Cigarette Smoke Inhalation Decreases α_1-Antitrypsin Activity in Rat Lung

Abstract. *Brief inhalation exposure of rats to three or six puffs of cigarette smoke significantly decreases elastase inhibitory capacity per milligram of α_1-antitrypsin in lung lavage fluid. This effect is not observed in ozone-tolerant rats and can be reversed by treating the lung lavage fluid from smoke-exposed rats with reducing agents. Samples of human serum obtained immediately after smoking also show decreased elastase inhibitory capacity per milligram of α_1-antitrypsin. Again, elastase inhibitory capacity can be restored by treatment with a reducing agent. Cigarette smoking may cause emphysema by inactivating α_1-antitrypsin through oxidation.*

Pulmonary emphysema is characterized anatomically by destruction of lung alveoli. A widely accepted explanation for this destruction holds that elastolytic proteases from lung leukocytes and macrophages attack alveolar connective tissue components (*1*). Normally, these components are protected against elastase-mediated attack by α_1-antitrypsin, a circulating antiprotease that is present in alveolar epithelial fluid (*2*) and that constitutes the predominant endogenous regulator of leukocyte elastase activity (*3*). Indeed, inherited deficiency of this antiprotease predisposes an individual to development of pulmonary emphysema (*4*). The majority of individuals affected by this disease, however, have normal concentrations of α_1-antitrypsin. The major risk factor for the development of emphysema appears therefore to be environmental rather than genetic, and cigarette smoking is strongly implicated (*5*). We recently suggested that the association of cigarette smoking with pulmonary emphysema in man might be due, in part, to local inactivation of lung α_1-antitrypsin during smoking (*6*). This suggestion was based on our observation that aqueous extracts of cigarette smoke inactivated human α_1-antitrypsin in vitro. Other recent data support this possibility (*7*, *8*). The present experiments were undertaken to determine whether inactivation of lung α_1-antitrypsin also occurs after inhalation of cigarette smoke in vivo.

Male Sprague-Dawley rats (150 to 300 g) were housed in a laminar flow hood (Lab Products) and given unrestricted access to a diet of Wayne Lab Blocks (Allied Mills) and water. The rats were exposed to smoke from an unfiltered Kentucky reference 2A1 cigarette; the smoke was generated by a Walton horizontal smoking machine (*9*). Each exposure lasted 30 seconds and consisted of a 35-ml "puff" that had been automatically diluted with ten parts of humidified air. Smoke was purged from the chamber by a 30-second flow of humidified air before introducing the next puff. Control animals were restrained in the machine holders for an equal time period but were not exposed to cigarette smoke. Immediately afterward, both the control and experimental animals were anesthetized with ketamine hydrochloride (300 mg per kilogram of body weight, intramuscularly; Bristol Laboratories) and exsanguinated by division of the abdominal aorta. The lungs were perfused through the right ventricle with cold, isotonic saline at a water pressure of 25 cm until free of visible blood, and then infused through an endotracheal cannula with the same solution at the same pressure. The saline was withdrawn and returned four times through a syringe attached to the cannula; then the lungs were reinfused with fresh saline and the lavaging was repeated. The infusion and lavaging procedure was done three times, after which the fluid from all three lavages from the same animal were pooled and centrifuged (1000*g*) at 4°C for 15 minutes to separate the cells. The supernatant fluid was transferred to dialysis tubes and concentrated by packing the dialysis bags in Aquacide IA (Calbiochem) for 6 hours at 4°C, and the concentrated fluid was stored at −80°C until ready for assay.

Table 1. Effect of exposure to cigarette smoke on the EIC of lung α_1-antitrypsin in normal rats in vivo.

