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Synergistic Inactivation of Plasma α_1-Proteinase Inhibitor by Aldehydes of Cigarette Smoke with Styrene Oxide and 1,2-Dichloroethane

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Abstract. Previous studies from this laboratory have shown that α_1-proteinase inhibitor (α_1-PI) can be inactivated by aldehydes found in the cigarette smoke as well as by industrial chemicals. Studies presented here demonstrate the synergistic inactivation of α_1-PI by styrene oxide or 1,2-dichloroethane when mixed with acrolein or pyruvic aldehyde and vice-versa. The data suggest that smokers exposed to chemicals in industry may be more prone to lung emphysema due to synergistic inactivation of α_1-PI by chemicals and cigarette smoke components.

A previous communication from this laboratory (Ansari *et al.* 1987) has shown that proteinase inhibitory activity of plasma or purified α_1-PI can be reduced by epoxides and 1,2-dihaloethanes in a dose-dependent manner under *in vitro* conditions. These studies raised the possibility that the decrease in proteinase inhibitory activity may be used as a marker of chemical exposure. Several investigators (Dooley and Pryor 1982; Travis *et al.* 1984; Laurent and Bieth 1985) have shown that components found in cigarette smoke inactivate the α_1-PI activity and may be a major risk factor in causing lung emphysema. In addition to the suggested mechanism that inactivation of α_1-PI is caused by the oxidation of methionine residues by the oxidants present in cigarette smoke, we have shown that inactivation of α_1-PI can also be caused by aldehydes present in cigarette smoke (Gan and Ansari 1986). Subsequently, it was shown that inactivation of α_1-PI by acrolein occurs through covalent modification of lysine and histidine while methionine remains unchanged as shown by amino acid analysis (Gan and Ansari 1987). These observations suggested that in workers, who are also smokers, exposure to these chemicals may result in an additive or synergistic effect on inactivation of α_1-PI. The present *in vitro* studies show that aldehydes of cigarette smoke, with styrene oxide and 1,2-dichloroethane cause synergistic inactivation of α_1-PI.

Materials and Methods

Pancreatic elastase, trypsin, human plasma α_1-proteinase inhibitor, N-succinyl-(Ala)$_3$-p-nitroanilide, α-N-benzoyl-DL-arginine-p-nitroanilide HCl and *p*-nitroaniline were purchased from Sigma Chemical Co., St. Louis, MO.; styrene oxide (97%), 1,2-dichlorethane (99%), acrolein (99%) and pyruvic aldehyde (40% aqueous solution) were purchased from Aldrich Chemical Co., Milwaukee, WI.

Incubation of α_1-Proteinase Inhibitor with Styrene Oxide or 1,2-Dichloroethane in the Presence of Acrolein or Pyruvic Aldehyde

Styrene oxide, acrolein, and 1,2-dicholoroethane solutions were prepared in ethanol (1:1, v/v). Pyruvic aldehyde was diluted in water to make a 20% solution. Aliquots of these chemicals, alone or in combination, were added to α_1-PI solution to the desired concentrations. The reaction mixture was incubated at 37°C for 2 hr. Two types of controls were also incubated at 37°C for 2 hr, one containing plasma or α_1-PI in 0.1 M phosphate buffer, pH 7.2 and the other containing the highest concentration of the particular chemical (in 0.1 M phosphate, pH 7.2) utilized in a given series of experiments. Appropriate aliquots were removed from the incubation mixtures for elastase inhibitory or trypsin inhibitory assays.

Enzyme Assays

Elastase was assayed by the method of Bieth *et al.* (1974). Ten μg of elastase (stock solution, 1 mg per ml of 0.05 M sodium acetate buffer, pH 4.0) in 10 μl was mixed with 0.2 ml of 0.1 M

sodium phosphate buffer, pH 7.2; thereafter, 2.9 ml of the phosphate buffer and 0.1 ml of succinyl-$(Ala)_3$-*p*-nitroanilide (stock solution, 4.5 mg per ml dissolved in dimethylsulfoxide) were added. The rate of change of absorbance at 410 nm was monitored on a Gilford Automatic Spectrophotometer (model 250) at 25°C.

