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Hepatocyte Uptake of α_1-Proteinase Inhibitor-Trypsin Complexes In Vitro: Evidence for a Shared Uptake Mechanism for Proteinase Complexes of α_1-Proteinase Inhibitor and Antithrombin III

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In vivo clearance studies have indicated that the clearance of proteinase complexes of the homologous serine proteinase inhibitors α_1-proteinase inhibitor and antithrombin III occurs via a specific and saturable pathway located on hepatocytes. In vitro hepatocyte-uptake studies with antithrombin III-proteinase complexes confirmed the hepatocyte uptake and degradation of these complexes, and demonstrated the formation of a disulfide interchange product between the ligand and a cellular protein. We now report the results of in vitro hepatocyte uptake studies with α_1-proteinase inhibitor-trypsin complexes. Trypsin complexes of α_1-proteinase inhibitor were prepared and purified to homogeneity. Uptake of these complexes by hepatocytes was time and concentration-dependent. Competition experiments with α_1-proteinase inhibitor, α_1-proteinase inhibitor-trypsin, and antithrombin III-thrombin indicated that the proteinase complexes of these two inhibitors are recognized by the same uptake mechanism, whereas the native inhibitor is not. Uptake studies were performed at 37°C with ^{125}I-α_1-proteinase inhibitor-trypsin and analyzed by sodium dodecyl sulfate-gel electrophoresis in conjunction with autoradiography. These studies demonstrated time-dependent uptake and degradation of the ligand to low molecular weight peptides. In addition, there was a time-dependent accumulation of a high molecular weight complex of ligand and a cellular protein. This complex disappeared when gels were performed under reducing conditions. The sole cysteine residue in α_1-proteinase inhibitor was reduced and alkylated with iodoacetamide. Trypsin complexes of the modified inhibitor were prepared and purified to homogeneity. Uptake and degradation studies demonstrated no differences in the results obtained with this modified complex as compared to unmodified α_1-proteinase inhibitor-trypsin complex. In addition, the high molecular weight disulfide interchange product was still present on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of solubilized cells. Clearance and clearance competition studies with α_1-proteinase inhibitor-trypsin, alkylated α_1-proteinase inhibitor-trypsin, antithrombin III-thrombin, and antithrombin III-factor IX$_a$ further demonstrated the shared hepatocyte uptake mechanism for all these complexes.

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Human α_1-proteinase inhibitor (α_1PI, formerly α_1-antitrypsin) is a single-chain glycoprotein of $M_r \sim 53{,}000$ [1], which is capable of inhibiting most serine proteinases, including trypsin, chymotrypsin, pancreatic and neutrophil elastase, thrombin, plasmin, factor X_a and factor XI_a [2–7]. These serine proteinases attack a reactive Met-Ser bond near the carboxyl terminus of α_1PI [8,9], leading to the formation of a 1:1 complex [10]. The precise nature of the interaction between the proteinase and α_1-proteinase inhibitor is unknown, although the complexes are not dissociated by sodium dodecyl sulfate (SDS), implying that it is a covalent bond [11]. The complex can be dissociated at high pH or in the presence of nucleophiles such as hydroxylamine [12,13]. The binding of proteinase to α_1-proteinase inhibitor is accompanied by conformational change, as evidenced by circular dichroism and ultraviolet difference spectroscopy [2].

The clearance of α_1PI-proteinase complexes has been examined in rats [14,15], and in mice [16]. These studies demonstrate rapid clearance of α_1PI-proteinase complexes, in contrast to the relatively slow clearance of native α_1PI. α_1PI-trypsin complexes are cleared from the murine circulation with a t½ of less than 20 min [16]. In addition, the clearance of α_1PI-trypsin can be blocked by simultaneous injection of a large molar excess of either α_1PI-trypsin or antithrombin III-thrombin (ATIII-thrombin), but not α_2-macroglobulin-methylamine (α_2M-CH_3NH_2) or native α_1PI [16]. Previous studies demonstrated that the uptake mechanism for the clearance of ATIII-proteinase complexes is located on hepatocytes, and that carbohydrate recognition is not involved in this clearance [17]. These studies indicate that proteinase complexes of the homologous proteinase inhibitors, α_1PI and ATIII, are cleared by the same uptake mechanism on hepatocytes, and that this pathway is distinct from the pathway utilized in the catabolism of α_2M-proteinase complexes.

