

Erythrocytes prevent inactivation of α_1 -antitrypsin by cigarette smoke

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ABSTRACT: Red blood cells (RBC) possess strong antioxidant activity. We tested whether this activity was sufficient to prevent the oxidative inactivation of α_1 -antitrypsin (α_1 -AT) by cigarette smoke. We found that RBC in physiological concentrations completely prevented the inactivation of α_1 -AT. The major erythrocytic antioxidants, catalase, superoxide dismutase and glutathione were then selectively inhibited and the RBC retested. Only the inhibition of catalase significantly impaired the protective ability of added erythrocytes. We suggest that RBC antioxidants may be an important variable in determining the degree of protection of α_1 -AT against oxidation.

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Among the mechanisms of tissue injury, two processes are considered to be of central importance: 1) excess proteolytic activity; and 2) oxidation by reactive oxygen species and other forms of free radicals. Of all human organs the lung alone is directly exposed to an oxygen-rich environment and, not surprisingly, many recent studies have provided evidence that oxidant-induced cellular damage underlies the pathogenesis of many pulmonary diseases, including the adult respiratory distress syndrome, gas inhalation, ischaemia-reperfusion and hyperoxic lung injury [1].

In 1963, LAURELL and ERIKSSON [2] described the association of α_1 -antitrypsin (α_1 -AT) deficiency and pulmonary emphysema. Fifteen years later, CARP and JANOFF [3] described the oxidative inactivation of α_1 -AT by cigarette smoke. These two observations became the cornerstone of the protease/antiprotease hypothesis of emphysema as a combined oxidative and proteolytic injury [4]: the oxidative inactivation of α_1 -AT, directly by cigarette smoke [5, 6] or indirectly by phagocyte-released free radicals [7], would leave leucocytic elastase uninhibited to act on lung structural proteins, such as elastin and collagen. Numerous investigations have since supported this hypothesis, also demonstrating a role of

antioxidants in protecting α_1 -AT against smoke [5, 6] and phagocyte-mediated inactivation [8].

Since red blood cells (RBC) have high antioxidant activity, we speculated that these cells in physiological concentrations might be sufficient to protect α_1 -AT against inactivation by cigarette smoke. Our results support this premise.

Methods

Inactivation of α_1 -AT by aqueous smoke extract

α_1 -AT was dissolved in 0.1 M Tris pH 8.0 to a final concentration of 2.5 mg·ml⁻¹. All reagents were from Sigma Chemical Co. if not otherwise specified. One mg of the inhibitor completely inactivated 5.4 U of porcine pancreatic elastase.

Aqueous buffered fresh smoke extract was prepared from unfiltered cigarettes of the same commercial brand. Two cigarettes were mounted into blue plastic Eppendorf pipette tips and connected with a T-piece to a 60 ml plastic syringe. Smoke was aspirated in 2 s by hand and expelled slowly into 0.1 M Tris pH 8.0 kept under

ice; 20 cigarettes were used to prepare a 2 ml smoke solution, the pH of which was adjusted to 7.5.

Increasing amounts of fresh smoke extract (10, 20, 30, 40 μ l) were then added to a constant amount of α_1 -AT solution (20 μ l) and brought to a constant final volume of 60 μ l using 0.1 M Tris buffer. After a 15 min period of incubation at room temperature, elastase inhibitory activity (EIA) was measured by adding porcine pancreatic elastase solution, 2.07 U in 100 μ l 0.1 M Tris pH 8.0, to a cuvette containing 30 μ l of the various α_1 -AT samples and mixed with 1 ml of Tris buffer. After incubation for 5 min at room temperature, 20 μ l of succinyl-(alanyl)3-p-nitroanilide (SAPNA), 10 mg in 1 ml of N-N-dimethyl-formamide, was added and hydrolysis of the substrate was followed in a Gilford spectrophotometer by the increasing optical density at 405 nm for at least 5 min.

The regression curves that fit the data best were determined by a curve fitting program using a Hewlett Packard 97 programmable calculator.

Inactivation of α_1 -AT in the presence of RBC

Increasing amounts of normal, packed RBC (washed five times in normal saline solution and free of EIA) were mixed with a constant volume of α_1 -AT (100 μ l) prior to the addition of the smoke extract (200 μ l). Samples were then centrifuged at 3,000 g for 10 min and the supernatant was evaluated spectrophotometrically for EIA.