Number of rats	Exposure	EIC*	Decrease from control value (%)
16	Restraint only	0.59 ± 0.06†	
9	3 puffs	0.42 ± 0.05	29‡
14	6 puffs	0.36 ± 0.06	39‡

*Values are expressed as milligrams of pancreatic elastase inhibited per milligram of total α_1-antitrypsin (measured immunologically) in lung lavage fluid ± 1 standard deviation from the mean. Lung lavage fluid was obtained approximately 1 hour after smoke inhalation. †The control value, 0.59 mg elastase inhibited per milligram of antigenic α_1-antitrypsin, exceeds the theoretically calculated ratio of 0.5 mg. The latter value is based on a 1:1 molar interaction between enzyme (molecular weight, 26,000) and inhibitor (molecular weight, 52,000). The discrepancy resulted from impurities in the commercial elastase preparation used in our experiments. ‡Significantly different from controls at $P < .01$ (*t*-test).

We measured the concentration of α_1-antitrypsin in rat bronchopulmonary lavage fluid by rocket immunoelectrophoresis (*10*), using antiserum (prepared by us) to purified α_1-antitrypsin. [The purification of α_1-antitrypsin from rat serum was done in our laboratory by a modification of the method of Rosenberg *et al.* (*11*).] Elastase inhibitory capacity (EIC) of all rat bronchopulmonary lavage fluid was measured against the same batch of porcine pancreatic elastase (114.5 U/mg; Elastin Products). Succinyl-L-alanyl-L-alanyl-L-alanyl-*p*-nitroanilide was used as the substrate (*12*).

A preliminary experiment was carried out to test the assumption that inhibition of pancreatic elastase by rat bronchopulmonary lavage fluid is due solely to α_1-antitrypsin in the fluid. A sample of antiserum to rat α_1-antitrypsin and a sample of serum obtained before immunization were treated with excess trypsin to bind and inactivate the endogenous proteinase inhibitors in these serums. Soybean trypsin inhibitor was then added to counteract unbound trypsin. As expected, the treated antiserum and the control serum contained no free, active trypsin, nor could these treated serums inactivate pancreatic elastase, which soybean trypsin inhibitor does not affect. The lavage fluids were then mixed with (i) treated antiserum or (ii) treated control serum. The EIC was eliminated in condition (i) but remained unchanged in (ii), indicating that antibody to α_1-antitrypsin eliminated all pancreatic elastase inhibitory activity of the lavage fluid. This finding is consistent with the results of a previous study (*13*) which showed that other bronchopulmonary inhibitors do not inhibit pancreatic elastase.

Experiments were then undertaken to examine the effects of a single, brief exposure to cigarette smoke on the EIC of α_1-antitrypsin in rat lung lavage fluid. The results are shown in Table 1. It can be seen that inhalation of three or six consecutive puffs of cigarette smoke caused a statistically significant decrease in the EIC per milligram of α_1-antitrypsin.

In an earlier study (*6*), we showed that inactivation in vitro of human α_1-antitrypsin by aqueous extracts of cigarette smoke is due to oxidation reactions, and that human α_1-antitrypsin can also be inactivated by oxygen free radicals (*14*). It therefore appeared that an oxidation-based mechanism might also be responsible for loss of rat lung α_1-antitrypsin ac-

0036-8075/79/1214-1313$00.50/0

tivity in vivo after the inhalation of cigarette smoke. To test this, we dialyzed concentrated bronchopulmonary lavage fluid from cigarette smoke-exposed rats against 0.2*M* tris-NaCl buffer (*p*H 8.1) containing 0.025*M* sodium metabisulfite for 24 hours at 4°C, followed by 3 hours of dialysis against fresh reagent at 37°C. We then reassayed the EIC per milligram of α_1-antitrypsin in the lavage fluid. Treatment with reducing agent led to a 75 percent recovery of normal EIC. Moreover, the recovered EIC was solely due to reactivated α_1-antitrypsin, since antibody to rat α_1-antitrypsin completely abolished the EIC of the reactivated samples. Similar treatment of control lavage fluids from sham-smoking rats did not affect their EIC (sodium metabisulfite does not inactivate pancreatic elastase). Thus, loss of EIC of rat lung α_1-antitrypsin after inhalation of cigarette smoke could be reversed by a reducing agent, suggesting that local oxidation of α_1-antitrypsin in the lung might have been the responsible mechanism.