In vitro uptake studies have demonstrated specific, saturable binding and subsequent degradation of ATIII-proteinase complexes by rat hepatocytes in primary culture [18]. In addition, the uptake of ATIII-trypsin complexes was accompanied by the formation of a disulfide interchange product between the ligand and a cellular protein [18]. A similar observation has been made with insulin, which undergoes disulfide interchange with the insulin receptor [19].

In the present report, the catabolism of α_1PI-trypsin complexes was studied in vitro using rat hepatocytes in primary culture. The uptake of α_1PI-trypsin was specific and saturable and was inhibited by large molar excesses of α_1PI-trypsin or ATIII-thrombin, but not by native α_1PI. The bound ligand formed a disulfide interchange product with a cellular protein and was degraded in a time-dependent manner. The role of the single cysteine residue in α_1PI [20] in the formation of this disulfide interchange product was examined by utilizing a derivative of α_1PI in which the cysteine residue was modified with iodoacetamide. The modified inhibitor retained 100% activity [21], and no difference in the uptake of trypsin complexes of the modified inhibitor by hepatocytes was detected. In addition, the disulfide intechange product was still observed, suggesting that the proteinase may provide the cysteine residues involved in the disulfide interchange product between proteinase complexes of α_1PI and ATIII and a cellular protein in hepatocytes.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA), iodoacetamide, dithiothreitol, and p-nitrophenyl guanidinobenzoate were obtained from the Sigma Chemical Co. Collagenase and TPCK-treated trypsin were from Worthington Biochemicals Corp. This trypsin was 50% active, as determined by active site titration [22]. ^{125}I, carrier-free, and lactoperoxidase, coupled to Sepharose, were obtained from New England Nuclear (Boston, MA) and P-L Biochemicals, Inc. (Piscataway, NJ), respectively. Human α_1PI, ATIII thrombin, and factor IX_a were prepared and assayed as previously described [16,17,23,24]. All other reagents were of the best commercial grade available.

Methods

Preparation of proteinase inhibitor-proteinase complexes. Complexes of α_1PI with trypsin, and ATIII with thrombin or factor IX_a were prepared by incubating equimolar amounts of proteinase inhibitor and active proteinase in 0.05M HEPES, 0.15 M NaCl, pH 7.40, at room temperature for 10 min. Prior to radiolabeling for use in the binding studies described below, the α_1PI-trypsin complexes were purified by chromatography on Sephacryl S-200 (Pharmacia). The purity and integrity of these complexes was assessed by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn [25]. These complexes were iodinated by the lactoperoxidase method of David and Reisfeld [26], and resolved from free ^{125}I on a Sepharose G-25 column (15×0.5 cm) and were used for experiments within 24 hr of labeling. The lead edge of the protein peak was pooled and the ^{125}I-α_1PI-trypsin was examined by SDS-polyacrylamide gel electrophoresis and autoradiography (as described below). By this technique, the ^{125}I-α_1PI-trypsin complex was homogeneous, with no detectable free iodine or other contaminants noted on the gel.

Reduction and alkylation of α_1PI. α_1PI (100 μM) was incubated with an equal volume of 7.5 mM dithiothreitol at 23°C for 1 hr in 0.05 M HEPES, 0.15 NaCl, pH 7.40. Iodoacetamide was then added to the reaction mixture to a final concentration of 10 mM and allowed to react for an additional 30 min. The reaction mixture was then dialyzed into 20 mM sodium phosphate, pH 7.40, with frequent changes of dialysis buffer. The extent of modification was determined by amino acid analysis. Duplicate samples of alkylated α_1PI and native α_1PI were hydrolyzed in vacuo for 24 hr at 110°C in 6 N HCl. Amino acid compositions were determined in a Beckman 120B amino acid analyzer. The results were compared to the known amino acid sequence of α_1PI [9], and the mole fraction of carboxymethylcysteine per mole α_1PI was determined. Trypsin complexes of the modified α_1PI were prepared, purified, and radiolabeled as described above, with essentially identical results as obtained with the unmodified α_1PI.

Isolation and maintenance of hepatocytes. Male Fischer 344 rats (Charles River Breeding Laboratories) were used for the preparation of hepatocytes by the two-step collagenase technique as previously described [18]. The viability of the isolated hepatocytes was 80–95% as assesed by trypan blue exclusion, with greater than 95% of the preparation as single cells. The isolated hepatocytes were diluted to 1 million cells/ml in Leibovitz L-15 medium (Gibco) supplemented with 10% fetal calf serum (Gibco), insulin 10^{-7} M, and gentamycin, 50 μg/ml. Collagen-coated

35-mm-diameter culture dishes (Falcon Plasticcs) were plated with 1 ml of cell suspension and incubated at 37°C for 2 hr. The medium was changed, omitting the fetal calf serum, and the cells were incubated overnight.