Trypsin was assayed by the procedure of Erlanger *et al.* (1961). Twenty five μg of trypsin in 25 μl of 0.0025 M HCl was added to 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.2. Three ml of 0.5 mM α-*N*-benzoyl-DL-arginine-*p*-nitroanilide in 0.1 M sodium phosphate buffer, pH 7.2 containing 0.005 M $CaCl_2$ was added, and the rate of change of absorbance at 410 nm was monitored as described for elastase.

Enzyme Inhibitory Assays

The reaction mixture for the elastase inhibitory assay consisted of samples containing native or chemically treated plasma (10 μl), a native or modified α_1-PI preparation (60 μg, stock solution, 1 mg/ml in 0.1 M sodium phosphate buffer, pH 7.2), 10 μg of elastase, and sufficient 0.1 M sodium phosphate, pH 7.2 to bring the volume to 0.2 ml. The mixture was incubated at room temperature for 15 min, followed by the addition of 2.9 ml of the sodium phosphate buffer and 0.1 ml of succinyl-$(Ala)_3$-nitroanilide substrate. The elastase (residual) activity was measured as described above.

The conditions for the trypsin inhibitory assays are the same as those for elastase, except for the substitution of appropriate enzyme (25 μg), inhibitor (60 μg), plasma (25 μl) and substrate (3.0 ml of 0.5 mM BAPNA).

Statistical comparisons were performed by Duncan's multiple range test, using a statistical package (SPSS) for personal computers.

Results

When α_1-PI was incubated at 37°C with increasing concentrations (0 to 10 mM) of styrene oxide both elastase inhibitory capacity (EIC) and trypsin inhibitory capacity (TIC) were reduced. For example[1], as indicated in Figure 1, at 10 mM a 67% and a 22%

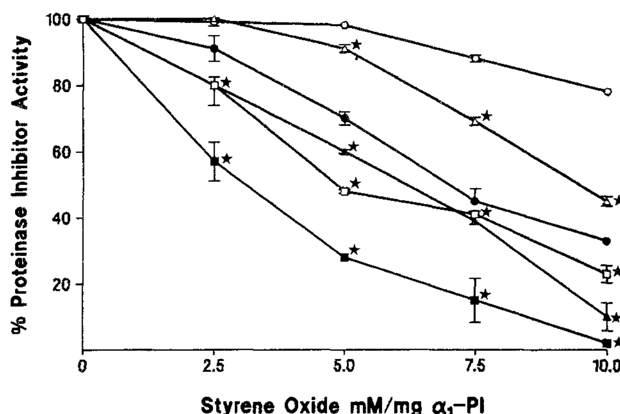

Fig. 1. Inactivation of the proteinase inhibitory activity of α_1-PI by styrene oxide. Disappearance of the EIC (●——●) and TIC (○——○) alone and in the presence of 0.05 mM acrolein (▲——▲, EIC; △——△, TIC) or 5 mM pyruvic aldehyde (■——■, EIC, □——□, TIC). Each value represents the mean ±S.D. of three determinations. ★ $p < 0.01$

of the EIC and TIC were lost, respectively. The loss of the proteinase inhibitory activities however, were exacerbated when 0.05 mM of acrolein (which by itself did not have any effect on the inhibitor) was included with styrene oxide in the incubation solutions. Thus, at 10 mM styrene oxide and 0.05 mM acrolein, 90% (instead of 67%) of EIC and 55% (instead of 22%) of TIC of α_1-PI were lost (Figure 1). Similar synergistic effects were observed when varying concentrations of styrene oxide and 5 mM pyruvic aldehyde were simultaneously used with α_1-PI (Fig 1). For instance, at 10 mM styrene oxide, and at 10 mM styrene oxide plus 5 mM pyruvic aldehyde, the EIC was further reduced from 67% to 99% while the TIC decreased from 22% to 78% (Figure 1), respectively.

Figure 2 shows the synergistic effects of styrene oxide (2 mM) or 1,2-dichloroethane (DCE, 100 mM) on the inactivation of α_1-PI at these concentrations. The synergistic effects are evident when they are mixed with various concentrations of pyruvic aldehyde (0 - 75 mM). Thus, at 75 mM pyruvic aldehyde alone, the loss of EIC of α_1-PI was 50%, but when it is combined with 2mM styrene oxide, the loss is now 67%. Similar results are obtained for the reduction of the TIC; for example, at 75mM pyruvic aldehyde the loss of TIC is 60% which increases to 78% by the addition of 2 mM styrene oxide.

