Cigarette Smoke Induces Bronchoconstrictor Hyperresponsiveness to Substance P and Inactivates Airway Neutral Endopeptidase in the Guinea Pig

Possible Role of Free Radicals

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Abstract

We examined the effects of acute exposure to cigarette smoke on the airway responses to substance P in anesthetized guinea pigs and on the activity of airway neutral endopeptidase (NEP). After exposure to air or to cigarette smoke we measured the change in total pulmonary resistance (R_L) induced by increasing concentrations of aerosolized substance P in the absence or presence of the NEP inhibitor phosphoramidon. In the absence of phosphoramidon the bronchoconstrictor responses to substance P were greater in cigarette smoke–exposed guinea pigs than in air-exposed animals. Phosphoramidon did not further potentiate the responses to substance P in smoke-exposed guinea pigs, whereas it did so in air-exposed animals. In the presence of phosphoramidon, bronchoconstrictor responses to substance P in animals exposed to air or to cigarette smoke were not different. Aerosols of SOD delivered before cigarette smoke exposures dramatically reduced smoke-induced hyperresponsiveness to substance P, whereas heat-inactivated SOD had no effect on smoke-induced hyperresponsiveness to substance P. Cigarette smoke solution inhibited NEP activity from tracheal homogenate in a concentration-dependent fashion, an inhibitory effect that was mostly due to the gas phase of the smoke, but not to nicotine. The mild chemical oxidant *N*-chlorosuccinimide mimicked the concentration-dependent inhibitory effect of smoke solution on airway NEP activity. We conclude that cigarette smoke causes enhanced airway responsiveness to substance P in vivo by inactivating airway NEP. We suggest that cigarette smoke–induced inhibition of airway NEP is due to effects of free radicals.

Introduction

Epidemiological and clinical studies have clearly established the role of tobacco smoke in the genesis of airway inflammation and bronchial hyperresponsiveness. Cigarette smoke is associated with a high risk of developing chronic bronchitis (1, 2). Furthermore, cigarette smoke causes an increase in airway resistance in normal volunteers (3) and bronchial hyperresponsiveness in chronic active or passive smokers (4, 5), and exacerbates symptoms of asthma in children whose parents are smokers (6, 7). Therefore, knowledge of the mechanisms of cigarette smoke–induced inflammation in the airways is important in relating smoking to the pathophysiology of disease.

Tachykinins, among which is substance P, are neuropeptides that are localized in the C fiber nerve endings in the airways (8) that produce a series of effects including neutrophil adhesion and chemotaxis (9), increased vascular permeability (10), cough (11), gland secretion (12), and smooth muscle contraction (13–16). These effects are termed neurogenic inflammation. Because cigarette smoke produces neurogenic inflammation in the airways by releasing endogenous tachykinins (10, 17, 18), these neuropeptides are likely to play an important role in cigarette smoke–induced airway inflammation.

Neutral endopeptidase (NEP),[1] also termed enkephalinase (EC 3.4.24.11), is a cell membrane–bound peptidase that is present in the lungs and airways of many species, including humans and guinea pigs (13, 14, 19–21). Recently, several studies have demonstrated that NEP is an important modulator of tachykinin-induced stimulation of cholinergic neurotransmission (15), smooth muscle contraction and bronchoconstriction (13–15, 22–24), mucus secretion (25), tachykinin-induced increase in capillary permeability (26), and cough (11). Because cigarette smoke is known to inactivate several enzymes (27–30), we hypothesized that it might also inactivate airway NEP and thereby magnify tachykinin-induced effects in the airways.

Therefore, the aim of this study was to examine whether cigarette smoke induces an increase in the bronchoconstrictor response to substance P, and if so, whether this was due to inactivation of airway NEP.

Methods

Animals

Pathogen-free male Hartley outbred guinea pigs (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing 500–600 g were used in this study.