Additional, albeit indirect, support for this conclusion was obtained by using rats that were rendered tolerant to oxidant-induced lung injury by prior exposure to ozone. In such animals, subsequent inhalation of six puffs of cigarette smoke failed to cause a significant decrease in EIC per milligram of α_1-antitrypsin in lung lavage fluid (Table 2). The hypothesis that oxidants in cigarette smoke are responsible for the inactivation in vivo of lung α_1-antitrypsin is consistent with the foregoing observation. Recent chemical studies (*8*) suggest that an active-site methionine residue in α_1-antitrypsin may be the critical portion of the molecule affected by oxidizing agents.

Mechanisms other than oxidation of α_1-antitrypsin could account for the partial loss of the EIC of this protein after cigarette smoke inhalation. For example, increased complex formation might occur between the inhibitor and lung proteases. This possibility was examined by crossed antigen-antibody electrophoresis, and no evidence of increased inhibitor-enzyme complex formation was found in the lung lavage fluid of smoke-exposed rats.

We also measured the EIC of circulating α_1-antitrypsin in human chronic smokers. Five males and 17 females (average age, 42 years; average smoking history, 27 years of smoking at least one pack per day) were asked to smoke one cigarette (personal brand) 10 minutes prior to venesection and serum collection. Total serum α_1-antitrypsin was determined immunologically, and the EIC of the serum was measured as before. [Ninety percent of human serum EIC is due to α_1-antitrypsin (*15*).] Compared to 13 nonsmoking subjects (five males and eight females; average age, 31 years), the smokers' serum showed a 20 percent reduction in EIC per milligram of α_1-antitrypsin, which was statistically significant ($P < .01$). Inhalation of cigarette smoke immediately prior to collection of test samples appeared to be necessary for statistically significant differences to be obtained.

Table 2. Effect of exposure to cigarette smoke on the EIC of lung α_1-antitrypsin in ozone-tolerant rats in vivo. Data are for five rats in each condition.

Preliminary treatment*	Exposure	EIC†	Decrease from control value (%)
Air	Restraint only	0.59 ± 0.07	
Air	6 puffs	0.40 ± 0.08	32‡
O_3	Restraint only	0.57 ± 0.05	
O_3	6 puffs	0.52 ± 0.05	9§

*Twenty rats were rendered tolerant to O_3 by exposure to O_3 (2 ppm) for 4 hours; control rats ($N = 20$) were exposed to air instead of O_3. Tolerance was confirmed 7 days later by exposing ten rats in each group to O_3 (9 ppm) for 4 hours. None of the controls survived the night; eight experimental rats survived. On days 9 to 12, the remaining animals were exposed to cigarette smoke or restraint only, as before. †Values are expressed as milligrams of pancreatic elastase inhibited per milligram of total α_1-antitrypsin (measured immunologically) in lung lavage fluid ± 1 standard deviation from the mean. ‡Significantly different from controls at $P < .01$. §Not significantly different.

To our knowledge, this is the first report of the inactivation of lung α_1-antitrypsin in an animal by a single exposure to inhaled cigarette smoke. Transient imbalance between lung proteases and lung protease inhibitors caused by smoking could lead to injury of alveolar walls, and repeated episodes of such protease-antiprotease imbalance could cause slow deformation of alveoli and resultant disease in some chronic smokers.

Several additional questions are raised by these studies. It is not known whether inactivation of α_1-antitrypsin takes place in a conducting air space or a respiratory air space. Our findings do not provide information on the duration of the inactivation or the effects of chronic smoking on α_1-antitrypsin. Nor is it clear why all cigarette smokers do not develop emphysema, or do so at different rates (differences in levels of endogenous antioxidants may affect the outcome). Finally, other mechanisms, such as stimulation of elastase secretion by alveolar macrophages (*16*), may play a role in the pathogenesis of pulmonary emphysema in cigarette smokers. Despite these questions, we believe that inactivation of lung α_1-antitrypsin by cigarette smoke is an important observation, worthy of further study.

