Amelioration of human neutrophil elastase-induced emphysema in hamsters by pretreatment with an oligopeptide chloromethyl ketone


ABSTRACT: Human neutrophils are a likely source of elastase in the pathogenesis of human pulmonary emphysema. A study was undertaken to determine whether emphysema, induced in hamsters by intratracheal treatment with human neutrophil elastase (HNE), could be ameliorated by intratracheal instillation of succinyl-alanyl-alanyl-prolyl-valine-chloromethyl ketone (CMK). One mg of CMK was given to hamsters 1 h before 360 or 360 μg HNE or 1 h or 4 h after 360 μg HNE. The animals were studied eight weeks after treatment. The CMK given 4 h after HNE did not ameliorate the emphysema. The CMK given 1 h before HNE, ameliorated the development of emphysema but not bronchial secretory cell metaplasia. A molar ratio of instilled CMK to HNE of 128 was required for 50% in vivo effectiveness in ameliorating emphysema. Clearance studies indicated that 6.9% of the instilled CMK could be lavaged from the lungs 1 h after instillation. Therefore, an 8.9 to 1 molar ratio of lavageable CMK to HNE, at the time of HNE instillation, resulted in 50% protection. Using an in vitro assay with 3H-elastin as substrate, a 3 to 1 molar ratio of CMK to HNE was required to inhibit 50% of the elastolytic activity; 14% of the activity remained with an 18 to 1 molar ratio of CMK to HNE. Study of the in vivo effectiveness of anti-elastases, given as pretreatment in ameliorating HNE-induced emphysema and secretory cell metaplasia, is a reasonable bioassay, which may be used as a step in evaluating such agents for possible use in the prevention of human disease.


It has been shown that intratracheal instillation of succinyl-alanyl-alanyl-prolyl-valine-chloromethyl ketone (CMK) moderates the emphysema induced in hamsters by intratracheal instillation of porcine pancreatic elastase (PPE) [1]. Other oligopeptide chloromethyl ketone compounds have also been found to be effective in ameliorating PPE-induced emphysema when given orally [2] or intraperitoneally [3, 4]. When a crude extract of human neutrophils was used to induce emphysema, an oligopeptide chloromethyl ketone compound, given by aerosol, ameliorated the emphysema induced [5].

Human neutrophil elastase (HNE) induces much less severe emphysema than an equivalent dose of PPE, whether equivalence is based on moles of elastase or in vitro elastolytic activity [6-8]. Both enzymes induce secretory cell metaplasia in hamsters [8, 9]. Using a living smooth muscle cell culture as a substrate, HNE exhibited a 10-fold lower elastolytic activity as compared with PPE [10]. With purified elastin substrates HNE and PPE exhibit different peptide bond specificities but similar elastolytic activity. In view of these results and the fact that human neutrophils are a probable source of elastase in the pathogenesis of emphysema, it seemed appropriate to determine whether emphysema and secretory cell metaplasia induced by purified HNE could be ameliorated by intratracheally administered CMK and whether the effectiveness of CMK in preventing elastolysis in vitro was helpful in predicting the in vivo effectiveness in the HNE-induced emphysema model.

Materials and methods

In vitro

Human neutrophil elastase (HNE) (29 kd) was purified from purulent sputum by the method of Martoglio et al. [11]. The amino acid composition of our preparations was similar to that reported by Tsuchida and Liener [12], with a mean deviation in residues per mole of 1.4. There was a single band with a molecular mass of 29 kd on a sodium dodecyl sulphate polyacylamide gel and there were 4 bands in close proximity that stained for protein, carbohydrate and elastase-like activity on an analytical disc gel (pH 4.5). Our preparation was 98±4% (mean±SEM, n=8) active as determined by active site titration [13].
The optical density at 280 nm was used to determine the enzyme concentration in solutions,

\[ E_{1}^{16} \text{ cm, 280 nm} = 9.85. \]

Porcine pancreatic elastase (PPE) was prepared according to the method of Sutoron [14] and assayed as previously described [15, 16]. It exhibited greater than 90% activity.