Effect of antioxidant-depleted RBC on inactivation of α_1 -AT by smoke solution

RBC were completely depleted of glutathione (GSH) by incubating a 25% (by volume of packed cells) erythrocyte suspension with 2 mM N-ethyl-maleimide (NEM) for 60 min. Superoxide dismutase was completely inhibited by incubating a 10% erythrocyte suspension with 50 mM diethyldithiocarbamate (DTCA) for 60 min. RBC catalase activity was reduced to 12% of its original activity by incubation of a 10% erythrocyte suspension with 50 mM 3-amino-1, 2, 4-triazole (AMT) for 12 h. All incubations were performed at 37°C and cells were washed four times in normal saline solution after each incubation. Glutathione concentration and activities of superoxide dismutase and catalase were measured spectrophotometrically [9].

Because of the high degree of haemolysis observed when antioxidant-depleted RBC were incubated with the smoke extract, we could not use the spectrophotometric assay for elastase. Instead we performed the measurement as described [10], except that exposed X-ray films were used in the place of Cornaflex membranes. For each treated RBC sample, a paired sample of untreated cells was used from the same stock RBC suspension. These control cells were also incubated as the treated samples, but using normal saline instead of the NEM, AMT or DTCA solutions.

The comparison between inactivation of α_1 -AT by smoke in the absence or in the presence of RBC (treated or untreated) was done with a paired t-test. AMT-treated, NEM-treated and untreated RBC were compared using an analysis of variance (ANOVA), followed by paired t-tests. DTCA-treated RBC were compared to untreated and AMT- and NEM-treated RBC with paired t-tests. Since seven t-tests were performed, a Bonferroni corrected p-value of $0.05/7 = 0.007$ was required for significance, two-tailed.

Inactivation of α_1 -AT by smoke alone was excluded from the ANOVA as being logically different from the other four. DTCA-treated RBC was excluded since only four experiments contributed data and the repeated measures ANOVA would only have used those four for all conditions. Bonferroni corrected t-tests were chosen because not all comparisons were of interest and because of unequal numbers in the conditions.

Fibrin-agarose immunoelectrophoresis of α_1 -AT

Fibrin-agarose immunoelectrophoresis was performed as described [11]. Ten mg of bovine fibrinogen in 1 ml of normal saline solution was added to 9 ml of hot agarose (1.25% in Tris buffer, 0.1 M, pH 8.65).

Results

To measure the inactivation of α_1 -AT by cigarette smoke extract, we incubated a standard amount of α_1 -AT in buffered Tris solution with increasing volumes of cigarette smoke extract. Figure 1A shows that there was a linear relationship ($r=-0.74$) between the amount of smoke extract added and the degree of inhibition of α_1 -AT achieved.

The effect of RBC on α_1 -AT inactivation was determined with increasing amounts of RBC prior to addition of a constant volume of smoke extract. There was a dose-dependent protective effect ($r=0.78$) of added RBC against smoke inactivation of α_1 -AT with complete protection of elastase inhibitory activity (EIA) at the highest concentration of erythrocytes (fig. 1B), ($p=0.00008$). This effect can be shown qualitatively by fibrin-agar electrophoresis. Figure 2 shows the loss of functional activity of α_1 -AT, while the antigenic reactivity is preserved. The protection of α_1 -AT by RBC is also clearly demonstrated. In a separate experiment (not shown) washed erythrocyte membrane ("ghosts"), prepared by osmotic lysis [12], and intact RBC added after the additional smoke extract did not provide any protection. This suggests that the oxidant scavenger is an endocellular, cytosolic constituent and that the oxidation of α_1 -AT is not reversible by RBC.