In vivo studies

Measurement of total pulmonary resistance (R_L). Animals were anesthetized using sodium pentobarbital (55 mg/kg i.p.; Abbott Laboratories, North Chicago, IL) and then ventilated artificially with a tracheal

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1. *Abbreviations used in this paper:* NEP, neutral endopeptidase; R_L, total pulmonary resistance.

cannula, using a constant-volume ventilator (model 680; Harvard Apparatus Co., Inc., S. Natick, MA) at a frequency of 90 breaths/min. The tidal volume was adjusted so that arterial blood gases (mean±1 SE) were 75±1.4 mmHg for Pa_{O_2}, 93.8±0.4% for Sa_{O_2}, and 43±1 mmHg for Pa_{CO_2}; pH was between 7.3 and 7.4. Airflow was monitored continuously using a pneumotachograph (Fleisch No. 000; oem Medical Inc., Richmond, VA) connected to a differential pressure transducer (model DP45; Validyne Engineering Corp., Northridge, CA). The tidal volume was obtained by electrical integration of airflow (model FV156; Validyne Engineering Corp.). A fluid-filled polyethylene catheter was introduced into the esophagus to measure the esophageal pressure as an approximation of pleural pressure. Intratracheal pressure was measured using a polyethylene catheter inserted into a short tube connecting the tracheal cannula to the pneumotachograph. The transpulmonary pressure (defined as the pressure difference between the intratracheal and the esophageal pressures) was measured with a differential pressure transducer (model 268 B; Sanborn Co., Waltham, MA). Output signals representing tidal volume, transpulmonary pressure, and airflow were amplified using amplifiers (model CD19; Validyne Engineering Corp.) and recorded on a polygraph recorder (model 1508C; Honeywell, Inc., Denver, CO). R_L was calculated as previously described (13). Aerosols of drugs (mass median aerodynamic diameter = 7.6 μm; geometric SD = 2.6; output, 350 μl/min) were generated by an ultrasonic nebulizer (model 25; DeVilbiss Co., Somerset, PA) and delivered to the airways by the ventilator.

Cigarette smoke exposure. Cigarette smoke was collected in a polypropylene syringe at a rate of 1 puff/min (each puff, 2-s duration, 35 ml; total of 10 puffs/cigarette) to a final butt length of 23 mm. The smoke was then slowly expelled into the inspiratory circuit of the ventilator. Thus, each puff of cigarette smoke was diluted in air by ~ 1/5 and was delivered to the animal during 30-s intervals followed by 30 s with air ventilation.

Effect of acute exposure to cigarette smoke on substance P–induced bronchoconstriction. To determine whether cigarette smoke exposure induces hyperresponsiveness to substance P we studied two groups of animals. One group of guinea pigs was studied after being exposed to the smoke of either one or two cigarettes delivered 15 min apart. In the other group animals had no cigarette smoke exposure and were ventilated with air only. Concentration-response curves measuring the effects of aerosol administration of increasing concentrations of substance P (ranging from 10^{-7} to 10^{-4} M; seven breaths at each concentration) on R_L were performed in both air- and cigarette smoke–exposed animals 15 min after smoke or air exposure.

Effect of exposure to capsaicin on substance P–induced bronchoconstrictor responses to substance P. To determine whether hyperresponsiveness to substance P was due to the irritant effect of smoke with subsequent neural release of tachykinins, we studied the effect of capsaicin (another irritant that releases tachykinins from sensory nerves) on the bronchoconstrictor response to substance P. Various doses of aerosolized capsaicin (10^{-4} M) ranging from 16 to 22 breaths were administered to obtain a reversible increase in R_L at least as large as that observed after cigarette smoke exposures. Concentration-response curves to substance P (ranging from 10^{-7} to 10^{-4} M; seven breaths at each concentration) were performed 15 min after capsaicin exposure, a time by which R_L had returned to baseline.

Effect of phosphoramidon on smoke-induced responses to substance P. To determine the effects of the NEP inhibitor phosphoramidon on cigarette-induced hyperresponsiveness to substance P, phosphoramidon (2 mg/kg) was administered intravascularly in a group of animals 5 min after they had received the smoke from two cigarettes and in a group of air-exposed animals. Concentration-response curves measuring the effects of aerosol administration of increasing concentrations of substance P (ranging from 10^{-7} to 10^{-4} M; seven breaths at each concentration) on R_L were determined in both groups (air- and cigarette-exposed) of guinea pigs 10 min after the administration of phosphoramidon.