Uptake and degradation experiments. The hepatocytes were equilibrated for 1 hr with L-15 medium containing insulin, gentamycin, 0.02 M HEPES (pH 7.40), and 10 mg/ml BSA. This medium was aspirated and replaced with 0.75 ml of medium containing radiolabeled ligand. Nonspecific uptake was determined as the uptake of ^{125}I-ligand in the presence of a 1,000-fold molar excess of unlabeled ligand as previously described for the uptake of ATIII-proteinase complexes in this system [18]. The cells were incubated under various conditions for various periods of time at 37°C. The reaction was terminated by aspirating the medium and washing the cells four times with 1 ml of cold Earle balanced salt solution with Ca^{+2} and Mg^{+2} (Gibco), 0.02 HEPES, pH 7.40. The cells were solubilized by incubating in a solution of 0.2M NaOH and 2.5% SDS at room temperature for 1 hr and counted in a gamma counter. Cell protein was determined as described by Peterson [27] and radioactivity normalized to cell protein.

In some studies, uptake was performed with 15 nM α_1PI-trypsin or ^{125}I-alkylated α_1PI-trypsin for time intervals between 0 and 7 hr. The cells were then washed as described above and solubilized in 2.5% SDS for 1 hr. Both supernatants and solubilized cells were then subjected to SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide gels [25] in the presence and absence of β-mercaptoethanol. In addition, supernatant culture medium from hepatocytes in culture for 24 hr was incubated with 15 nM ^{125}I-α_1PI-trypsin for 7 hr at 37°C, and samples subjected to SDS-polyacrylamide gel electrophoresis as described above. The gels were then processed as previously described [18] with glutaraldehyde to prevent loss of peptides as follows: (1) 50% methanol, 10% acetic acid for 30 min; (2) 5% methanol, 7% acetic acid for 30 min; (3) 10% glutaraldehyde for 30 min; (4) four washes in water followed by staining with Coomassie brilliant blue in the usual manner. All gels were then subjected to autoradiography employing Kodak XAR-2 film (Cardinal X-ray, Winston-Salem, NC) with an image intensifying screen for various time periods at −70°C.

In vivo plasma elimination studies. Clearance studies were performed as previously described [16] using CD-1 female mice (Charles River Breeding Laboratories). For some studies, complexes of ATIII with either thrombin or factor IX_a were prepared as described above, utilizing ^{125}I-ATIII, prepared as described [26], or ^{125}I-factor IX_a prepared using IODOBEADS™ (Pierce Chemical Co.) as previously described [24].

RESULTS

In Vitro Hepatocyte Uptake of α_1PI-Trypsin Complexes

α_1PI-trypsin complexes, purified as described above, were homogeneous, and SDS-polyacrylamide gel electrophoresis of the complex after incubation at 37°C for 7 hr showed no breakdown of the complex (Fig. 1). Time-dependent uptake of the α_1PI-trypsin by hepatocytes was studied by incubating cells with 1 to 15 nM concentrations of complex over 7 hr (Fig. 2). Uptake of the ligand was time-dependent, and a steady state was achieved by 5 hr. Binding of ligand to collagen-coated plates in the absence of cells was less than 1% of the total added ligand at every concentration.

