Effect of Smoking on Functional Activity of Plasma α₁-Protease Inhibitor*

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We determined the levels of α₁-protease inhibitor, the plasma trypsin-inhibiting capacity (TIC), and elastase-inhibiting capacity (EIC) in 29 nonsmokers and 30 smokers, who were healthy volunteers matched for age (mean age, 39 ± 12 years [±SD]). The functional activity of plasma α₁-protease inhibitor (in micrograms of enzyme inhibited per microgram of α₁-protease inhibitor) was slightly but significantly lower in smokers, compared with nonsmokers, both for TIC and EIC (smokers' TIC and EIC were 88.0 ± 16.2 percent [±SD] and 90.4 ± 17.9 percent of the respective mean values in nonsmokers; p<0.05). Among smokers, there was a significant negative correlation (r = −0.37; p<0.05) between the average number of cigarettes smoked per day and the functional activity of plasma α₁-protease inhibitor; the seven subjects who smoked 40 or more cigarettes per day had significantly lower EIC and TIC than the remaining smokers. In 12 smokers tested before and after a two-hour period of intense smoking of eight cigarettes, there was a statistically significant decrease (p<0.05) in EIC one hour after smoking to 93.1 ± 2.5 percent (±SE) of the initial value prior to smoking. It is concluded that there is a slight but significant decrease in the functional activity of plasma α₁-protease inhibitor in smokers, both for TIC and EIC.

The current hypothesis of the pathogenesis of emphysema is that it results from proteolytic pulmonary injury caused by an imbalance between proteases and antiproteases. Janoff and Carp demonstrated that tobacco smoke in vitro inhibited the functional activity of α₁-antitrypsin, more properly termed α₁-protease inhibitor, and suggested that this may be a potential pathogenetic mechanism in emphysema; however, there is controversy regarding the activity of α₁-protease inhibitor in smokers compared with nonsmokers. The initial reports that α₁-protease inhibitor in bronchoalveolar lavage of smokers had only about 50 to 60 percent of the activity in nonsmokers were not confirmed by other studies. Serum trypsin-inhibiting capacity (TIC) was reported to be decreased in smokers compared with nonsmokers in one study, however, in another study, the ratio of TIC to elastase-inhibiting capacity (EIC) was used to indicate inactiva-

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Materials and Methods

Subjects

We studied healthy male volunteers, who also took part in our study on plasma elastase. The study was approved by the University Ethics Committee on Human Experimentation, and informed consent was obtained. To determine the long-term effect of smoking on plasma TIC, EIC, and TIC/EIC ratio, we compared 30 healthy male smokers with 29 healthy male subjects who had never smoked and who were matched for age (mean age, 39 ± 12 years [±SD]). The smokers had been smoking for a mean of 22 ± 12 years (±SD) and were smoking an average of 27.3 ± 10.8 cigarettes per day (±SD). On the day of study, prior to blood sampling, which was done between 9 and 10:30 A.M., the smokers had smoked a mean of 5.4 ± 3.4 cigarettes (±SD); the mean interval between the last cigarette smoked and sampling of blood was 30.0 ± 39.8 minutes (±SD).

In order to determine the short-term effect of intense smoking, we studied 12 volunteer smokers before and after a two-hour period of intense smoking, during which they deeply inhaled smoke from eight medium-tar filter cigarettes; the same brand (16 mg of tar; 1.1 mg of nicotine) was used in all. The subjects were studied in the morning after at least eight hours of abstinence from smoking; samples of blood were taken prior to smoking, immediately after completion of smoking, and 0.5, 1, and 2 hours later.
In all subjects, blood was taken into two tubes with edetic acid (EDTA) as anticoagulant. One tube was used for determination of the leukocyte count and carboxyhemoglobin level, and the other tube was used to obtain plasma, which was stored in aliquots at -70°C for later assays of protease inhibitor levels, TIC, and EIC. Levels of carboxyhemoglobin were determined by spectrophotometry\textsuperscript{8} using commercially prepared plates and standards (Behring Diagnostics); \( \alpha_1 \)-protease inhibitor was determined by radial immunodiffusion\textsuperscript{9} using commercially prepared plates and standards (Behring) and purified \( \alpha_1 \)-protease inhibitor\textsuperscript{10} as standard.