Effect of SOD on the response to substance P. To assess whether SOD reduces the effects of cigarette smoke on the response to substance P, animals were exposed to the smoke from two cigarettes delivered in the same manner as described previously, except that each cigarette smoke exposure was preceded (by 3 min) by the administration of an aerosol of SOD (90 breaths). Different concentrations of SOD (7,500, 15,000, or 30,000 U/ml) were tested. Because boiling SOD is known to inactivate the enzyme (31), we also exposed a group of animals to aerosolized SOD at a concentration of 30,000 U/ml that was maintained in boiling water for 20 min before its administration. After a waiting period of 15 min after cigarette smoke exposure, concentration-response curves measuring the effects of aerosol administration of increasing concentrations of substance P (ranging from 10^{-7} to 10^{-4} M; seven breaths at each concentration) on R_L were determined and compared with the responses obtained in the absence of SOD.

Each successive concentration of substance P was delivered after R_L had reached its maximum effect. Responses were evaluated as the maximum R_L values after each concentration of substance P and were expressed as the percent of the response to an aerosol of 0.9% NaCl solution (seven breaths) given before the administration of substance P.

In vitro studies

Measurement of NEP-like activity. To determine the effects of cigarette smoke on airway NEP-like enzyme, guinea pigs were anesthetized with pentobarbital (55 mg/kg i.p.) and the trachea was removed. Tissues were stored at −70°C for later determination of NEP activity using the enkephalin degradation method of Llorens et al. (32). Briefly, the tissues were minced and homogenized in assay buffer containing 125 mM NaCl and 50 mM Hepes buffer, pH 7.4, and diluted as necessary. Duplicate samples of 50 μl of homogenates were incubated at 37°C for 40 min with ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin (20 nM; final volume, 100 μl) in the presence of the aminopeptidase inhibitor bestatin (10^{-6} M). Phosphoramidon (10^{-6} M) was added to parallel duplicate samples. Blanks were obtained by using assay buffer (50 μl) instead of tracheal homogenates under identical experimental conditions. Cleaved ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin was separated chromatographically from uncleaved substrate and counted in a scintillation counter. NEP-like activity was assessed by calculating the ratio of cleaved substrate to total ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin under conditions of initial velocity. Substrate degradation inhibited by 10^{-6} M phosphoramidon was considered specific for NEP-like activity.

Preparation of cigarette smoke solution. Water-soluble extract of cigarette smoke (gas phase + tar) was prepared as follows. Cigarette smoke was collected in a polypropylene syringe at a rate of 1 puff/min (each puff, 2-s duration, 35 ml; total of 10 puffs/cigarette) to a final butt length of 23 mm. Each puff of cigarette was bubbled slowly through the assay buffer solution (125 mM NaCl, 50 mM Hepes buffer; pH 7.4). The smoke solution was then titrated to pH 7.4. Smoke solution of the gas phase was obtained in the same manner except that the smoke was drawn through a filter pad (Cambridge Filter Corp., Syracuse, NY). The smoke solutions were kept on ice and used within 30 min of their preparation.

Effect of cigarette smoke solution on airway NEP-like activity. The cleavage of ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin by tracheal homogenates (50 μl) was determined after a preincubation for 15 min at room temperature with various concentrations of cigarette smoke solution (25 μl at each concentration) using either the gas phase or the combined gas phase and tar smoke solution.

Effects of nicotine and of the oxidant N-chlorosuccinimide on airway NEP-like activity. To determine whether nicotine by itself influenced the activity of airway NEP-like enzyme we measured the degradation of ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin by tracheal homogenates (50 μl) after preincubation for 15 min at room temperature with different concentrations of nicotine (25 μl).

To examine whether the chemical oxidant *N*-chlorosuccinimide inhibits airway NEP-like enzyme, we measured the cleavage of ([^{3}H]-Tyr,D-Ala^2)-leucine enkephalin by tracheal homogenates (50 μl) after a preincubation for 15 min at room temperature with various concentrations of *N*-chlorosuccinimide (25 μl at each concentration).

Nicotine and *N*-chlorosuccinimide were diluted with assay buffer and titrated at pH 7.4.

Reagents. We used research grade 3A1 cigarettes, which are low nicotine content cigarettes developed by the University of Kentucky Tobacco and Health Research. Each cigarette contained 31.1 mg tar and 0.27 mg nicotine.

