**Alpha_1*-protease inhibitor moderates human neutrophil elastase-induced emphysema and secretory cell metaplasia in hamsters**


**ABSTRACT:** A study was undertaken to determine whether emphysema and airway secretory cell metaplasia, induced in hamsters by intratracheal treatment with human neutrophil elastase (HNE), could be moderated by pretreatment with human alpha_1*-protease inhibitor (API). API (4.9 mg) was given intratracheally to hamsters 1 h before 0.3 mg HNE. Eight weeks later, lung volumes and pressure-volume relationships were measured in the anaesthetized animals. Mean linear intercepts and secretory cell indices were measured in lung sections. API given 1 h before HNE moderated the development of bronchial secretory cell metaplasia. The severity of emphysema was reduced by 75%. Clearance studies indicated that 80% of the functional activity of instilled API could be lavaged from the lungs after 1 h, indicating a 4 h half-life in the lavageable compartment of the lungs. We calculate that for 50% protection from emphysema the molar ratio of lavageable API to HNE at the time of HNE instillation was 4.8 as compared with 0.78 for 50% inhibition of elastolytic activity in vitro, indicating that API is only 16% as efficient in vivo as compared with its in vitro HNE inhibitory effectiveness. Nevertheless, we conclude that human API given intratracheally is efficacious against HNE-induced emphysema and secretory cell metaplasia.


Experimental emphysema can be induced in animals with enzymes that possess elastolytic activity, e.g. papain, porcine pancreatic elastase and human neutrophil elastase (HNE) [1]. In 1973, Kaplan et al. [2] found that induction of emphysema in hamsters with porcine pancreatic elastase was inhibited by mixing the elastase with normal human serum (PiM) but not with human alpha_1*-protease inhibitor (API) deficient (PiZ) serum. Purified API has also been given to hamsters intratracheally 40–60 min before exposure to an aerosol of 3% papain and found to moderate the induction of emphysema [3]. However, there have been no reported studies where human API was used in an animal model to inhibit the action of human neutrophil elastase (HNE), which is considered to be the major enzyme in the protease-antiprotease hypothesis of the pathogenesis of human emphysema.

Intratracheal administration of purified HNE induces emphysema and bronchial secretory cell metaplasia (SCM) in hamsters [4, 5]. The present experiments were designed to test the hypothesis that intratracheally administered API moderates the emphysema and SCM induced in hamsters by intratracheally instilled HNE. We also wished to compare human API with other elastase inhibitors in the hope of gaining further insight into the relationship between the in vitro and in vivo behaviour of anti-elastases with HNE.

**Methods**

**In vitro studies**

HNE (29 kDa) was purified from purulent sputum by a modification of the method of Martodam et al. [6]. The composition, properties and activity of the HNE have previously been described [4, 7, 8] and found to be 98±4% (mean±SE, n=8) by active-site titration [8].

Human API (53 kDa) was purified from plasma using a modification of the method of Pannel et al. [9] as described previously [10]. The final step involved dialysis against 1 mM ammonium carbonate buffer, pH
Three methods were used to measure the HNE inhibitory activity of API. Firstly, residual HNE activity was measured by $^3$H-elastin solubilization as described previously [8]; API addition preceded that of HNE. The second method used the synthetic substrate, succinyl trialanine p-nitroanilide (SAPNA) [14]. The third method was a $^{125}$I-HNE binding assay [14]. Briefly, a known amount of API was incubated with a 50–250% molar excess of $^{125}$I-HNE. The mixture was subjected to molecular sieve chromatography at 4°C to isolate and quantify API-$^{125}$I-HNE. The amount of $^{125}$I-HNE bound to the API was calculated from the known specific radioactivity of $^{125}$I-HNE. Correction was made for losses on the column of $^{125}$I-HNE (21±4%, n=2). Functionally active API was defined as API capable of binding and inactivating HNE. Complexes of API-HNE contain API and HNE in a 1:1 molar ratio [15].