AMT-treated, NEM-treated and untreated RBC clearly differed in their protection of EIA against smoke inactivation (repeated measures ANOVA, $F(2,10)=6.11$, $p=0.018$). Bonferroni corrected t-tests (using the mean square error from the ANOVA in order to increase the degrees of freedom) showed a clear difference between

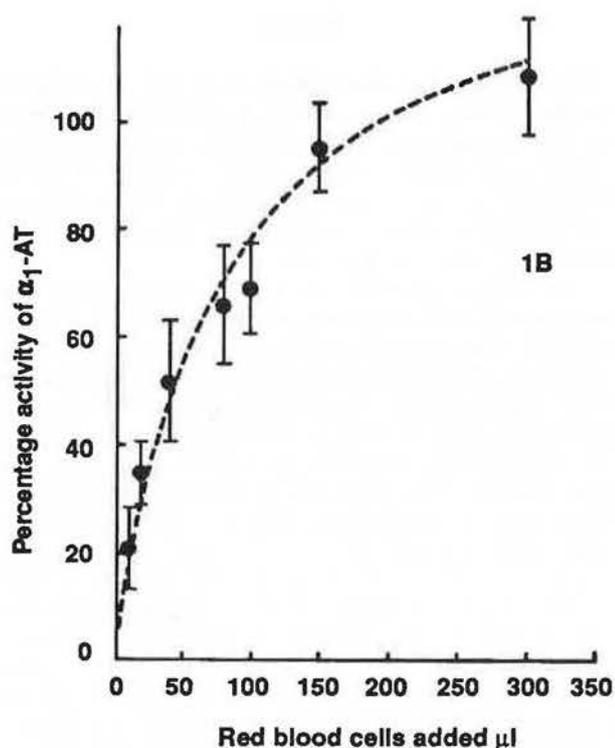
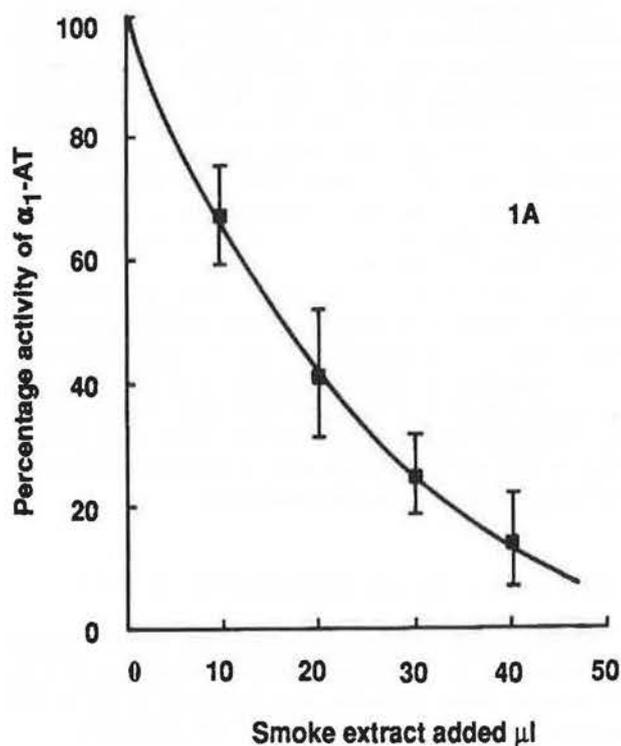


Fig. 1. - Smoke inactivation of α_1 -AT and its protection by red blood cells (RBC). 1A: increasing amounts of cigarette smoke extract inactivate the elastase inhibiting function of α_1 -AT. 1B: protection of α_1 -AT is shown by increasing amounts of normal packed RBC. The protection was dose-dependent and was complete at the highest concentrations of erythrocytes used, corresponding to a 50% haematocrit. Results are expressed as mean \pm SEM. α_1 -AT: alpha₁-antitrypsin.

AMT-treated and NEM-treated RBC, with AMT-treated being lower ($t(10)=3.16, p=0.005$), and between AMT-treated and untreated RBC ($t(10)=3.295, p=0.004$), again with AMT-treated being lower than DTCA-treated RBC ($p=0.012$, not meeting the Bonferroni criterion). The three other comparisons were not statistically significant (two-tailed p 's > 0.10) (table 1).