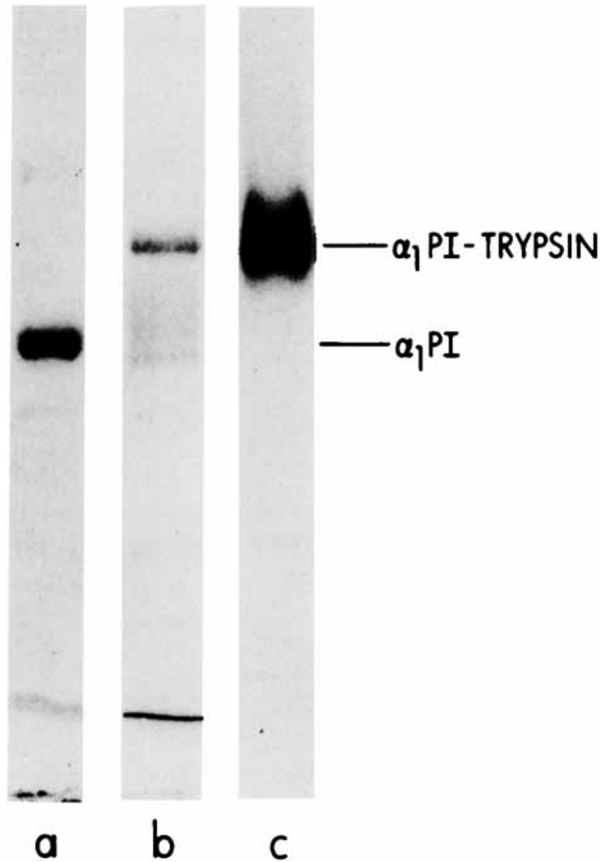

Fig. 1. SDS-polyacrylamide gel electrophoresis and autoradiography of ^{125}I-α_1PI-trypsin. SDS-polyacrylamide gel electrophoresis was performed under nonreducing conditions on a 7.5% acrylamide gel. Lane a is α_1PI; lane b is the purified α_1PI-trypsin complex; lane c is an autoradiogram of the ^{125}I-α_1PI-trypsin preparation incubated at 37°C for 7-hr prior to electrophoresis to demonstrate ligand stability under the experimental conditions. The autoradiogram was overexposed to examine the preparation for degradation products. The dye front is evident on the Coomassie blue-stained gels a and b. There is no evidence of free ^{125}I-peptides at the dye front in lane c.

Specific uptake was determined by subtracting the nonspecific uptake in the presence of 1,000-fold molar excess of unlabeled complex from total uptake as previously described in this system [18]. The nonspecific uptake was approximately 30–40% of the total uptake at each concentration of ligand studied.

Uptake of α_1PI-Trypsin in the Presence of Competitive Doses of Unlabeled Ligands

The 6-hr uptake of 1 nM α_1PI-trypsin was studied with increasing concentrations of unlabeled ligands (Fig. 3). The uptake of α_1PI-trypsin was progressively inhibited with increasing concentrations of unlabeled ligand as previously described for uptake of ATIII-proteinase in this system [18]. Similar results were obtained with unlabeled ATIII-thrombin as the competing ligand. This cross-competition indicates

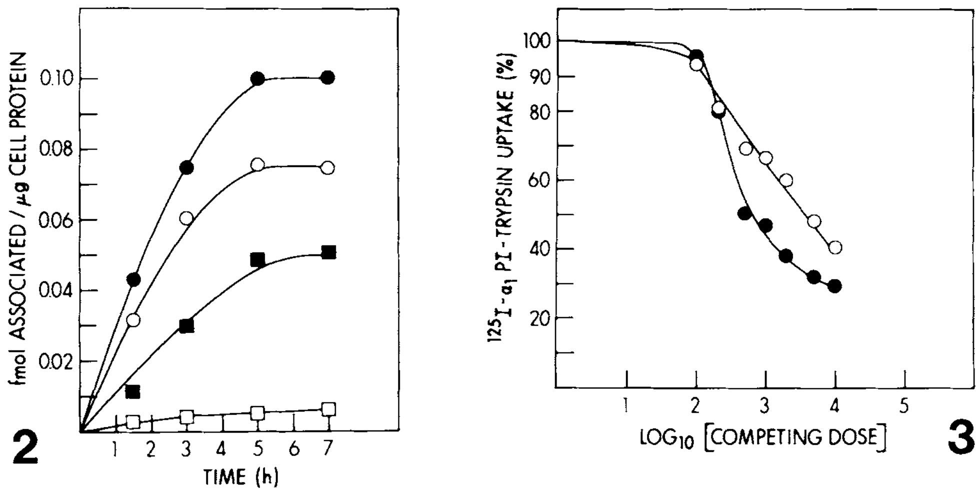

Fig. 2. Specific uptake of ^{125}I-α_1PI-trypsin at various concentrations as a function of time. The concentrations employed were 1 nM (□), 5 nm (■), 10 nM (○), 15 nM (●). All points were performed in duplicate.