Determinations of EIC and TIC for smokers were always paired with simultaneous assays for nonsmokers in order to minimize technical variability in assays between the two groups. The EIC was determined using purified porcine pancreatic elastase (Elastin Products, St. Louis) and succinyl-tri-alanyl \( p \)-nitroanilide\textsuperscript{11} as substrate, as previously described.\textsuperscript{12} For determination of TIC,\textsuperscript{13} varying amounts of plasma were incubated with 48\( \mu \)g of porcine pancreatic trypsin (Sigma Chemical Co., St. Louis) for ten minutes at 25°C in a total volume of 200\( \mu \)l of 0.1 M phosphate buffer (pH 7.0), followed by the addition of 3 ml of 0.5 mM N-benzoyl-L-arginine ethyl ester in the same buffer as substrate,\textsuperscript{17} and were assayed as a change in absorbance per minute at 253 nm. All assays for both EIC and TIC were done in duplicate using at least three concentrations of plasma, and linear inhibition plots with a maximum of about 80 percent inhibition of enzymatic activity were obtained. To avoid any bias, least-squares linear regression was used to calculate the volume of plasma completely inhibiting enzyme activity. The EIC and TIC were then expressed as micrograms of enzyme inhibited per microgram of immunologically determined \( \alpha_1 \)-protease inhibitor.

Statistical analyses

Statistical significance\textsuperscript{18} was assayed by the unpaired \( t \)-test for comparison of results between smokers and nonsmokers. We used repeated measures analysis of variance and the Bonferroni correction for multiple comparisons for determining the statistical significance of results obtained after intense smoking. The relationship between different variables was evaluated by least-squares linear regression.

RESULTS

Table 1 compares levels of plasma \( \alpha_1 \)-protease inhibitor, TIC, EIC, and TIC/EIC in smokers and nonsmokers. Levels of plasma \( \alpha_1 \)-protease inhibitor were significantly higher (p<0.01) in smokers than in nonsmokers, similar to the previously reported differences in population surveys.\textsuperscript{19,20} Our plasma levels of \( \alpha_1 \)-protease inhibitor in both nonsmokers and smokers, quantitated with a purified preparation of \( \alpha_1 \)-protease inhibitor,\textsuperscript{14} are lower than in the previous studies\textsuperscript{19,20} but similar to the mean value of 1.32 mg/ml obtained by Jeppsson et al.\textsuperscript{21} These authors\textsuperscript{21} pointed out that previously published values of circulating levels of \( \alpha_1 \)-protease inhibitor were overestimated because of the lack of uniform standards for quantitation of \( \alpha_1 \)-protease inhibitor. The levels of \( \alpha_2 \)-macroglobulin and inter-\( \alpha_1 \)-trypsin inhibitor were similar in smokers and nonsmokers.

Both TIC and EIC in micrograms of enzyme inhibited per microgram of plasma \( \alpha_1 \)-protease inhibitor were slightly but significantly (p<0.05) lower in

<table>
<thead>
<tr>
<th>Data</th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Age, yr</td>
<td>39.6±12.5</td>
<td>39.2±12.0</td>
</tr>
<tr>
<td>Alpha-( \alpha_1 )-protease inhibitor, mg/ml</td>
<td>1.11±0.18</td>
<td>1.41±0.25</td>
</tr>
<tr>
<td>Alpha-( \alpha_2 )-macroglobulin, mg/ml</td>
<td>2.50±0.49</td>
<td>2.63±0.57</td>
</tr>
<tr>
<td>Inter-( \alpha_1 )-trypsin inhibitor, mg/ml</td>
<td>0.567±0.052</td>
<td>0.569±0.070</td>
</tr>
<tr>
<td>TIC, mg/ml</td>
<td>0.807±0.108</td>
<td>0.710±0.131</td>
</tr>
<tr>
<td>EIC, mg/ml</td>
<td>0.615±0.113</td>
<td>0.556±0.110</td>
</tr>
<tr>
<td>TIC/EIC</td>
<td>1.315±0.166</td>
<td>1.309±0.200</td>
</tr>
</tbody>
</table>