**In vivo studies**

Thirty one male, Syrian golden hamsters, *Mesocricetus auratus* (Engle Laboratory Animals Inc., Farmersburg, IN) were placed into four weight-matched groups and given two transoral intratracheal injections 1 h apart. The animals were anaesthetized by CO$_2$ inhalation before each administration. Two groups received 1.0 ml of saline followed either by 0.5 ml saline (SAL-SAL, n=10) or by 0.3 mg of HNE in 0.5 ml of saline (SAL-HNE, n=7). The other two groups of animals received 4.9 mg of API in 1 ml of saline followed either by 0.5 ml of saline (API-SAL, n=6) or by 0.3 mg of HNE (API-HNE, n=8) in 0.5 ml saline.

All animals were studied 56 days after treatment. Lung volumes and quasi-static deflation pressure-volume relationships were measured in animals anaesthetized with sodium pentobarbital. The procedures have been published previously [4, 5]. After completion of the physiological measurements, the animals were exsanguinated. The lungs were inflated fixed, in situ, by injecting 5 ml of fixative (4% formalin - 1% glutaraldehyde). Transverse sections of the lungs were embedded in paraffin and 5–6 μm sections of the left lungs were stained for microscopic examination with haematoxylin-eosin (HE) or with the alcian blue/priodic acid-Schiff (AB-PAS) reaction. The secretory cell index (SCI), a semi-quantitative grading from 0–4 of secretory cell metaplasia [16], and the mean linear intercept (MLI) were determined on the AB-PAS and HE stained slides, respectively, using methods described previously [16, 17].

The effectiveness of API in inhibiting emphysema induction was assessed by calculating the index of effectiveness using a 50% weighting for anatomical severity of emphysema (determined by the MLI) and 50% weighting for functional abnormalities compatible with emphysema (determined by functional residual capacity and vital capacity) [17]. The following equation was used, with the means of the various groups. The groups are indicated in the subscripts.

$$\text{Index of effectiveness} = \frac{VC_{\text{API-HNE}}}{VC_{\text{API-SAL}}} \times 25 + \frac{FRC_{\text{API-HNE}}}{FRC_{\text{API-SAL}}} \times 25 + \frac{MLI_{\text{API-HNE}}}{MLI_{\text{API-SAL}}} \times 50$$

Retention of functionally active API in the lavageable compartment of the lungs was studied in three hamsters that had been instilled with 0.5 ml of saline containing 2.5 mg of API; three hamsters received saline only. One hour after instillation, each hamster was anaesthetized with sodium pentobarbital and the lungs lavaged with three 5 ml aliquots of saline. Bronchoalveolar lavage fluid (BAL) was centrifuged to remove the cells and the
Supernatant was assayed for HNE inhibitory activity using \(^{3}H\)-elastin. The amount of functionally active API present in the BAL supernatant was estimated by comparing inhibition of HNE with a standard curve obtained using varying amounts of stock API solution diluted in saline. The values were corrected for the almost negligible inhibition measured in the BAL of saline-treated hamsters. The half-life of API in the BAL was calculated using second order disappearance kinetics [7].

The SCI data were analysed statistically by the nonparametric Kruskal Wallis test [18]. Differences among means of the other data were analysed by analysis of variance [19]. Probability values of \(p<0.05\) were considered significant. Values are expressed as mean±se.

**Results**

**In vitro**

Titration of API with active-site titrated trypsin (81±2%, \(n=2\), active) indicated that 0.9±0.1 moles (n=4) of API per mole of active trypsin were required for complete inhibition of the trypsin-catalysed hydrolysis of TAME. The HNE inhibitory activity of API measured with \(^{3}H\)-elastin or SAPNA as substrates is presented in Figure 2. The elastolytic activity was inhibited in a linear, dose-response fashion, except below 20% residual elastolytic activity (fig. 2). A straight line extrapolation of the linear portion of this titration plot intersects the abscissa at an API/HNE molar ratio of 1.60 and 1.51 for the \(^{3}H\)-elastin and SAPNA assays, respectively (fig. 2). The HNE-API binding studies indicated that for each 1.56±0.12 (n=2) moles of API in the initial incubation mixture, 1.0 mole of API was recovered in the isolated API-\(^{3}H\)-HNE complex.