Fig. 3. Concentration-dependent competition of ^{125}I-α_1PI-trypsin uptake. ^{125}I-α_1PI-trypsin, 1 nM, was incubated for 6 hr with increasing doses (0–10,000 fold molar excess) of unlabeled α_1PI-trypsin (●) or ATIII-thrombin (○). The uptake in the absence of competing ligand (0.008 fmol associated ligand/μg cell protein) was taken as 100%.

that α_1PI-trypsin and ATIII-thrombin complexes are catabolized by the same saturable process as previously reported in an in vivo system [16,24]. This is in marked contrast to the results obtained with native α_1PI, where even at 10,000-fold molar excess, hepatocyte uptake of α_1PI-trypsin was still greater than 85% of the uptake in the absence of excess α_1PI (data not shown).

SDS-Polyacrylamide Gel Eletrophoresis and Autoradiographic Analysis of Uptake and Degradation of ^{125}I-α_1PI-Trypsin Complexes by Hepatocytes

Figure 4 is an autoradiograph derived from an SD-polyacrylamide gel (7.5%) of solubilized hepatocytes incubated with 15 nM ^{125}I-α_1PI-trypsin for up to 7 hr at 37°C. This study demonstrates time-dependent accumulation of ^{125}I-α_1PI-trypsin in the hepatocytes with some accumulation of radiolabel at molecular weights lower than the intact complex. The peptides at the dye front possess an M_r of at least 10,000 as determined from calibration standards (lysozyme, ribonuclease, and cytochrome C) and cannot represent free ^{125}I or ^{125}I-tyrosine. In addition, there is a time-dependent accumulation of a high molecular weight complex that just enters the 7.5% gel (Fig. 4A). When identical samples were electrophoresed under reducing conditions, the high molecular weight component is greatly diminished, and the amount of radiolabel migrating near the dye front is greatly increased, along with intact α_1PI-trypsin complex (Fig. 4B). When electrophoresed at concentations over 100-fold higher than ligand in any experimental lane (Fig. 1), the stock ^{125}I-α_1PI-trypsin preparation demonstrated no evidence of label at the dye front; therefore, the peptides must arise from incubation of the ligand with the cells.

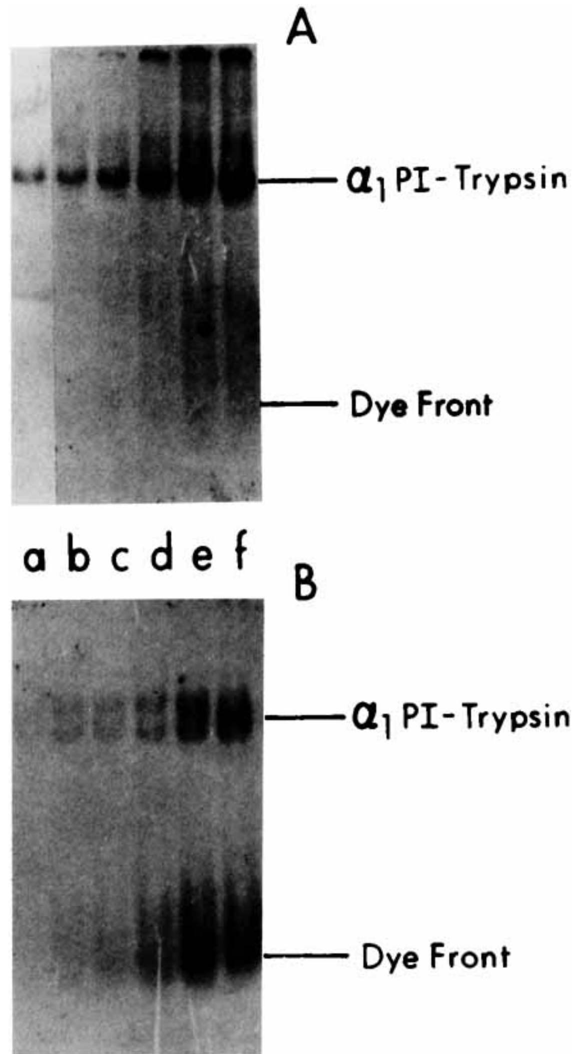

Fig. 4. SDS-polyacrylamide gel electrophoresis and autoradiography of cell-associated ^{125}I-α_1PI-trypsin. A) SDS-polyacrylamide gel electrophoresis under nonreducing conditions after incubation of 15 nM ^{125}I-α_1PI-trypsin with hepatocytes at 37°C for 0 (a), 30 (b), 60 (c), 90 (d), 180 (e), and 300 (f) min. B) SDS-polyacrylamide gel electrophoresis under reducing conditions at the same time points. The position of the ^{125}I-α_1PI-trypsin was determined by electrophoresis of ^{125}I-α_1PI-trypsin complexes. Identical sample loads were applied to each lane.

