS Institute). Data were log transformed when variances differed by more than threefold. A type I error less than or equal to 0.05 was considered significant.

RESULTS

Baseline C_{dyn} of SS-exposed guinea pigs was 17% above that in FA-exposed animals (1.11 ± 0.06 vs 0.95 ± 0.05 ml/cm H_2O/kg body wt for the SS- and FA-exposed groups, respectively, mean ± SEM, $p = 0.05$). Baseline R_L was not different in the SS- compared with the FA-exposed animals (0.21 ± 0.01 vs 0.22 ± 0.01 cm H_2O/ml/sec for SS- and FA-exposed groups, respectively, mean ± SEM, $p = 0.61$).

In both SS- and FA-exposed guinea pig lungs, capsaicin (the C-fiber stimulant) and SP (one of the C-fiber neurotransmitters) increased R_L and decreased C_{dyn} ($p < 0.001$ for both dose effects, Figs. 1 and 2). However, capsaicin caused less of an increase in R_L in the lungs from animals which had been chronically exposed to SS (Fig. 1). At the final dose of $10^{-8.125}$ mol capsaicin, the R_L in the SS-exposed lungs was only 40% of that in the FA-exposed lungs. Similarly, SS exposure reduced the capsaicin-induced decrease in C_{dyn} (Fig. 1). In contrast, SS- and FA-exposed guinea pig lungs were equally responsive to SP (Fig. 2).

Exposure to SS did not change lung morphology (Fig. 3), collagen or elastin deposition, fixed lung volume, specific surface area or total surface area, or mean linear intercept length of alveolar airspaces (Table 1). Prominent bands of collagen were present around the airways and blood vessels of the lungs. Elastin distribution was similar to that of collagen but the volume of elastin compared with that of collagen was less. Elastin was more evident within the smooth muscle layers of blood vessels compared to the connective tissues of airways. Collagen and elastin within the lung parenchyma was scant with fine, thin wisps of fibrillar material most evident in the septal tips of alveoli lining the ducts. No obvious differences in the relative amounts or distribution of collagen and elastin were noted between exposed and control animals.

DISCUSSION

We conclude that SS exposure to developing guinea pigs (1) increased lung compliance without affecting alveolar size or elastin deposition and (2) decreased the airway reactivity of the C-fiber system without changing reactivity to one of its neurotransmitters, SP.

The male guinea pigs in this study were exposed to SS from 1 week of life at which time they weighed about 140 g to 6 weeks of life at which time they weighed about 400 g. Guinea pigs have been known to live to 8 years of age and growth of male guinea pigs is not complete until 9 months of age at which time they weigh about 1000 g. Their age of puberty is 5–10 weeks (UC Davis School of Veterinary Medicine, 1990). Thus, the developmental stage of these guinea pigs would be similar to human childhood.

Our most significant finding is that SS exposure diminished lung responses to a stimulant of C-fibers, capsaicin, indicating a downregulation of the local C-fiber reflex response.

Chronic mainstream smoke exposure may also lead to decreased responsiveness of the C-fiber system. Swanny *et al.* (1993) reported that rats exposed to mainstream smoke for 4–8 weeks developed a shorter apnea and diminished tachypneic response to acute inhalation of mainstream smoke. Since apnea and rapid shallow breathing are classic C-fiber responses, it is likely that C-fibers or their central processing were downregulated. C-fibers have been shown to become tachyphylactic to other stimulants such as capsaicin (Fujimura *et al.*, 1993; Hua and Yaksh, 1992) and phenyldiguanide (Bonham and Joad, 1991). On the other hand, Karlsson *et al.* (1991) reported that adult guinea pigs exposed to mainstream smoke for 2 hr/day for 2 weeks