*Table values are means±SD.
Abbreviations used: TIC = trypsin inhibiting capacity; EIC = elastase inhibiting capacity.
\(*\)Significantly different from nonsmokers (p<0.01).
\(\dagger\)In micrograms of trypsin per microgram of \( \alpha_1 \)-protease inhibitor.
\(\ddagger\)Significantly different from nonsmokers (p<0.05).
\(\S\)Micrograms of pancreatic elastase per microgram of \( \alpha_1 \)-protease inhibitor.

smokers than nonsmokers (mean TIC and EIC in smokers were, respectively, 88.0±16.2 percent [±SD] and 90.4±17.9 percent of mean values of nonsmokers); however, the ratio of TIC/EIC was not significantly different between the two groups. Both EIC and TIC in smokers showed a negative correlation with the average number of cigarettes smoked per day (for EIC, \( r = -0.391 \) and \( p = 0.033 \); for TIC, \( r = -0.350 \) and \( p = 0.051 \)) but were not significantly related to carboxyhemoglobin levels or the number of cigarettes smoked on the day prior to blood sampling. The seven smokers who smoked 40 or more cigarettes per day (mean, 44.3±5.3 [±SD]) had significantly lower EIC (0.471±0.046 [±SE] vs 0.581±0.020; \( p<0.02 \)) and TIC (0.625±0.073 [±SE] vs 0.735±0.020; \( p<0.05 \)) than the remaining smokers (mean, 22.1±5.3 cigarettes per day [±SD]).

Figure 1 shows the effect of intense smoking in the 12 smokers studied after eight hours of abstinence from smoking. Following the two-hour period of intense smoking of eight cigarettes, the carboxyhemoglobin level in the blood increased markedly, as expected. Levels of plasma \( \alpha_1 \)-protease inhibitor (data not shown) were unchanged immediately after smoking and tended to be higher (but not statistically significantly so) at one and two hours after smoking compared with initial values (mean levels were 99.6±1.19 percent [±SE], 103.1±2.3 percent, and 106.4±3.4 percent of initial, respectively). Plasma EIC and TIC were not changed immediately after smoking (Fig 1) and at 0.5 hour after smoking (data were obtained in the first six subjects and not shown). There was a slight but statistically significant (p<0.05) decrease in EIC one hour after completion of smoking to 93.9±2.5 percent (±SE) of initial EIC; TIC at one hour was 95.3±3.2 percent (±SE) of initial TIC, but this decrease was not
smoking, as indicated by a significant decrease in plasma EIC. Smoking did not affect the ratio of TIC/EIC.

We used both TIC and EIC to evaluate the effect of smoking on the functional activity of plasma α₁-protease inhibitor because of the reported difference in results between Chowdhury et al., who found decreased serum TIC in smokers, and Beatty et al., who assumed that TIC was not affected by smoking and used the TIC/EIC to indicate inactivation of α₁-protease inhibitor in smokers. Studies in subjects with α₁-protease inhibitor deficiency have demonstrated that EIC is more specific than TIC in evaluating α₁-protease inhibitor, because trypsin is also inhibited by plasma protease inhibitors other than α₁-protease inhibitor; however, we found the level of two other plasma trypsin inhibitors, α₂-macroglobulin and inter-α-trypsin inhibitor, to be equal in smokers and nonsmokers, making it unlikely that differences in TIC between the two groups were due to differences in other protease inhibitors. Thus the decreased TIC in smokers suggests inactivation of α₁-protease inhibitor. This conclusion is supported by the decreased EIC in smokers, since pancreatic elastase is presumably inhibited only by α₁-protease inhibitor in plasma and by the decrease in EIC after intense smoking (Fig 1).