As a further control, supernatants from the cell uptake studies were examined by these same techniques with no evidence of ligand degradation. Also, when 15 nM ^{125}I-α_1PI-trypsin was incubated with conditioned medium obtained from 24-hr hepatocyte cultures, there was no evidence of ligand degradation (data not shown). These studies indicate that the peptides found within hepatocytes (Fig. 3) resulted from degradation of ^{125}I-α_1PI-trypsin complexes after uptake. It is concluded that this material represents radiolabeled peptides derived from degradation of internalized ligand.

Uptake of Trypsin Complexes of Alkylated α_1PI

Amino acid analysis was employed to determine the extent of carboxamidomethylation of cysteine residues in the ligand preparation utilized for the binding studies. These analyses, performed in triplicate, indicated a yield of 1.08 μmol carboxamidomethyl cysteine per μmol of α_1PI. These data, in comparison with amino acid analysis of a control preparation of α_1PI and the known amino acid sequence [9] indicate 100% derivatization of the sole cysteine in the molecule. In addition the preparation was assayed for trypsin inhibitory capacity, as described above, demonstrating retention of 100% activity. This observation is consistent with a previous report [21].

Uptake studies with trypsin complexes of alkylated α_1PI were performed as described above. These studies showed time-dependent uptake of 15 nM ^{125}I-alkylated α_1PI-trypsin, essentially identical to the results obtained with unmodified α_1PI-trypsin complexes (Fig. 5). These studies indicate that the sole cysteine residue in α_1PI is not involved in the uptake of α_1PI-trypsin complexes by hepatocytes.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiographic Analysis of Uptake and Degradation of ^{125}I-Alkylated α_1PI-Trypsin Complexes by Hepatocytes

In order to further assess the role of the single cysteine residue in α_1PI in the uptake and degradation of α_1PI-trypsin, and in the formation of the high molecular weight complex which is sensitive to reduction, samples of solubilized hepatocytes that had been incubated with 15 nM ^{125}I-alkylated α_1PI-trypsin complexes for up to 7 hr at 37°C were subjected to SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions, with autoradiographic analysis as described above (Fig. 6). These results are essentially identical to those obtained with unmodified α_1PI-trypsin complexes, indicating that the sole cysteine residue in α_1PI is not involved in

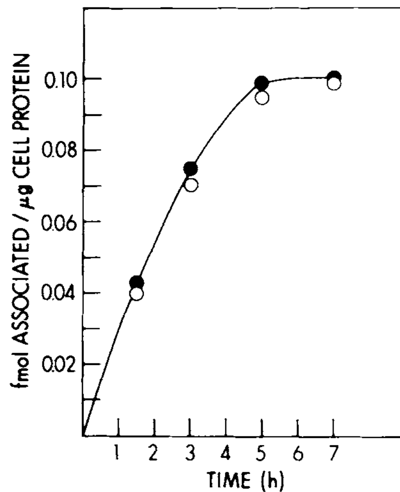

Fig. 5. Specific uptake of ^{125}I-alkylated α_1PI-trypsin complexes at 15 nM concentration as a function of time (○). The uptake of unmodified α_1PI-trypsin complexes at 15 nM concentration is shown for comparison (●).