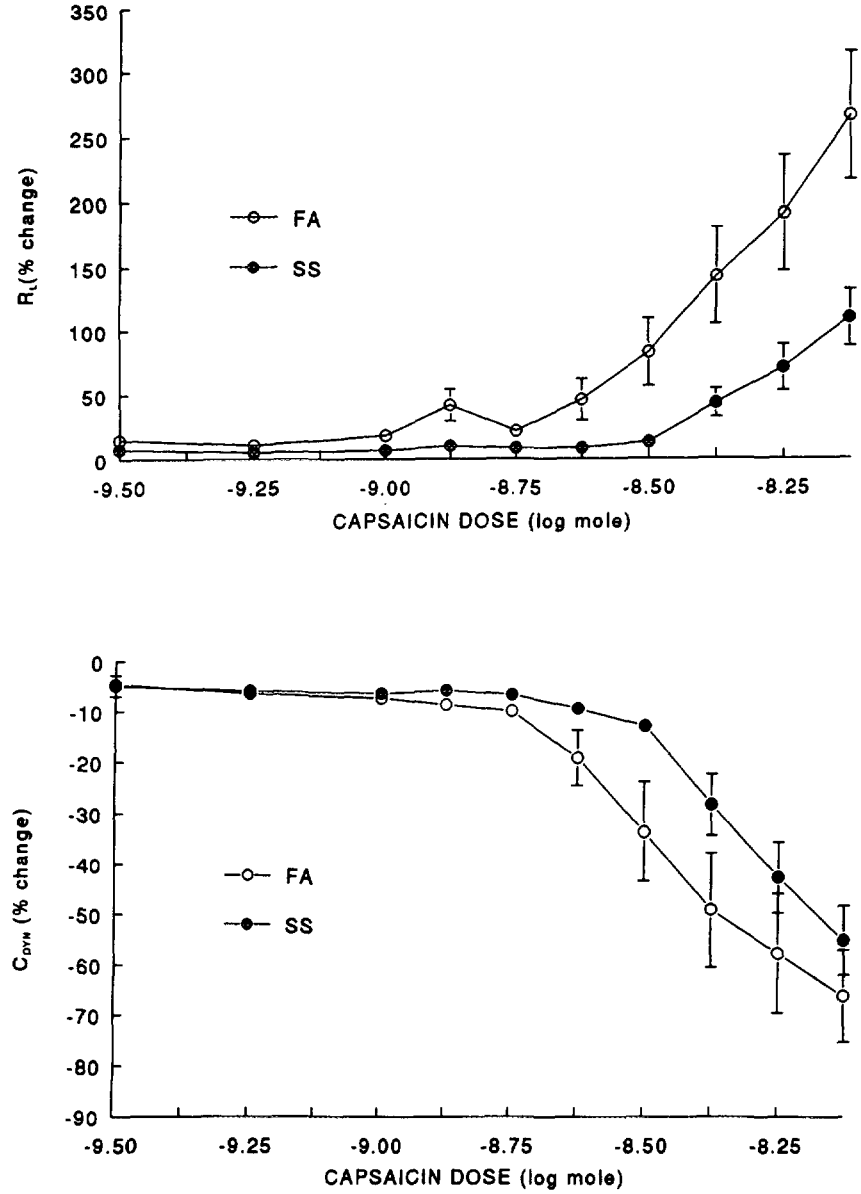

FIG. 1. Capsaicin-induced changes in R_L and C_{dyn} in isolated lungs from guinea pigs exposed to filtered air (FA, open circles) or sidestream smoke (SS, solid circles) from Day 8 of life to Day 37–48 of life. (Top) Capsaicin injected into the pulmonary artery increased R_L ($p = 0.001$). However, the increase in R_L was significantly diminished by SS exposure ($p = 0.02$). (Bottom) Capsaicin injected into the pulmonary artery decreased C_{dyn} ($p = 0.0001$). The decrease in C_{dyn} was also significantly diminished by SS exposure ($p = 0.04$). Statistics one-way multivariate repeated measures ANOVA on the log-transformed data.

developed increased rather than decreased cough and C-fiber neurotransmitter release when challenged with capsaicin.

Theoretically, the C-fiber axon reflex could be downregulated by (1) preventing activation of the axons, propagation of the impulse along the axon, and/or release of tachykinins from the axon; (2) enhancing metabolism of tachykinins; or (3) decreasing the affinity or number of NK_1 (SP) or NK_2 (NKA) tachykinin receptors and/or responsiveness of the end organ. We are able to begin to determine where in the reflex SS exposure may have caused its effects by comparing the lung reactivity to capsaicin with that to SP.

SP introduced via the vasculature is metabolized by neutral endopeptidase which also metabolizes NKA and by angiotensin-converting enzyme which only metabolizes SP (Shore *et al.*, 1992). Since airway reactivity to SP was not changed by chronic SS exposure, it is unlikely that either enzyme was affected. In contrast, acute exposure to mainstream smoke has been shown to decrease neutral endopeptidase activity (Dusser *et al.*, 1989). However, acute expo-