**In vivo**

The intratracheal instillation of HNE caused emphysema. Hamsters given saline followed 1 h later by 0.3 mg HNE (positive control group), had an increase of 40% in MLI above the mean for control animals receiving saline followed 1 h later by saline (fig. 3A). The volume of air in the lungs at 25 cmH\(_{2}\)O transpulmonary pressure (total lung capacity (TLC)) increased by 18% and the functional residual capacity (FRC) increased by 79% (fig. 3B).

Pretreatment of hamsters with 4.9 mg of API 1 h before 0.3 mg human neutrophil elastase (HNE), on the mean±se values for mean linear intercept for four groups of hamsters. In the labels of the groups given on the abscissa, the initial treatment is given above, followed 1 h later by the treatment given below. The asterisks indicate a significant difference from the SAL-1 h-HNE control group. In the labels of the groups given on the abscissa, the initial treatment is given above, followed 1 h later by the treatment given below. The asterisks indicate a significant difference from the SAL-1 h-HNE control group. SAL: saline; API: alpha, protease inhibitor; SAPNA: succinyl trialanine p-nitroanilide.
and by other investigators for porcine pancreatic elastase of 1.5-1.8 previously reported by us for HNE [1, 14] in moderating HNE-induced emphysema in hamsters [24]. The reasons for the differences are not clear.

The API clearance studies indicated that 2.0±0.2 mg (n=3) of API were recovered in functionally active form in lavage fluid 1 h after the instillation of 2.5 mg of API. This is an 80% recovery. The calculated half-life in the lavageable compartment of the lungs is 4 h.

Fig. 4. - The secretory cell index values for the central intrapulmonary airways of the left lung of hamsters. Treatment groups are as in figure 3. The SCI values were assigned based on the appearance of the airway epithelium of three sections of the left lung. Zero represents no stained secretory material, 1 represents slight, 2 moderate, 3 severe and 4 very severe increase in the amount of stained secretory material in the airway epithelium. An asterisk indicates a significant difference from the SAL-1 h-HNE control group (Kruskal-Wallis test). SAL: saline; API: alpha_1-proteinase inhibitor; HNE: human neutrophil elastase.

Discussion

Three methods were used to determine the molar ratios of API to HNE that should result in complete inhibition of HNE. The mean of the three values was 1.56±0.03 (mean±SE). This compares with molar ratios of 1.5-1.8 previously reported by us for HNE [11, 14] and by other investigators for porcine pancreatic elastase [20-22]. The excess of API required has been attributed to nonproductive proteolysis of API by elastase rather than the presence of inactive API in the purified preparation [20]. In contrast, some workers have reported that only 1.0 mole of API per mole HNE is required to inhibit HNE under similar conditions [23, 24]. The reasons for the differences are not clear.

The present study showed API to be effective in vivo in moderating HNE-induced emphysema in hamsters when given intratracheally 1 h before the HNE. The assumed mechanism of emphysema-inhibition when API is given before elastase is that a sufficient quantity of active API remains in the lung fluids to inactivate the instilled HNE [17]. From our clearance data, at least 80% of the instilled API was still present 1 h later. This is a minimum value since there was probably more API in the lung than came out in the BAL.

The index of effectiveness for 4.9 mg of API given 1 h before 0.3 mg (10.3 nmol) HNE was 75%. Assuming a linear relationship between this point and the origin (0%), the amount of API needed for an index of effectiveness of 50% would be 3.3 mg. It can be calculated from the clearance data that at the time of HNE instillation, 2.6 mg (49.8 nmol) of functionally active API was present in the lavageable compartment. This is a molar ratio of API to HNE of 4.8.

The relative efficiency of an inhibitor in vivo vs in vitro can be expressed as the molar ratio of inhibitor to HNE for 50% inhibition in vitro divided by the molar ratio for 50% protection in vivo x100%. API has an efficiency of 0.78/4.8x100% or 16%. By comparison eglin-c has an efficiency of 40% [17], succinyl-Ala-Ala-Pro-Val chloromethyl ketone (CMK) has an efficiency of 34% [25] and secretory leucocyte protease inhibitor has an efficiency of 24% (unpublished data). The lower in vivo than in vitro effectiveness of these agents may be due, in part, to inhomogeneous distribution of the instillates in hamsters’ lungs [5].