Our findings that plasma EIC and TIC were about 10 percent lower in smokers than nonsmokers indicate slight inactivation of plasma α₁-protease inhibitor in smokers and support the findings of Chowdhury et al. that TIC was lower in smokers than nonsmokers. Despite a possible bias because the subjects they studied were patients, their data suggested partial inactivation of plasma α₁-protease inhibitor in smokers, as the decreased TIC in smokers was related to the intensity of smoking. In contrast, Beatty et al. used the TIC/EIC ratio to indicate α₁-protease inhibitor inactivation, and found it increased in smokers. The use of the TIC/EIC ratio was based on TIC/EIC determinations on oxidized α₁-protease inhibitor and on kinetic studies showing that oxidized α₁-protease inhibitor had lost its EIC while retaining its TIC, although the rate of association with trypsin was reduced about tenfold. The difference from our results which showed that smokers' EIC and TIC decreased in the same proportion so that TIC/EIC ratios were similar in smokers and nonsmokers, may be due to differences in the conditions of the TIC assay.

Three more recent studies did not find significant differences in functional activity of circulating α₁-protease inhibitor in smokers compared with nonsmokers. The difference in results between those studies and ours may be due to differences in selection of subjects or methodologic differences as discussed subsequently. Our use of plasma for assays did not affect results, for we found in several smokers...
and nonsmokers that EIC and TIC were similar in plasma and serum; this was also shown by Cox and Billingsley.26 Our data suggest that inactivation of α1-protease inhibitor in smokers is likely to be observed only if heavy smokers (40 or more cigarettes per day) are included.

Cox and Billingsley26 found no difference in TIC, EIC, and TIC/EIC between 26 smokers and 26 age-matched nonsmokers; however, 60 percent of their smokers were female subjects, while all of our subjects were male; differences in the effects of smoking on plasma α1-protease inhibitor in male and female subjects may be a factor. Lellouch and co-workers27 did not find a difference in the functional activity of serum α1-protease inhibitor between smokers and nonsmokers in a population survey of 719 men; however, these investigators27 determined EIC in duplicate using only one concentration of serum, which resulted in only about 40 percent inhibition of pancreatic elastase, as estimated from their data and the equation they used to calculate the functional activity of α1-protease inhibitor.27 Determination of EIC from only partial inhibition of pancreatic elastase using only one concentration of serum may not be sensitive enough to detect the 10 percent decrease in EIC that we observed in smokers. Bridges et al28 did not find significant differences in EIC or TIC between 50 nonsmokers and 50 age-matched cigarette smokers. The difference in results between our study and theirs28 may be due to our subjects being slightly older than theirs (mean age, 39 years vs 32 years), with probably a greater proportion of heavy smokers (mean cumulative cigarette consumption in all smokers, 31.8 pack-years vs 19 pack-years).

In our previous studies on bronchoalveolar lavage,8 we found that functional activity of lavage α1-protease inhibitor in smokers after overnight abstinence from smoking was similar to nonsmokers but found slight (10 percent) inactivation in a group of smokers lavaged one hour after smoking two cigarettes. Inactivation may be due not only to the direct effect of tobacco smoke but to the release of oxidants from neutrophils and macrophages.29,30 Our present data suggest that inactivation of α1-protease inhibitor in the lung after smoking, although slight, may be continuing for a sufficient time so that enough α1-protease inhibitor is inactivated and exchanged with plasma to be reflected by slight but detectable changes in plasma activity. The concept that inactivation of pulmonary α1-protease inhibitor by smoking may be reflected by changes in plasma α1-protease inhibitor is supported by our finding of slight inactivation of plasma α1-protease inhibitor following a two-hour period of intense smoking. Even though the degree of overall inactivation of α1-protease inhibitor may be slight, there may be more intense but localized inactivation of α1-protease inhibitor at critical areas such as the region of the respiratory bronchiole, as suggested by Janoff et al,31 to be of pathogenetic significance.

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