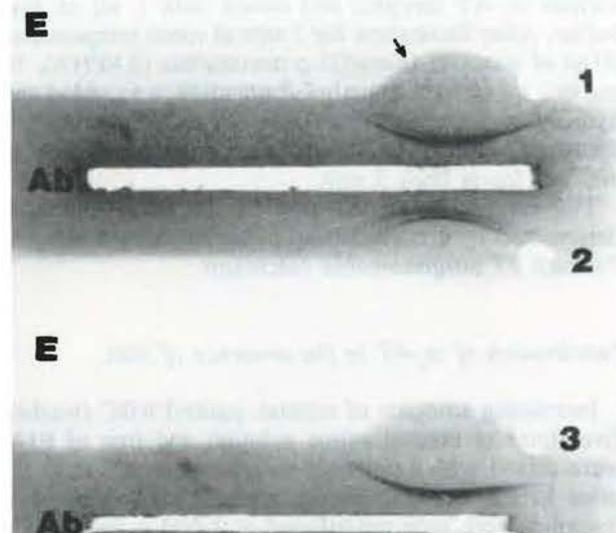


Fig. 2. - Fibrin-agarose immunoelectrophoresis of α_1 -AT. Electrophoresis was for 40 min. Anode is at the left. Troughs labelled E contain elastase ($2.07 \text{ U}\cdot\text{ml}^{-1}$). Troughs labelled Ab contain antibody to α_1 -AT. Well 1: sample of α_1 -AT incubated with smoke extract in the presence of $300 \mu\text{l}$ of washed and packed RBC/haematocrit 50%. Note the preservation of elastase inhibition by α_1 -AT, as evidenced by the semicircular area of undigested fibrinogen (indicated by the arrow). Well 2: α_1 -AT incubated with smoke extract without erythrocytes. There is almost complete absence of elastase inhibition whilst the antigenic specificity is preserved. Well 3: Untreated α_1 -AT. α_1 -AT: alpha₁-antitrypsin, RBC: red blood cells.

Table 1. - Reduction of α_1 -AT EIA by aqueous smoke solution and its protection by intact and antioxidant-depleted RBC

Test conditions (substances added to α_1 -AT)	α_1 -AT EIA %
Smoke	55.0 ± 2.49 (6)
Smoke + RBC	94.8 ± 4.60 (6)*
Smoke + RBC + AMT	84.1 ± 3.06 (5)**
Smoke + RBC + NEM	93.1 ± 2.49 (6)
Smoke + RBC + DTCA	94.2 ± 3.32 (4)

In all experiments using RBC, the final haematocrit was 50%. Results are expressed as mean \pm SEM (number for determinations in parenthesis). *: value significantly different ($p < 0.005$) from the value obtained with smoke alone; **: value significantly different ($p < 0.01$) from values obtained with untreated RBC and NEM-treated RBC; α_1 -AT: alpha₁-antitrypsin; EIA: elastase inhibitory activity; RBC: red blood corpuscles; AMT: 3-amino-1, 2, 4-triazole; NEM: N-ethyl-maleimide; DTCA: diethyldithiocarbamate.

Discussion

The inactivation of α_1 -AT by cigarette smoke and phagocyte-produced reactive oxygen species has been well established since first described by CARP and JANOFF [3]. This inactivation is based on the oxidation of a critical methionine residue in position 358 causing a major reduction in the anti-elastase activity of the molecule. Several antioxidants have prevented this phenomenon when added *in vitro* to systems using either cigarette smoke [5, 6] or stimulated phagocytes [8] as oxidizing agents. We report new experimental evidence indicating that RBC may be another important variable in this oxidant/antioxidant balance: in our *in vitro* model, physiological concentrations of RBC completely prevented the inactivation of α_1 -AT by cigarette smoke.

The role of RBC as possible somatic scavengers has received attention only in the last few years. Although it has long been known that antioxidants are abundant in erythrocytes, their major function was thought to be the reduction of haemoglobin-bound iron. Our results suggest that their scavenging effect goes well beyond the erythrocytic intracellular content and extends to plasma proteins, and possibly to other blood cells and surrounding tissues. This is in accord with recent investigations that have shown RBC capable of metabolizing reactive oxygen species in the extracellular environment, and also of preventing or attenuating oxidant injury to other cells. For example, RBC were able to inhibit the oxidative intracellular killing of bacteria [13], greatly reduce extracellular hydrogen peroxide concentration [14, 15], scavenge hydrogen peroxide released by stimulated phagocytes [16], prevent oxidative damage of murine leukaemia cells [17], protect pulmonary artery endothelial cells against hydrogen peroxide [18] and improve survival of rats exposed to 95% oxygen when pre-insufflated in their trachea [19].