The calculation of relative effectiveness can also take into account the duration of the inhibitor in the lavageable compartment of the lungs in a functionally active form 1 h after instillation of the inhibitor. This is the moment that HNE is instilled. For this calculation of “persistent effectiveness” the percentage efficiency is multiplied by the fractional amount of functional inhibitor remaining in the lavageable compartment 1 h after instillation. For API this value is 0.8, so the percentage persistent effectiveness is 13% as compared with 14% for SLPI, 17% for eglin-c and 2% for CMK.

HNE and other serine proteases cause SCM when given intratracheally to hamsters [4, 26, 27]. The enzymes must be enzymatically active to have this effect [26]. API given 1 h before HNE is completely effective in moderating HNE-induced SCM. There is probably more homogeneous distribution of the antiprotease on the bronchial mucosa of the large airways than further out in the lung parenchyma. Eglin-c given 1 h before HNE has also been shown to significantly moderate HNE-induced SCM [4]. CMK did not have this effect [25]. The lack of effectiveness of CMK with regard to SCM may relate to its low molecular weight and, hence, its rapid absorption and clearance from the airway surface [25].

A variety of antielelastases including API are currently undergoing investigation as possible agents to be used in the prevention of emphysema. It has been shown that weekly intravenous infusions of API into API-deficient individuals can restore blood and BAL values of API to an average level that is computed to be well within the normal range and more than twice as high as the
threshold value that is estimated on epidemiological grounds, to be necessary for protection of the lungs against the development of emphysema [28]. The results of the experiments described here support the hypothesis that intratracheally administered API moderates the emphysema and SCM induced in hamsters by intratracheally instilled HNE. However, it is not known whether the neutrophil elastase, which putatively gives rise to human emphysema, is released while the neutrophil is adherent to the alveolar capillary endothelium, while the neutrophil is migrating through the alveolar wall into the alveolar space, or after the neutrophil has reached the alveolar space; our experiments pertain primarily to the last possibility. We think it likely that if HNE plays a role in human SCM, the enzyme reaches the secretory cells from the airway lumen. It has been shown that administration of recombinant API by aerosol was effective in augmenting the lower respiratory tract concentration of API in API-deficient individuals [29]. In view of the foregoing considerations, administration of an aerosol of API seems entirely rational as replacement therapy designed to prevent further development of emphysema and bronchial SCM in severely API-deficient individuals. Its potential role as preventive therapy for persons with adequate protective levels of API who cannot stop smoking is much less clear.

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References


RÉSUMÉ: Cette étude a été entreprise pour déterminer si l'émphyséme et la métaplasie des cellules sécrétoires des voies aériennes, induits chez les hamsters par un traitement intra-trachéal au moyen d'élastase neutrophilique humaine (HNE), pouvaient être modérés par un traitement préalable au moyen d'inhibiteur de l'alpha2-protease humaine (API). API (4.9 mg) a été administré par voie intra-trachéale à des hamsters 1 h. avant 0.3 mg de HNE. Huit semaines plus tard, les volumes pulmonaires et les rapports pression-volume ont été mesurés chez les animaux anesthésiés. L'on a mesurés, dans les coupes de poumon, les intercepts linéaires moyens et les indices de cellules sécrétoires. API, donné 1 h. avant HNE, modère le développement de la métaplasie des cellules sécrétoires bronchiques. La gravité de l'émphyséme est réduite de 75%. Les études de clearance indiquent que 80% de l'activité fonctionnelle de l'API instillé pouvaient être lavés des poumons après 1 h., ce qui indique une demi-vie de 4 h. dans le compartiment lavable des poumons. Nous calculons que pour une protection de 50% à l'égard de l'émphyséme, le rapport molaire du API/HNE lavable au moment de l'instillation de HNE, est de 4.8 par comparaison avec 0.78 pour une inhibition de l'activité elastolytique in vitro. Ceci indique que API n'a in vivo qu'une efficacité de 16% par comparaison à son efficacité inhibitrice de HNE in vitro. Néanmoins, nous concluons que l'API humaine donné par voie intra-trachéale est efficace contre l'émphyséme induit par HNE, et contre la métaplasie des cellules sécrétoires. *Eur Respir J.*, 1990, 3, 673-678.