Drugs and chemicals were obtained from the following sources: substance P, phosphoramidon, and bestatin (Peninsula Laboratories, Inc., Belmont, CA); SOD from bovine liver, nicotine (L-1-methyl-2-[3-pyridyl]pyrrolidine), and *N*-chlorosuccinimide (Sigma Chemical Co., St. Louis, MO); and ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin (CEA, Gif-sur-Yvette, France). All drugs for in vivo studies were prepared in 0.9% NaCl solution on the day of experiment.

Statistical analysis. Data are expressed as mean±1 SE. Two-way analysis of variance was used to compare mean R_L values of the effects of different doses of cigarette smoke, capsaicin, phosphoramidon, and SOD on the concentration-response curves to substance P. The effects of cigarette smoke, phosphoramidon, and SOD on baseline R_L were studied by a one- or two-way analysis of variance. Multiple comparisons among means were performed by the Neuman-Keuls test (33).

Results

In vivo studies

Effect of acute exposure to cigarette smoke on substance P–induced bronchoconstriction. In air-exposed animals substance P induced a very weak increase in R_L that was not statistically significant ($P > 0.09$; Fig. 1). An increase in R_L was observed during cigarette smoke exposure, but this increase was rapidly reversible. Thus, R_L returned to a stable baseline within the 15-min resting period after the smoke exposure; baseline R_L after the smoke exposure from one or two cigarettes was not different (0.17±0.02 vs. 0.16±0.02 cm $H_2O \times ml^{-1} \times s^{-1}$, respectively; $P > 0.3$). After exposure to cigarette smoke, substance P generated concentration-dependent increases in R_L ($P < 0.001$), and substance P–induced responses were greater than in air-exposed animals ($P < 0.001$). Cigarette smoke–induced hyperresponsiveness to substance P increased with the number of cigarettes to which the animals were exposed ($P < 0.001$; Fig. 1).

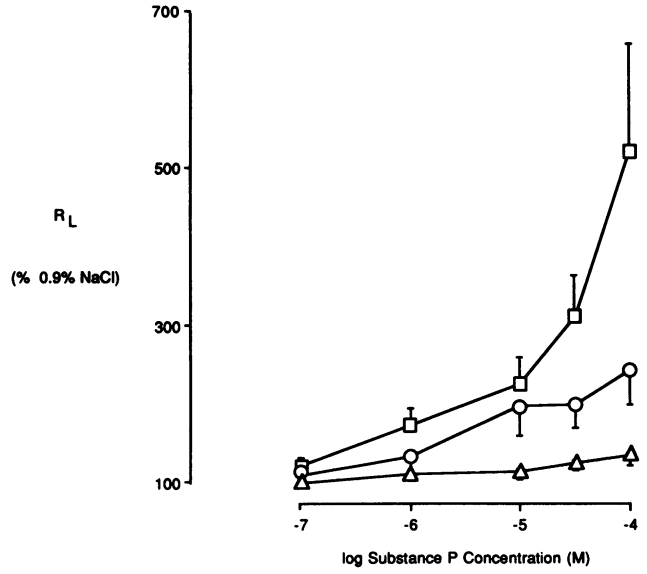

Figure 1. Concentration-response curves to aerosolized substance P (seven breaths) after exposure to air (*triangles*) or after acute exposure to the smoke from one (*circles*) or two (*squares*) cigarettes in anesthetized guinea pigs. Each curve represents data collected from five different animals. R_L is expressed (mean±1 SE) as the percent of the response to aerosolized 0.9% NaCl solution (seven breaths) given before the administration of substance P. After exposure to cigarette smoke substance P–induced responses were greater than in air-exposed animals ($P < 0.001$). Cigarette smoke–induced responses to substance P after exposure to two cigarettes was greater than after one cigarette ($P < 0.001$).

Effect of exposure to capsaicin on substance P–induced bronchoconstrictor responses to substance P. Exposure to aerosolized capsaicin produced a reversible increase in R_L (mean increase, 438% of baseline R_L, ranging from 300 to 617%) that was at least as large as that produced by cigarette smoke exposure (mean increase, 286% of baseline R_L, ranging from 139 to 529%). However, after capsaicin exposure aerosolized substance P had no significant effect on R_L at concentrations as high as 10^{-4} M ($n = 5$, $P > 0.3$).