Cigarette smoke is a complex mixture of several reactive compounds that can interact with α_1 -AT. This reaction can be divided into a fast and a slow portion. Short-lived free oxygen radicals, present in the gas phase of smoke, are responsible for the initial fast loss of functional activity of α_1 -AT [20]. This reaction could not have been responsible for the inactivation seen in our experiments because we have used aqueous smoke extract that was 10–30 min old when utilized for inhibition studies. More slowly reacting compounds are known to be present in aqueous extracts. Hydrogen peroxide is slowly formed [21] and can probably account for a substantial portion of the α_1 -AT inactivation. The protective effect of catalase in our study argues in favour of this possibility. PRYOR *et al.* [5] also showed that the addition of catalase was able to completely eliminate the reduction of EIA caused by a cigarette smoke solution while the protection afforded by superoxide dismutase was only partial. Similar results were obtained by JAMES and COHEN [6], indicating that indeed hydrogen peroxide is responsible for most of the smoke-inactivation of α_1 -AT.

The majority of the studies referenced above have identified catalase as the most active "somatic" erythrocytic antioxidant. AGAR *et al.* [17] showed that depletion of glutathione or inhibition of superoxide dismutase did not alter the protection provided by murine erythrocytes to leukaemia cells exposed to hydrogen peroxide. However, the inactivation of catalase greatly diminished this protection whilst its subsequent addition restored it. WINTERBOURN and STERN [14] also found catalase to be the most active scavenger of extracellular reagent hydrogen peroxide whilst in a different experimental model VAN ASBECK *et al.* [19] demonstrated that only the depletion of glutathione diminished the protective effect of RBC insufflated intrabronchially into rats exposed to 95% oxygen. In this experiment the inhibition of catalase and superoxide dismutase did not alter the protection provided by the insufflated RBC.

While these studies cannot easily be compared to ours, they suggest a scavenging role of RBC in situations of oxidative stress. In our model catalase appears to be the most active antioxidant. Considering that this enzyme is extremely active, even 90% inactivation (as obtained in our experiments) might be insufficient to cause a substantial reduction in the protective effect exerted by added erythrocytes. This could explain the limited (but still significant) decrease in protection when catalase-depleted RBC were used.

It is uncertain whether RBC in the vascular compartment can adequately protect the alveolar and interstitial α_1 -AT against oxidative damage. However, circulating RBC have been shown to undergo a compensatory increase in their antioxidant activity when measured in healthy cigarette smokers compared to controls [22] and this could be viewed as a protective response to an oxidative challenge. The presence of RBC in the blood and their absence in alveoli and interstitium could also explain the difficulty in consistently demonstrating oxidized α_1 -AT in peripheral blood as compared to alveolar fluid. Based on our data it could be argued that α_1 -AT cannot be effectively oxidized in the presence of physiological concentrations of RBC.

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Les érythrocytes préviennent l'inactivation de l'alpha₁ antitrypsine par la fumée de cigarette: S. Mangione, F. Kueppers, C. Puglia, L.W. Greenspon.

RÉSUMÉ: Les globules rouges sanguins (RBC) ont si cette activité était une puissante activité anti-oxydante. Nous avons étudié si cette activité était suffisante pour prévenir l'inactivation oxydative de l'alpha₁-antitrypsine (α₁-AT) par la fumée de cigarette. Nous avons trouvé que les globules rouges en concentrations physiologiques préviennent complètement l'inactivation de α₁-AT. Les anti-oxydants majeurs des érythrocytes (catalase, superoxyde dismutase et glutathione) ont été ensuite inhibés sélectivement, et les globules rouges soumis à un nouveau test. Seule l'inhibition de la catalase a réduit significativement la capacité protectrice de l'addition d'érythrocytes. Nous suggérons que les anti-oxydants des globules rouges peuvent être une variable importante pour déterminer le degré de protection de l'alpha₁-antitrypsine à l'égard de l'oxydation.

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