The succinyl-alanyl-alanyl-prolyl-valine-chloromethyl ketone (CMK) (488 daltons) was synthesized according to published procedures [17]. It readily dissolved in 0.15 M saline and was used within 30 min.

The in vitro inhibitory effectiveness of the CMK was assessed using a modification of a standard elastolytic assay procedure, omitting sodium dodecyl sulphate [18]. Briefly, culture tubes containing 5 mg of 3H-insoluble ligamentum nuchae elastin, an excess of substrate that produced a near maximal rate, and 4 ml of filtered (0.22 \( \mu \)m) assay buffer (50 mM sodium phosphate, pH 7.35, 140 mM sodium chloride, 0.5 mg/ml bovine serum albumin) were incubated for 4 h at 37°C, alone (blank) or with 10 \( \mu \)g of HNE or 10 \( \mu \)g of PPE. To other tubes, increasing amounts of CMK were added before the elastases.

After incubation the supernatants were filtered and assessed for 3H-elastin peptides by liquid scintillation spectrometry. The concentration of CMK resulting in 50% inhibition (IC\(_50\)) of the HNE was determined by interpolation. In the absence of inhibitor, 1 nmol of HNE solubilized 407 \( \mu \)g h\(^{-1}\) of elastin, whereas 1 nmol of PPE solubilized 186 \( \mu \)g h\(^{-1}\) of elastin. The stability of the CMK in the assay system was assessed by preincubating CMK (384 nmol) for 1 h at 37°C in the culture tube containing elastin and buffer before adding HNE and incubating for an additional 4 h. Controls included tubes to which CMK was added followed immediately by HNE and a 4 h incubation period.

\(^{14}\)C-CMK (2.58 mCi mmol\(^{-1}\); MW of 490) was prepared by reaction of H-alaala-pro-val-CH\(_2\)Cl with \(^{14}\)C-succinic anhydride [17] and used for active site titration of the elastases as follows. Three mg of HNE or PPE was incubated with 1.5 mg of \(^{14}\)C-CMK in assay buffer without albumin at 4°C for 16 h, then at 37°C for 1 h, and dialysed at 4°C. The concentration of elastase was determined from the absorption of light at 280 nm. Aliquots were assessed for radioactivity and elastolytic activity.

Alkylolation of bovine serum albumin by CMK was assessed by incubating 15 nmol of albumin with 239 nmol of \(^{14}\)C-CMK at 15 min at 37°C in 1 ml of column buffer (0.05 M tris, pH 7.6, 0.6 M NaCl, 0.05% NaN\(_3\)). The solution was loaded on a column packed with Sephadex G-100 that had been calibrated with molecular weight standards. Eluted material was collected using a fraction collector and fractions were assessed for radioactivity. Non-specific alkylolation of HNE that had been previously treated with unlabelled CMK, dialysed and lyophilized (HNE-CMK) was assessed by incubating 17 nmol of HNE-CMK with 220 nmol of \(^{14}\)C-CMK and chromatographing as above.

**In vivo**

Retention in the lungs of functionally active CMK after instillation was studied as follows. Three hamsters were instilled with 0.5 ml of saline containing 1 mg of freshly dissolved CMK. Another three hamsters received saline only. One hour after instillation, each hamster was anaesthetized with sodium pentobarbital and the lungs lavaged three times with 5 ml aliquots of saline as previously described [15]. Bronchoalveolar lavage (BAL) fluid was centrifuged to remove the cells. The amount of functionally active CMK present in the BAL supernatant was estimated by comparing inhibition of HNE with a standard inhibitor curve after correction for inhibition obtained with the control BAL (almost negligible). These data were used to calculate the half-life of CMK in the BAL assuming second order disappearance kinetics as we have previously found for PPE-CMK [15] and eglin [19]. Second order kinetics uses the relationship:

\[ \frac{1}{C} - \frac{1}{C_0} = k \cdot t \]

where \( C_0 \) is the amount at the start, \( C \) is the amount t min later and \( k \) is the constant.