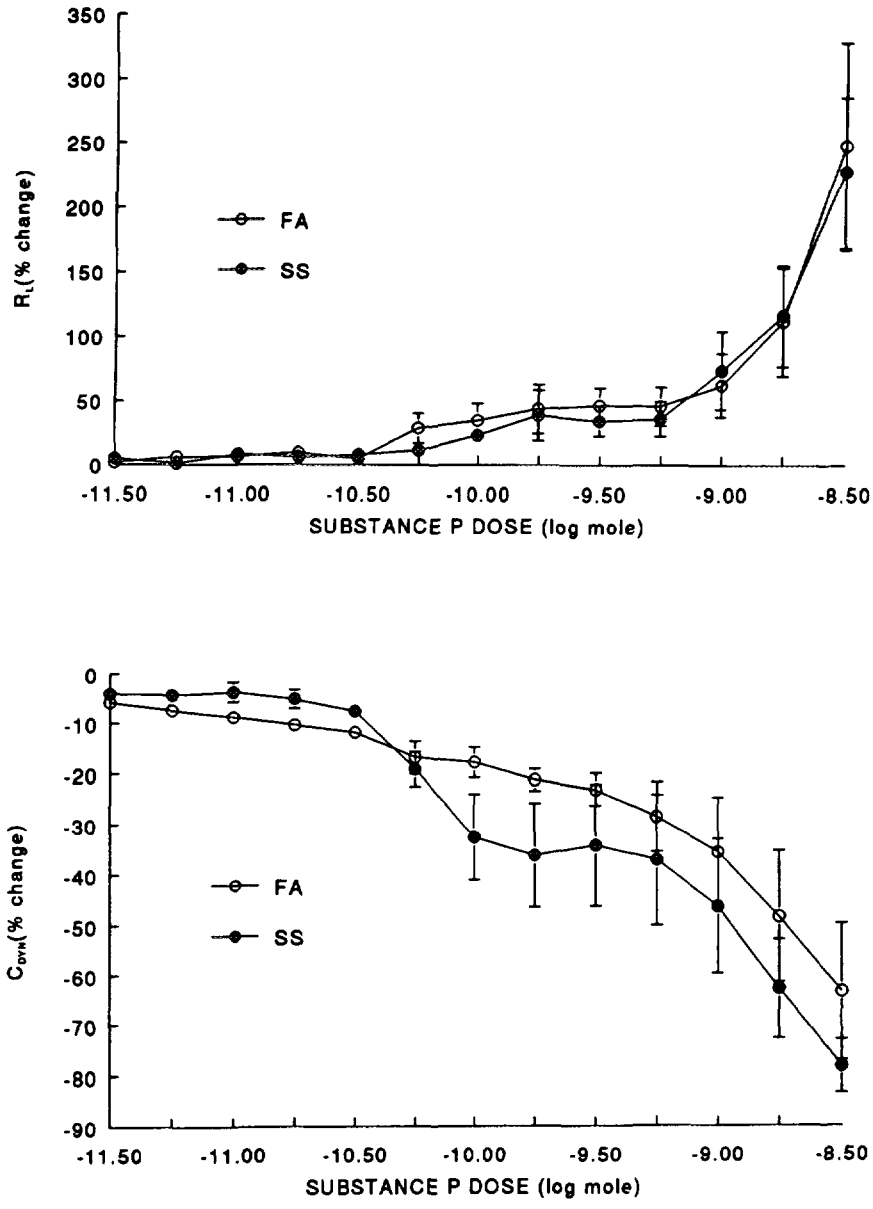

FIG. 2. SP-induced changes in R_L and C_{dyn} in isolated lungs from guinea pigs exposed to filtered air (FA, open circles) or sidestream smoke (SS, solid circles) from Day 8 to Day 37–48 of life. (Top) SP injected into the pulmonary artery increased R_L ($p = 0.001$). The difference between FA-exposed lungs and SS-exposed lungs was not statistically significant ($p = 0.66$, dose × exposure interaction). (Bottom) SP injected into the pulmonary artery decreased C_{dyn} ($p = 0.0001$). The difference between FA-exposed lungs and SS-exposed lungs was not statistically significant ($p = 0.50$ dose × exposure interaction). Statistics one-way multivariate repeated measures ANOVA on the log-transformed data.

sure to mainstream smoke is probably very different from chronic exposure to SS smoke.

Although the NK_1 receptor system can be downregulated by chronic exposure to agonists (Mussap *et al.*, 1993), the NK_1 receptors were apparently unaltered by SS exposure, since reactivity to SP was unchanged.

It is therefore likely that some aspect of the C-fiber axon itself or the NK_2 receptor system was downregulated.

Baseline R_L was not changed by SS exposure. SS exposure, however, increased C_{dyn} by 17%. This was a very modest, but statistically significant effect. We evaluated morphologically and morphometrically whether SS increased compliance by causing loss of alveolar tissue, by decreasing elastic fibers, or by enhancing lung growth. We found that SS exposure did not cause measurable loss of alveolar tissue, since the surface area and mean linear intercept length were not different from control lungs (Table 1). Also, SS exposure did not appear to reduce elastin or collagen. Finally, SS exposure did not appear to increase lung growth since fixed lung volume and total surface area were not