Effect of phosphoramidon on cigarette smoke–induced responses to substance P. Administration of phosphoramidon did not change baseline R_L in either the air-exposed (0.10±0.05 vs. 0.11±0.01 cm $H_2O \times ml^{-1} \times s^{-1}$, with and without phosphoramidon, respectively; $P > 0.5$) or cigarette smoke–exposed animals (0.16±0.01 vs. 0.16±0.02 cm $H_2O \times ml^{-1} \times s^{-1}$, with and without phosphoramidon, respectively; $P > 0.5$). Phosphoramidon markedly potentiated the responses to substance P in air-exposed animals ($P < 0.001$), whereas in animals exposed to the smoke from two cigarettes concentration-response curves to substance P in the absence or presence of phosphoramidon were not statistically different ($P > 0.25$; Fig. 2). Thus, after phosphoramidon the concentration-response curves to substance P in animals exposed to air and to the smoke from two cigarettes were not different ($P > 0.5$; Fig. 2).

Effect of SOD on the response to substance P. Administration of SOD at different concentrations did not induce changes in baseline R_L ($P > 0.5$). However, in animals exposed to the smoke from two cigarettes substance P–induced responses were reduced by pretreatment with SOD ($P < 0.001$). The attenuation of smoke-induced hyperresponsiveness was greater with increasing concentrations of SOD from 7,500 to 30,000 U/ml ($P < 0.001$; Fig. 3). At a concentration of 60,000 U/ml SOD did not show a greater effect than at 30,000 U/ml (results not shown). When SOD (30,000 U/ml) was heat inactivated it no longer prevented the cigarette smoke–induced response. Thus, cigarette smoke–induced inhibition of substance P responses after delivery of heat-inactivated SOD was similar to the responses observed in the absence of pretreatment with SOD ($P > 0.3$; Fig. 3).

In vitro studies

Effect of cigarette smoke solution on airway NEP-like activity. Because the in vivo studies suggested that cigarette smoke potentiated substance P–induced bronchoconstriction by inhibiting NEP in airways, we studied the effect of cigarette smoke on airway NEP activity in vitro. Airway NEP activity was inhibited by cigarette smoke solutions in a concentration-dependent fashion ($P < 0.001$). The inhibition of airway NEP activity by smoke solution was accounted for mostly by gas phase constituents (see data on filtered cigarettes in Fig. 4). However, the smoke solution from nonfiltered cigarettes (tar + gas phase) was slightly but significantly more potent ($P < 0.002$) in inhibiting airway NEP activity (Fig. 4).

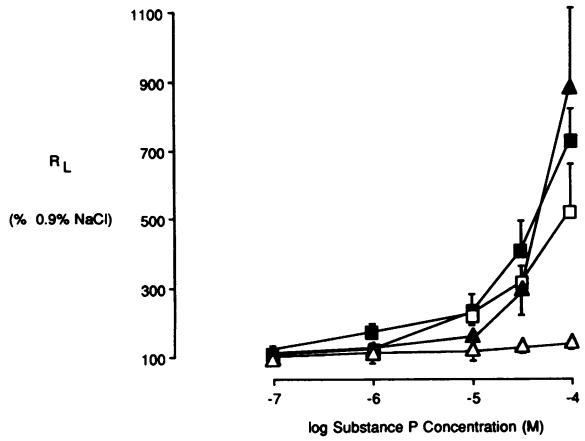

Figure 2. Comparison of bronchoconstrictor effects of aerosolized substance P (seven breaths) in anesthetized guinea pigs exposed to air only (*triangles*) and to smoke from two cigarettes (*squares*) in the absence (*open symbols*) or presence (*closed symbols*) of phosphoramidon (2 mg/kg intravascularly). Each curve represents data from five different animals. R_L is expressed (mean±1 SE) as the percent of the response to aerosolized 0.9% NaCl solution (seven breaths) given before the administration of substance P. There was no significant increase in R_L (bronchoconstriction) in response to substance P in air-exposed animals in the absence of phosphoramidon (*open triangles*). In the presence of phosphoramidon substance P caused a dose-related increase in R_L in air-exposed animals (*solid triangles*). In animals exposed to cigarette smoke alone (*open squares*) responses to substance P were significant and were not different from the responses of air-exposed animals given phosphoramidon. In cigarette smoke–exposed animals the presence of phosphoramidon (*closed squares*) did not increase bronchomotor responses further. Air-exposed animals given phosphoramidon and cigarette-exposed animals not given phosphormidon had similar responses.