Similar to styrene oxide, DCE also demonstrated a synergistic effect with pyruvic aldehyde for the inactivation of α_1-PI (Figure 2); the extent of further losses of the EIC and TIC of α_1-PI are in close agreement with those caused by styrene oxide (Figure 2).

[1] The rate of hydrolysis of succinyl-$(Ala)_3$-p-nitroanilide by elastase (10 μg) was 39.2 nmoles of p-nitroanaline per min, while in the case of trypsin (25 μg) the rate of hydrolysis of α-N-benzoyl-DL-arginine-*p*-nitroanilide was 18.8 nmoles per min,. The loss of the proteinase inhibitory activities of the α_1-PI or plasma due to chemical treatment was calculated as follows: [Rate of hydrolysis (control, *i.e.*, no inhibitor present)-rate of hydrolysis in the presence of native inhibitor, or modified inhibitor, or untreated plasma, or chemically treated plasma] ÷ rate of hydrolysis (control) × 100. For examples, the elastase inhibitory capacity (EIC) at 10 mM styrene oxide (Figure 1, is [(39.2-12.9) ÷ 39.2] × 100 = 67%, and the trypsin of inhibitory capacity (TIC) is [(18.8-14.7) ÷ 18.8] × 100 = 22%, while in the presence of native α_1-PI (60 μg, the same amount used in the chemically treated protein) the EIC is: [39.2-0.3) ÷ 39.2] × 100 = 99%.

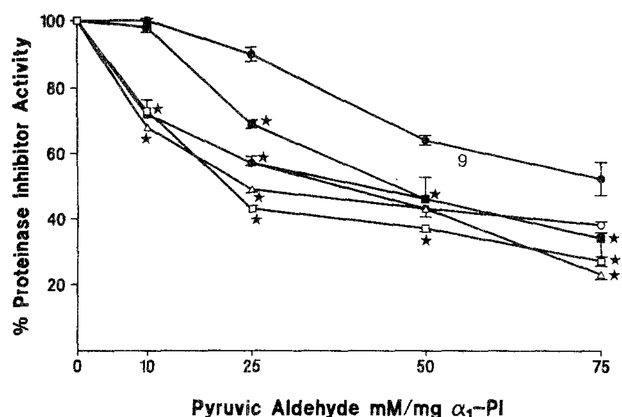

Fig. 2. Inactivation of the proteinase inhibitory activity of α_1-PI by pyruvic aldehyde (●——●, EIC; ○——○, TIC) alone and in the presence 2 mM styrene oxide (▲——▲, EIC; △——△, TIC) or 100 mM DCE (■——■, EIC; □——□, TIC). Each value represents the mean ±S.D. of three determinations. ★ p <0.01

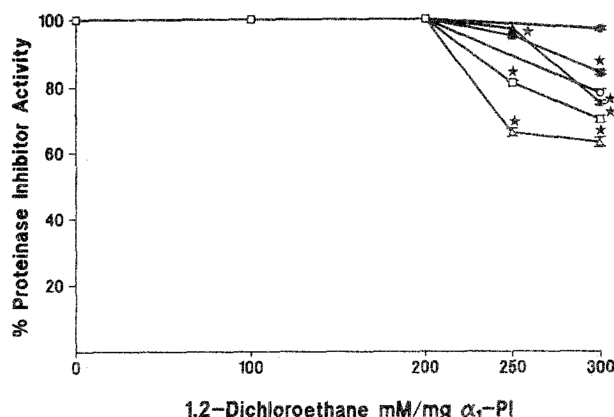

Fig. 4. Inactivation of proteinase inhibitory activity of α_1-PI by DCE alone (●——●, EIC; ○——○, TIC) and in the presence of 0.05 mM acrolein (▲——▲, EIC; △——△, TIC) or 5 mM pyruvic aldehyde (■——■, EIC; □——□, TIC). Each value represents the mean ±S.D. of three determinations. ★ p <0.01