A. JANOFF
H. CARP
D. K. LEE

Department of Pathology,
State University of New York,
Stony Brook 11794

R. T. DREW

Medical Department,
Brookhaven National Laboratory,
Upton, New York 11973

References and Notes

1. C. Mittman, Ed., *Pulmonary Emphysema and Proteolysis* (Academic Press, New York, 1972).
2. W. C. Tuttle and S. C. Westerberg, *Proc. Soc. Exp. Biol. Med.* **146**, 232 (1974).
3. K. Ohlsson, in *Neutral Proteases of Human Polymorphonuclear Leukocytes*, K. Havemann and A. Janoff, Eds. (Urban & Schwarzenberg, Balitmore, 1978), pp. 167–177.
4. S. Eriksson, *Acta Med. Scand. Suppl.* **177** (No. 432), 1 (1965).
5. O. Auerbach, E. C. Hammond, L. Garfinkel, C. Benante, *N. Engl. J. Med.* **286**, 853 (1972).
6. H. Carp and A. Janoff, *Am. Rev. Respir. Dis.* **118**, 617 (1978).
7. S. Satoh, T. Kurecki, L. F. Kress, M. Laskowski, Sr., *Biochem. Biophys. Res. Commun.* **86**, 130 (1979); A. B. Cohen, *Am. Rev. Respir. Dis.* **119**, 953 (1979).
8. D. Johnson and J. Travis, *J. Biol. Chem.* **253**, 7142 (1978); *ibid.* **254**, 4022 (1979).
9. The cigarettes were kept at 4°C and reconditioned before use by storage for 48 hours in a chamber equilibrated to 60 percent relative humidity. Kentucky reference 2A1 cigarettes have a low nicotine content (0.51 mg per cigarette) and are therefore especially suitable for use with rodents, which are sensitive to nicotine. They are otherwise quite similar to the average commercial cigarette, and have a tar content of 36.13 mg per cigarette. For details of the dosimetry and inhalation exposure of rodents to cigarette smoke generated by the Walton horizontal smoking machine, see M. R. Guerin *et al., J. Natl. Cancer Inst.* **63**, 441 (1979).
10. C. B. Laurell, *Scand. J. Clin. Lab. Invest. Suppl.* **29** (No. 124), 21 (1972).
11. M. Rosenberg, V. Roegner, F. F. Becker, *Am. Rev. Respir. Dis.* **113**, 779 (1976).
12. J. Bieth, B. Spiess, C. Wermuth, *Biochem. Med.* **11**, 350 (1974). Porcine pancreatic elastase may appear to be a nonphysiologic test protease; however, changes in the functional activity of α_1-antitrypsin can be assessed with any enzyme and still be relevant in the context of this investigation. Moreover, our earlier studies [A. Janoff and H. Carp, *Am. Rev. Respir. Dis.* **116**, 65 (1977)] showed that the inhibitory activity of α_1-antitrypsin against either porcine pancreatic elastase or human leukocyte elastase was equally suppressed by exposure to components of cigarette smoke in vitro.
13. H. Schiessler, K. Hochstrasser, K. Ohlsson, in *Neutral Proteases of Human Polymorphonuclear Leukocytes*, K. Havemann and A. Janoff, Eds. (Urban & Schwarzenberg, Balitmore, 1978), pp. 195–207.
14. H. Carp and A. Janoff, *J. Clin. Invest.* **63**, 793 (1979).
15. N. Heimberger, H. Haupt, H. Schwick, in *Proceedings of the International Research Conference on Proteinase Inhibitors*, H. Fritz and H. Tschesche, Eds. (de Gruyter, Berlin, 1971), pp. 1–21.
16. R. J. Rodriguez, R. White, R. W. Senior, E. A. Levine, *Science* **198**, 313 (1977).
17. I thank J. Wyatt for supplying the Kentucky reference 2A1 cigarettes and the Council for Tobacco Research-U.S.A. for lending us a Walton horizontal smoking machine. Supported by PHS grant HL-14262, Council for Tobacco Research-U.S.A. grant 1143, and Department of Energy contract EY-76-C-02-0016.

5 July 1979; revised 26 September 1979