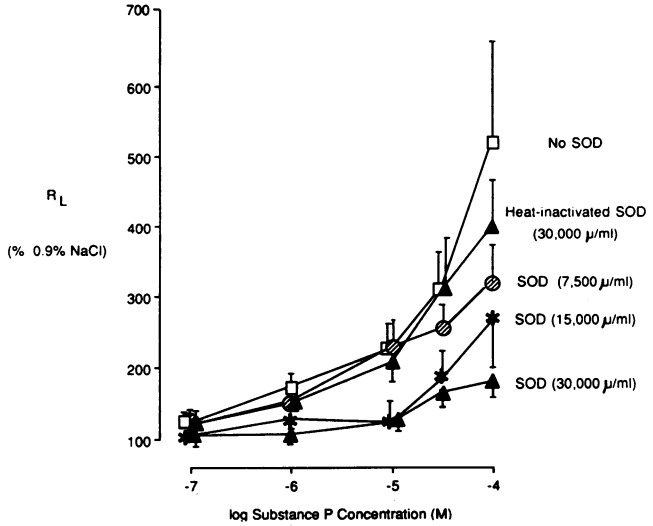

Figure 3. Concentration-response curves to aerosolized substance P (seven breaths) in anesthetized guinea pigs exposed to the smoke from two cigarettes, in the absence of or after pretreatment with aerosolized SOD (90 breaths before the exposure to each cigarette). Each curve represents data collected from five different animals. The curves show the responses observed in the absence (*open squares;* data are the same as in Fig. 1) and after the following concentrations of aerosolized nontreated SOD: 7,500 U/ml (*hatched circles*); 15,000 U/ml (*asterisks*); and 30,000 U/ml (*stippled triangles*); or after aerosol administration of heat-inactivated SOD at the concentration of 30,000 U/ml (*solid triangles*). R_L is expressed (mean±1 SE) as the percent of the response to aerosolized 0.9% NaCl solution (seven breaths) given before the administration of substance P. The attenuation of smoke-induced hyperresponsiveness was increased with increasing concentrations of SOD ($P < 0.001$) but was unaffected by heat-inactivated SOD ($P > 0.3$).

Effects of nicotine and the oxidant N-chlorosuccinimide on airway NEP-like activity. Nicotine at concentrations as high as 10^{-2} M did not decrease the cleavage of ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin (87.4±5.8% of the cleavage by NEP from tracheal homogenate alone; $n = 5$).

The fact that SOD inhibited cigarette smoke–induced potentiation of substance P–induced bronchoconstriction in vivo suggested that free radical generation from smoke was the causative agent. The studies with NEP inhibitors implicated NEP inhibition as the mechanism, and we hypothesized that the free radicals had their effect by inactivating NEP. Therefore, we examined the effect of the chemical oxidant *N*-chlorosuccinimide on NEP activity. As was the case for airway NEP when the tissues were exposed to smoke solution, *N*-chlorosuccinimide inhibited NEP activity from tracheal homogenate in a concentration-dependent fashion ($P < 0.001$; Fig. 5). The concentration of *N*-chlorosuccinimide that produced 50% inhibition of airway NEP activity (IC_{50}) was $3.2\pm0.6 \times 10^{-5}$ M ($n = 5$).

Discussion

Our study demonstrates that exposure to cigarette smoke leads to an enhanced bronchoconstrictor response to substance P, an effect that was observed as soon as 15 min after smoke exposure. The hyperresponsiveness to substance P was related to the amount of smoke exposure: the more cigarettes smoked,