To determine the protective effects of CMK against HNE-induced emphysema, 84 male, Syrian, golden hamsters, *Mesocricetus auratus* (Engle Laboratory Animals, Inc., Farmersburg, IN) were given two transoral intratracheal injections, 1 or 4 h apart. The animals were anaesthetized by CO\(_2\) inhalation before each administration of either 0.5 ml of saline or a saline solution containing 1 mg of CMK, 300 \( \mu \)g of HNE or 360 \( \mu \)g of HNE.

These experiments were carried out in two parts. In the first protocol the effects of CMK given 1 h before, 1 h after or 4 h after 360 \( \mu \)g of elastase were studied. There was a high mortality among the animals given 360 \( \mu \)g of HNE followed in 1 h by CMK (6 out of 8 died) or by saline (4 out of 8 died). There were also two deaths among the eight animals given 360 \( \mu \)g of HNE followed in 4 h by CMK. Another hamster died after receiving 360 \( \mu \)g of HNE 1 h after CMK. All deaths were due to pulmonary haemorrhage and occurred within 4 h of the second treatment. In the second protocol only the effect of pretreatment with CMK 1 h before HNE was investigated and the dose of elastase was lowered to 300 \( \mu \)g in order to reduce the probability of death. There were no deaths in this protocol.

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. Details of the procedure have been published elsewhere [8, 20]. After completion of the physiological measurements, the animals were
extrapolations of alanyl-prolyl-valine-chloromethyl ketone. The activity of the elastolytic phospholipase A2; PJ>E:

Fig. 1. - Examples of the titration of 10 μg HNE (open triangles) and PPE (open circles) by CMK, showing the percentage inhibition of elastolytic activity against 1H-elastin substrate. The dash lines are linear extrapolations of the steep portions of the plots. HNE: human neutrophil elastase (HNE), on the mean±SEM values of mean linear intercept (MLI) for six groups of hamsters. The data are from two experiments using two different doses of HNE. The values for the three groups in the protocol using 300 μg of HNE are represented by the open bars. The diamond indicates a significant difference of the saline-1 h-HNE groups from the saline-1 h-saline groups and the CMK-1 h-HNE groups. Sample sizes are in parentheses.

Fig. 2. - Bar diagram showing the effect of treatment with 1.0 mg of succ-ala-ala-pro-val-chloromethyl ketone (CMK, 1 h before human neutrophil elastase (HNE), on the mean±SEM values of mean linear intercept (MLI) for six groups of hamsters. The data are from two experiments using two different doses of HNE. The values for the three groups in the protocol using 300 μg of HNE are represented by the open bars. The diamond indicates a significant difference of the saline-1 h-HNE groups from the saline-1 h-saline groups and the CMK-1 h-HNE groups. Sample sizes are in parentheses.

Results

In vitro

CMK was stable in our in vitro assay. When CMK was incubated in the presence of substrate and buffer for 1 h before adding the HNE, there was no detectable loss of inhibitory activity as compared to the standard procedure of adding CMK immediately before the HNE (54.4±0.7% inhibition, n=3, as compared with 58.8±2.4% inhibition, n=3, respectively).

There appears to be a sharp inflection in the curves relating percentage inhibition to a molar ratio (fig. 1). For HNE this inflection occurred at a molar ratio of about 4 (60% inhibition), and for PPE at a molar ratio of about 1.6 (80% inhibition). At higher molar ratios the increase in inhibition with added inhibitor was much less. For PPE there was complete inhibition at a molar ratio of about 7.7; CMK appeared unable to completely inhibit HNE even at CMK to HNE molar ratios as high as 18 to 1.