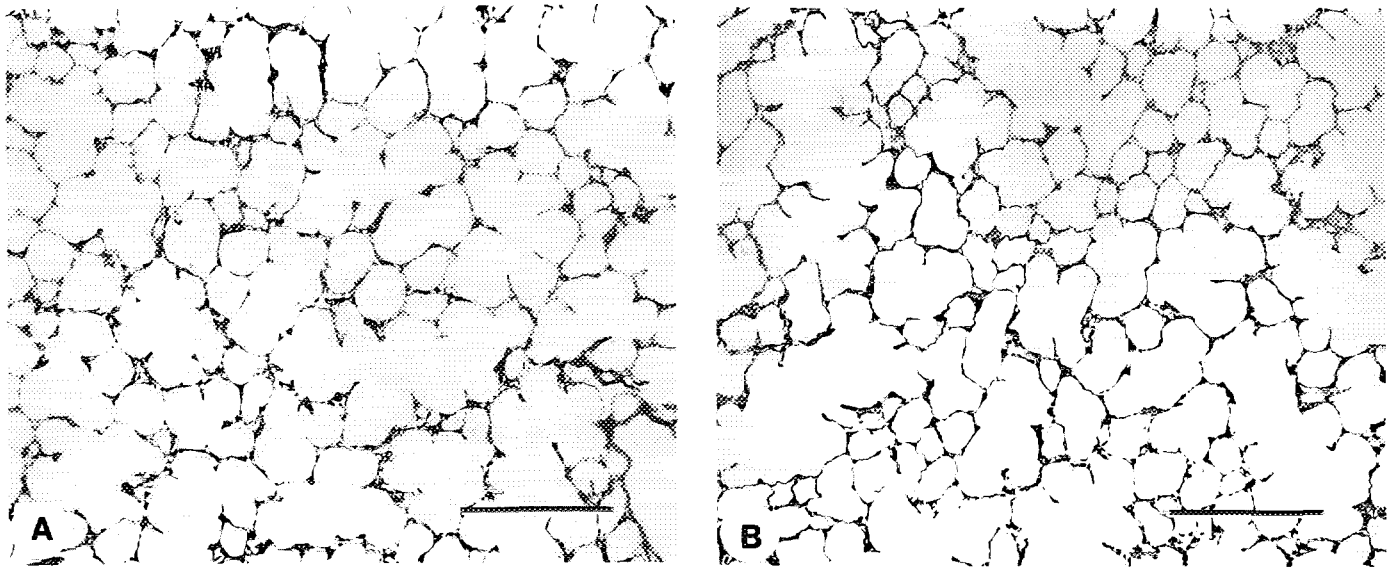

FIG. 3. Light micrographs of the lung parenchyma from guinea pigs exposed to filtered air (A) or sidestream smoke (B) from Day 8 to Day 37–48 of life. Airspace size and alveolar tissue wall thickness were similar in these lungs fixed by vascular perfusion. Scale bar is 250 μm.

affected. Thus, the mechanism of the small increase in C_{dyn} remains to be determined.

The SS exposure concentration used in this study (1 mg/m^3 RSP) was high, in the range of those reported for smoky bars (0.085 to 1.32 mg/m^3 RSP) rather than those usually reported for homes (0.036–0.70 mg/m^3 RSP) (U.S. Department of Health and Human Service, 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 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 fathers' smoking (Cook *et al.*, 1994), and the urinary cotinine levels in bottle-fed infants of smoking mothers are greater than that of adults exposed to ETS (Schulte-Hobein *et al.*, 1992). Although these findings could also be explained by altered nicotine metabolism in infants compared with adults, or greater absence of the noncaretaker from the home, they support the possibility that children receive more exposure to ETS than usually reported.

In summary, we have shown that chronic exposure of the developing guinea pig to SS results in an increase in lung compliance and a downregulation of the local C-fiber system probably involving the nerve itself or the NK_2 receptor system. The downregulation of the local C-fiber system may result in loss of an important defense response. Recent *in vivo* studies suggest that when the C-fiber responses are reduced, agents such as endotoxin (Long *et al.*, 1993), SO_2 (Long and Shore, 1993), and ozone (Sterner-Kock *et al.*, 1993) cause more inflammation. Thus, greater access of pollutants and viruses to the deep lung might explain some of the respiratory symptoms and the increase in respiratory infections (Wright *et al.*, 1991; Forastiere *et al.*, 1992; Ekwo *et al.*, 1983) reported in children of smokers.

TABLE 1
Effect of Sidestream Smoke Exposure on Alveolar Lung Morphometry[a]

	FA	SS	*p*
Fixed lung volume (ml)	15.2 ± 0.4	16.5 ± 0.6	0.13
Total surface area (cm^2)	4820 ± 242	5304 ± 265	0.23
Specific surface area (cm^2/cm^3)	256 ± 8	261 ± 17	0.82
Mean linear intercept length (μm)	104 ± 4	102 ± 6	0.82

[a] Lungs from guinea pigs exposed to filtered air (FA) or sidestream smoke (SS) from Day 8 of life to Day 37–48 of life and evaluated morphometrically (see text). Values are mean ± SEM, *p* value by *t* test.

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