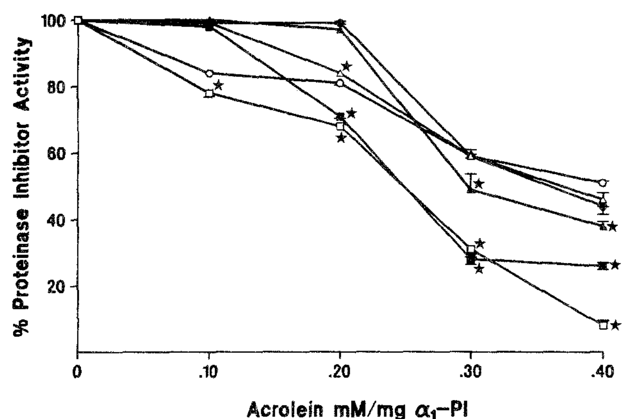

Fig. 3. Inactivation of proteinase inhibitory activity of α_1-PI by acrolein alone (●——●, EIC; ○——○, TIC) and in the presence of 2 mM styrene oxide (▲——▲, EIC; △——△, TIC) or 100 mM DCE (■——■, EIC; □——□, TIC). Each value represents the mean ±S.D. of three determinations. ★ p <0.01

The synergism of styrene oxide (2 mM) or DCE (100 mM) with varying amounts of acrolein in inactivating α_1-PI is summarized in Figure 3. The synergistic effects become more pronounced at higher concentrations of acrolein.

Figure 4 shows the synergistic effects of acrolein (0.05 mM) or pyruvic aldehyde (5 mM) with DCE in the inactivation of α_1-PI. This combination is not as effective as those depicted in Figures 1, 2, and 3.

Discussion

In earlier studies, it was shown that α_1-PI can be inactivated by epoxides and 1,2-dihaloethanes (Ansari *et al.* 1987). Among epoxides, styrene oxide was found to be the most potent one while 1,2-dichloroethane was more potent than 1,2-dibromoethane. It was also observed that carbonyl compounds found in cigarette smoke can also inhibit α_1-PI (Gan and Ansari 1986). It was interesting to note that aldehydes (acrolein, pyruvic aldehyde, and glyoxal) were effective in inactivating α_1-PI while diketones (2,3-butane-and pentane-dione) were virtually ineffective. Since acrolein and pyruvic aldehyde were the most effective aldehydes in inhibiting α_1-PI, we examined the synergistic effect of these compounds on styrene oxide (most potent epoxide in inhibiting α_1-PI) and 1,2-dichloroethane (most potent 1,2-dihaloethane in inhibiting α_1-PI).

Styrene oxide at the concentration of 10 mM does not modify any amino group (Ansari *et al.* 1986) of α_1-PI, but decreases some of the EIC and TIC activity, which may be the result of physical changes in the inhibitor caused by the chemical. However, a 0.05 mM concentration of acrolein modifies a lysine residue (Gan and Ansari 1987). Combination of the two causes synergistic inactivation of α_1-PI (Figure 1). A concentration of 25 mM causes a loss of 10–13 lysine residues while no loss of amino group is seen with 2 mM of styrene oxide. Both combinations indicate that inactivation is probably due to both amino acid modification as well as changes in the physical properties of the α_1-PI.

Although it is speculated that pyruvic aldehyde may be modifying α_1-PI through the Schiff's base formation (Schauenstein *et al.* 1977), definitive data to support this is not available. There is a possibility that the synergistic inactivation of α_1-PI by pyruvic aldehyde and styrene oxide could be due to

the combination of chemical and physical modification.

The effect of acrolein and pyruvic aldehyde on inactivation of α_1-PI by DCE is not as dramatic as in the case of styrene oxide but shows some synergistic activation.

These results indicate that α_1-PI can undergo synergistic inactivation by the chemicals and components of cigarette smoke. Therefore, when α_1-PI measurement is used as a marker of chemical exposure, a knowledge of smoking habits of the exposed personnel is also an important consideration. The data also indicate that smokers working in the chemical industry may be more prone to develop pulmonary emphysema, since inactivation of α_1-PI has been proposed as a key factor in the etiology of the disease (Kueppers and Bearn 1966).

These *in vitro* studies with purified α_1-PI need to be confirmed in the whole plasma as well as under *in vivo* conditions in animal models.

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