A least squares analysis of the steep portion of the plots, indicates that 261±53 nM CMK (mean of 2 determinations) was required to inhibit 50% of the elastolytic activity of 88 nM HNE (a 3 to 1 molar ratio of CMK to HNE). For PPE a molar ratio of 0.7 was sufficient for 50% inactivation. The regression line extrapolated to 100% inhibition with a CMK/HNE molar ratio of 7.4 (r=-0.99) and a CMK/PPE molar ratio of 1.3 (r=-0.99).

Incubation of HNE with 14C-CMK resulted in a 14C radioactivity peak that co-eluted with HNE during chromatography. With saturating amounts of 14C-CMK the HNE-14C-CMK complex had a specific radioactivity of 2.12±0.04 mCi·mmol-1 (n=4), 82±2% of that of the 14C-CMK preparation. The HNE-14C-CMK complex was
devoid of elastolytic activity. The $^{14}$C-labelled PPE exhibited specific radioactivity of $2.01\pm0.23\text{ mCi}\cdot\text{mmol}^{-1}$ ($n=2$) and no measurable elastolytic activity. The irreversibility as well as the specificity of the labelling of HNE by CMK was also demonstrated by the following experiment. When HNE-CMK was incubated with $^{14}$C-CMK and chromatographed, no measurable $^{14}$C radioactivity co-eluted with the HNE-CMK. In the experiment with albumin only 0.5% of the radioactivity co-eluted with the albumin.

In vivo

One hour after instillation of CMK, 6.9% could be lavaged out as functionally active. This gives a 4.5 min half-life for CMK in the lavage compartment using second order disappearance kinetics:

$$t_{1/2}=(1/50\%-1/100\%)/2.25\times10^3\cdot\text{min}^{-1}\cdot\text{m}^{-1}.$$

The intratracheal instillation of either 300 or 360 μg of HNE caused emphysema. Hamsters given saline, followed in 1 h by 360 μg of HNE, had an approximate 63% increase in MLI above the mean for control animals receiving saline followed in 1 h by saline (fig. 2). The total lung capacity at 25 cmH$_2$O transpulmonary ($TLC_{25}$) increased by about 24% and the functional residual capacity (FRC) increased by approximately 60% (fig. 3). Hamsters given 300 μg of HNE 1 h after saline had a 34% increase in MLI (fig. 2), a 15% increase in $TLC_{25}$, and a 43% increase in FRC (fig. 3). All of these differences were significant.

Pretreatment of hamsters with CMK 1 h before 300 or 360 μg of HNE ameliorated the emphysema. The mean values for MLI, FRC and residual volume at a transpulmonary pressure of -20 cmH$_2$O ($RV_{-20}$) were significantly less in the CMK followed in 1 h by HNE (360 and 300 μg) groups than in the saline followed in 1 h by HNE (360 and 300 μg) groups, respectively (figs 2 and 3).
functionally active form in BAL 1 h after CMK instillation, a molar ratio of 8.9 was apparently present at the time of HNE administration.

Treatment with CMK 4 h after 360 µg of HNE had no measurable effect on the severity of emphysema (fig. 4). Since there were only two surviving animals in the group given 360 µg of HNE followed in 1 h by CMK, statistical analysis was not attempted.

The intratracheal instillation of 300 µg of HNE caused secretory cell metaplasia in the hamster (fig. 5). Treatment of the hamsters with CMK 1 h before HNE instillation resulted in a small but insignificant decrease of the SCI compared to the group receiving saline followed in 1 h by HNE (fig. 5). SCI measurements were not made on the animals in the first protocol given 360 µg of HNE.

Discussion

Peptide chloromethyl ketones are widely utilized in vitro as irreversible inhibitors of serine proteases [23]. Inhibition occurs by formation of covalent bonds between the active site histidine (His-57) and serine (Ser-195) of the enzyme and the methylene group and ketonic carbonyl group, respectively, of the inhibitor. CMK is an excellent inhibitor of HNE and PPE in vitro, although the reaction with the latter is 2–3 fold slower [17, 24].