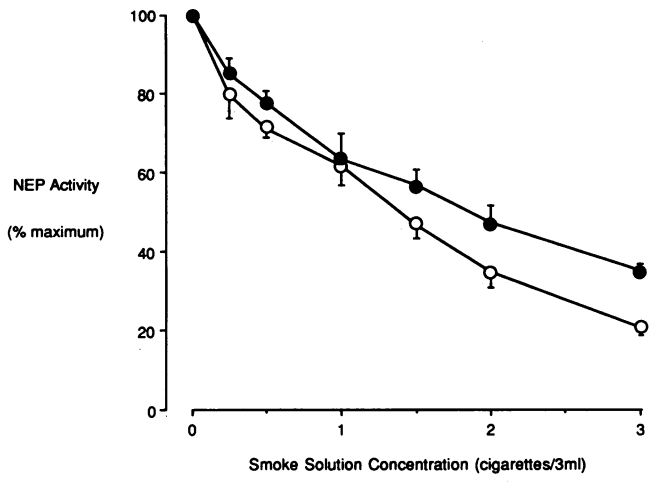

Figure 4. Effects of increasing concentrations of smoke solution obtained from nonfiltered (gas phase + tar; *open circles*) or filtered cigarettes (gas phase only; *closed circles*) on the activity of NEP in guinea pig tracheal homogenates. Each curve represents the data collected from five animals. NEP activity is expressed (mean±1 SE) as the percent of the cleavage (inhibitable by phosphoramidon) of ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin by tracheal homogenate in the absence of smoke solution. The inhibition of airway NEP by the smoke solution containing the tar + gas phase was slightly but significantly ($P < 0.002$) more potent than by the smoke solution containing the gas phase only.

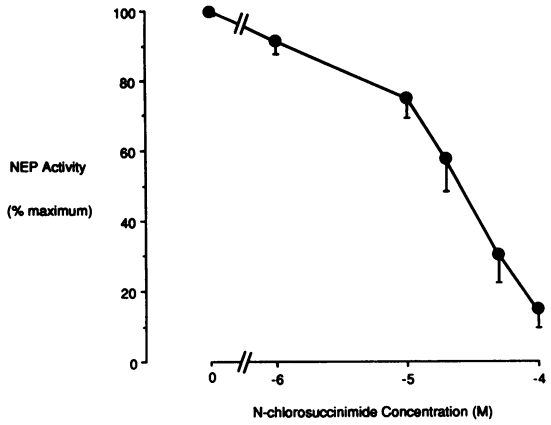

Figure 5. Effect of increasing concentrations of the oxidant *N*-chlorosuccinimide on the activity of NEP from guinea pig tracheal homogenates. Each curve represents the data collected from five animals. NEP is expressed (mean±1 SE) as the percent of the cleavage (inhibitable by phosphoramidon) of ([^{3}H]Tyr,D-Ala^2)-leucine enkephalin by tracheal homogenate in the absence of *N*-chlorosuccinimide. *N*-chlorosuccinimide inhibited airway NEP in a concentration-dependent fashion.

the greater the bronchoconstrictor responses to substance P. We attempted to study the mechanism by which cigarette smoke produces airway hyperresponsiveness to substance P. Exposure to another airway irritant, capsaicin, a drug known to release endogenous tachykinins from sensory nerves similar to cigarette smoke, did not produce subsequent potentiation of bronchoconstrictor effects of substance P. Therefore, a mechanism related to the release of endogenous tachykinins by an irritant of the airway mucosa cannot explain smoke-induced hyperresponsiveness to substance P.

From our evidence we suggest that a decrease in the enzymatic breakdown of substance P in airway tissue by NEP is the cause of the hyperresponsiveness. The hypothesis is based on the following information. Substance P can be inactivated by various enzymes including NEP, angiotensin-converting enzyme, serine proteases, aminopeptidases, and acetylcholinesterase (34, 35, and reviewed in references 15 and 22). However, previous studies have shown that NEP, an enzyme present in the airway tissues (14, 19), is responsible for the great majority of inactivation of tachykinins in the airways. Thus, NEP inhibitors increase markedly airway smooth muscle responses to tachykinins both in vivo and in vitro (13–15, 22, 23), whereas inhibitors of other proteases did not potentiate tachykinin-induced airway smooth muscle contraction (14, 22, 23).

Our in vivo studies suggest that a decrease in NEP activity after cigarette smoke is the cause of the augmented substance P responses. This is based on the findings that (*a*) pretreatment with phosphoramidon did not further potentiate the bronchoconstrictor responses to substance P in animals exposed to the smoke from two cigarettes, whereas phosphoramidon did increase the responses in air-exposed guinea pigs; and (*b*) in the presence of phosphoramidon, responses to substance P in both cigarette smoke– and air-exposed animals were not different. Phosphoramidon is a potent and selective inhibitor of NEP with a nanomolar affinity constant on purified NEP (36) as well as on NEP-like activity in guinea pig airway (13). Therefore, by preventing the degradation of tachykinins in the airways, phosphoramidon should potentiate substance P–induced effects, except when NEP-like activity has already been decreased so that it is unable to degrade tachykinins significantly.