In vivo studies

The present study showed CMK to be effective in vivo in ameliorating the induction of HNE-induced emphysema in hamsters when given intratracheally 1 h before the HNE. The assumed mechanism of emphysema inhibition when CMK is given 1 h before elastase is that a sufficient quantity of active CMK remains in the lung fluids to inactivate free HNE or alpha 2-macroglobulin bound HNE [26] before the HNE has the opportunity to cause elastolytic damage.

As with PPE-induced emphysema [1], HNE-induced emphysema was not ameliorated by treatment with CMK given 4 h after intratracheal instillation of HNE. We interpret this to mean that the enzymatic damage that leads to emphysema is largely complete by 4 h after intratracheal instillation of HNE. We assume that because of the extensive lung haemorrhage caused by HNE, the hamsters could not tolerate a second injection, 1 h after the HNE.

HNE, as well as other serine proteases, causes secretory cell metaplasia when given intratracheally to hamsters [8, 27, 28]. We know that HNE treated with CMK before intratracheal instillation, does not cause a lesion [27]. When CMK was given intratracheally 1 h before the HNE, some animals appeared to be protected whereas others were not; this variability may relate to distribution of instilled CMK to different regions of the lungs than the instilled HNE.

By comparison with our published values for eglin-c, a polypeptide elastase inhibitor, CMK is not as efficient a moderator of emphysema and secretory cell metaplasia induction. With a 1 h interval between instillations, the at the active site with complete and irreversible loss of elastolytic activity. The CMK did not react significantly with albumin during the course of a 15 min incubation. However, previous studies have shown that a related chloromethyl ketone will react with nucleophiles such as glutathione [17] and more slowly with peptides or free amino acids [25] with destruction of the inhibitor. The reaction rate of a related chloromethyl ketone with glutathione is 1800-fold slower than the elastase inhibition rate [23]. We observed no loss of CMK inhibitory activity when the CMK was preincubated with elastin.

A molar ratio of CMK to PPE of 0.7 was required for 50% inactivation, an almost stoichiometric relationship (fig. 1). By comparison, a 3-fold molar excess of CMK was required to inhibit 50% of the elastolytic activity of HNE, under the conditions we employed. The reasons for this are not clear but include the possibility that the reaction with HNE in the presence of elastin is not complete by 4 h.

CMK is a 2–3-fold faster inhibitor of PPE than HNE in the absence of elastin. The presence of elastin decreases the HNE inhibition rate of CMK by over 7-fold and the PPE rate by only 2-fold [24]. This may explain why CMK is a more effective inhibitor of PPE than HNE in the presence of elastin, as observed in figure 1.

In vivo studies

The present study showed CMK to be effective in vivo in ameliorating the induction of HNE-induced emphysema in hamsters when given intratracheally 1 h before the HNE. The assumed mechanism of emphysema inhibition when CMK is given 1 h before elastase is that a sufficient quantity of active CMK remains in the lung fluids to inactivate free HNE or alpha 2-macroglobulin bound HNE [26] before the HNE has the opportunity to cause elastolytic damage.

As with PPE-induced emphysema [1], HNE-induced emphysema was not ameliorated by treatment with CMK given 4 h after intratracheal instillation of HNE. We interpret this to mean that the enzymatic damage that leads to emphysema is largely complete by 4 h after intratracheal instillation of HNE. We assume that because of the extensive lung haemorrhage caused by HNE, the hamsters could not tolerate a second injection, 1 h after the HNE.
molar ratio of CMK to HNE required for an index of effectiveness of 50% was 128, which is considerably higher than the molar ratio of 3.3 for eglin-c [13], under comparable conditions. This 40-fold lower efficiency of CMK in vivo compared to eglin-c is not fully explained by the inhibitory capacities of the agents seen in vitro. At IC50, the inhibitor/HNE molar ratio was 3.0 for CMK, compared to 0.5 for eglin-c [13], only a 6-fold change.