Because our in vivo studies with inhaled cigarette smoke suggested that inhibition of NEP activity occurred during cigarette smoke exposure, we studied the effects of cigarette smoke solutions on recombinant NEP activity in vitro, recognizing that in vivo and in vitro conditions differed. In our experiments performed in vitro we found that gas phase cigarette smoke solution exerted the largest inhibitory effects on airway NEP activity, although the inactivation of the enzyme was slightly enhanced when the tar was associated with the gas phase. Our finding that cigarette smoke solution inhibits airway NEP activity in vitro indicates that smoke (mainly the gas phase) contains products capable of inactivating NEP. Therefore, these results are also compatible with our hypothesis that cigarette smoke can inactivate NEP.

The mechanism by which cigarette smoke inactivates NEP is not known. Inactivation of airway NEP is not due to nicotine, because it was unable to alter significantly the enzyme activity even at a concentration of 10^{-2} M, which corresponds to approximately six times the concentration of nicotine in the highest dose of cigarette smoke solution that we have tested (three cigarettes/3 ml). Our results suggest that the inactivation of airway NEP activity by cigarette smoke is due to free radicals. This conclusion is based on the finding that pretreatment with SOD dramatically reduced cigarette smoke–induced hyperresponsiveness to substance P in vivo, whereas heat-inactivated SOD was unable to prevent smoke-induced hyperresponsiveness to substance P. Cigarette smoke contains a large number of oxidizing free radicals both in the gas phase and in the tar (37). The protective effects of SOD suggest that superoxide radicals that are present in cigarette smoke (38) play a role in the smoke-induced inactivation of airway NEP. We cannot exclude the possibility that the mechanism of inactivation of NEP observed in vivo after cigarette smoke exposure and in vitro with smoke solutions was different. However, the possible role of free radicals in the inactivation of airway NEP by cigarette smoke in vivo is compatible with the effects of smoke solution on NEP activity because previous studies indicated that smoke solutions prepared in a similar manner are capable of inhibiting enzyme activities and generating oxidants for several hours (27–29).

Previous studies performed on purified NEP (39–41) have shown that this enzyme is inhibited by thiol and metal chelators such as EDTA and *o*-phenanthroline. Because our results suggested that oxidation (by free radicals) was a possible mechanism for NEP inactivation, we performed experiments in vitro using the chemical oxidant *N*-chlorosuccinimide to determine whether airway NEP could also be inactivated by oxidative processes. Our data show that airway NEP can be inactivated by oxidation. Under the conditions of our experiments *N*-chlorosuccinimide is known to be capable of oxidizing methionine as well as tryptophan and cysteine residues (42). Although the molecule of NEP has recently been sequenced (43), the relationship between the amino acids that constitute the primary structure of the molecule and the enzymatic activity of this peptidase is not yet known, so the exact relationship between the structure and oxidative inactivation remains obscure.

The sensory nerves in the airways contain substance P–im-

munoreactivity (8), and airway irritation causes the release of substance P from these nerves. The released substance P (and other tachykinins) causes a bronchoconstrictor response (14–16). Among the stimuli that trigger this neurogenic inflammation is cigarette smoke (10, 17, 18). Our present studies show that inhalation of cigarette smoke exaggerates the response to substance P, an important tachykinin released during neurogenic inflammation. The results suggest that smoke has its effect by inhibiting the enzyme NEP (which normally cleaves tachykinins and thereby limits their effects). Our results further suggest that smoke has its effect by the generation of free radicals. This effect of cigarette smoke may be an important mechanism by which cigarette smoking causes chronic inflammation of the airways. Free radicals are also produced as a result of the activation of phagocytes as a part of inflammatory responses (44), and they are produced in the atmosphere (e.g., by ozone). All of these sources may result in inactivation of NEP and thereby set up the airway to respond in an exaggerated fashion to irritation, thus producing a hyperresponsive state.

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