Possible explanations for the lower than expected in vivo efficiency of CMK compared to eglin-c include the more rapid clearance of the much smaller CMK molecule from the lungs during the 1 h interval between administration of inhibitor and HNE; the inactivation of CMK in the lungs by peptidases; or alkylation reactions and destruction of the inhibitor by reaction with physiological nucleophiles. Both ends of the CMK molecule are blocked and it should thus be inert to carboxypeptidases and aminopeptidases. An elastase could cleave the ala-ala bond of CMK, but the presence of the pro residue practically precludes this possibility. CMK has a 4.5 min functional half-life in the lavageable compartment of the lungs as compared with more than 35 min for eglin-c [19].

Using the BAL clearance data to estimate the amount of CMK present in the lungs 1 h after instillation, the molar ratio of CMK to HNE required in the lung at the time of HNE instillation for 50% protection from emphysema induction was 8.9. The inefficiency of the CMK in vivo as compared to in vitro can be expressed as the above mentioned molar ratio divided by the molar ratio of CMK to HNE required for 50% in vitro inhibition of elastolysis; for CMK the inefficiency is 8.9/3.0=2.9 as compared to 1.25/0.5=2.5 for eglin-c [19].

Oligopeptide chloromethyl ketones are highly reactive and potentially toxic molecules [29] that will never find a use as prophylactic agents in human emphysema and airway hypersecretion. Their irreversible inactivation of HNE is useful in exploring the in vivo behaviour of anti-elastases and modes of therapeutic intervention in animal models of emphysema [24, 30]. The current study and a previous one [19] indicate that post-treatment with an anti-elastase is not an effective protocol for the measurement of in vivo effectiveness of anti-elastases against HNE. Study of the in vivo effectiveness of anti-elastases, given as pretreatment in ameliorating HNE-induced emphysema and secretory cell metaplasia is a reasonable bioassay, which may be used as a step in evaluating such agents for possible use in the prevention of human disease.

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References


RÉSUMÉ: Les neutrophiles humains sont une source possible d’élâstase responsable de la pathogénèse de l’emphysème pulmonaire humain. Une étude a cherché à déterminer si l’emphysème, induit chez les hamsters par un traitement intratrachéal au moyen d’élâstase neutrophile humaine (HNE), pouvait être amélioré par l’instillation intratrachéale de succinyl-alanyl-alanyl-prolyl-valine-chlorométhyl cétoné (CMK).

Un mg de CMK a été donné aux hamsters 1 heure avant l’administration de 300 ou 360 µg de HNE ou 1 heure ou encore 4 heures après 360 µg de HNE. Les animaux ont été étudiés 8 semaines après le traitement. CMK donné 4 heures après HNE n’améliore pas l’emphysème. Le CMK donné 1 heure avant HNE limite le développement de l’emphysème, mais non pas la métaplasie des cellules sécrétaires bronchiques. Une relation molaire de CMK/HNE instillés de 128 est nécessaire pour une efficacité de 50% dans l’amélioration de l’emphysème in vivo. Les études de clearance ont indiqué que 6.9% de CMK instillés pouvaient être lavés à partir des poumons 1 heure après l’instillation. Dès lors, une relation de 8.9 sur 1 molaire de CMK sur HNE lavable au moment de l’instillation de l’HNE entraîne une protection de 50%. Au cours d’expérimentations in vitro utilisant la 3H-élastine comme substrat, une relation 3 sur 1 molaire de CMK sur HNE s’avère nécessaire pour inhiber 50% de l’activité élastolytique; 14% de l’activité persistant lorsqu’une relation molaire CMK sur HNE de 18 sur 1 est observée. L’étude de l’efficacité in vivo des anti-élâstases, donnés comme prétraitement pour améliorer l’emphysème induit par HNE et la métaplasie des cellules glandulaires, est une expérimentation raisonnable, qui peut être utilisée comme étape dans l’évaluation de ces agents, en vue d’une utilisation possible pour la prévention de la maladie humaine. Eur Respir J., 1989, 2, 421-427.