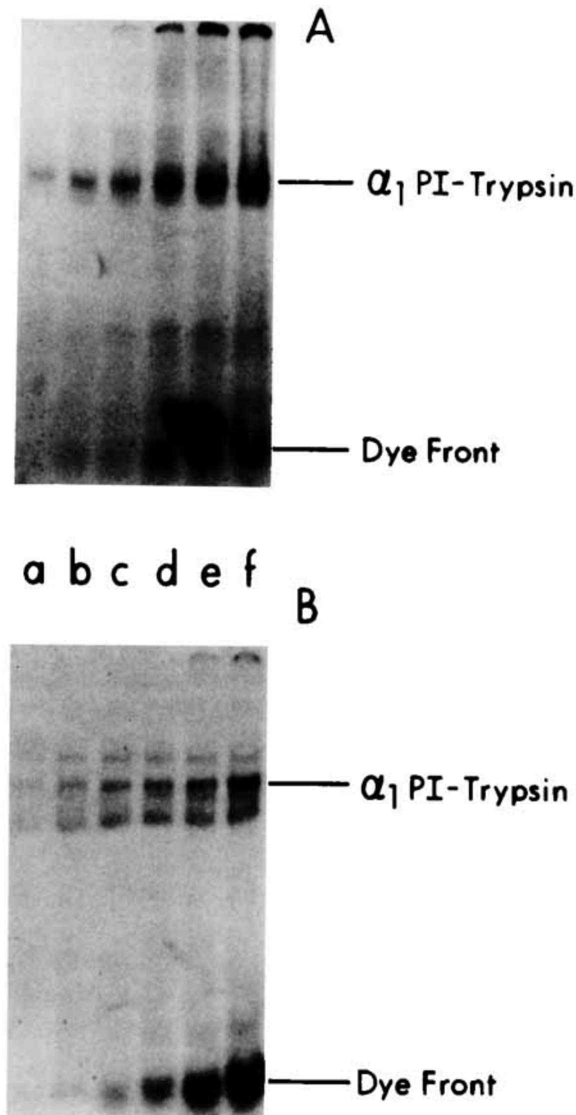

Fig. 6. SDS-polyacrylamide gel electrophoresis and autoradiography of cell-associated ^{125}I-alkylated α_1PI-trypsin. A) SDS-polyacrylamide gel electrophoresis under nonreducing conditions after incubation of 15 nM ^{125}I-alkylated α_1PI-trypsin with hepatocytes at 37°C for 0 (a), 30 (b), 60 (c), 90 (d), 180 (e) and 300 (f) min. B) SDS-polyacrylamide gel electrophoresis under reducing conditions at the same time points. The position of ^{125}I-alkylated α_1PI-trypsin was determined by electrophoresis of ^{125}I-alkylated α_1PI-trypsin complexes. Identical samples were applied to each lane.

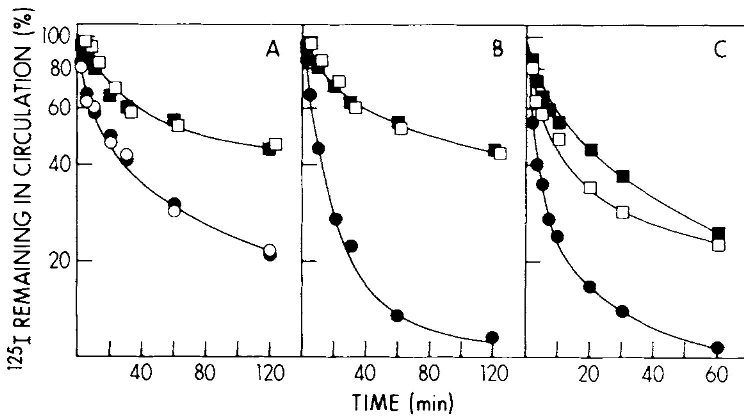

Fig. 7. Clearance of intravenously injected proteinase complexes of α_1PI and ATIII. ^{125}I-labeled complexes were injected into mice and blood samples obtained at intervals. Blood obtained at 5–10 sec was used as 100%. A) Clearance of α_1PI-trypsin (●), alkylated α_1PI-trypsin (○); clearance of α_1PI-trypsin in the presence of 2000-fold excess of either unlabeled α_1PI-trypsin (■) or ATIII-thrombin (□). B) Clearance of ATIII-thrombin (●); clearance of ATIII-thrombin in the presence of 2000-fold molar excess of either unlabeled ATIII-thrombin (■) or α_1PI-trypsin (□). C) Clearance of ATIII-factor IX_a (●); clearance of ATIII-factor IX_a in the presence of 2000-fold molar excess of either unlabeled ATIII-thrombin (■) or α_1PI-trypsin (□).

uptake or degradation of α_1PI-trypsin complexes by hepatocytes, and that it is also not involved in the formation of the high molecular weight disulfide interchange product seen in autoradiographs of solubilized cells.

In Vivo Plasma Elimination Studies of Proteinase Complexes of α_1PI and Antithrombin III

In order to further confirm and extend the results described above for in vitro uptake of ^{125}I-alkylated α_1PI-trypsin complexes, the clearance of these complexes was investigated using our previously described mouse model (Fig. 7A). These studies demonstrate that the clearance of ^{125}I-alkylated α_1PI-trypsin from the murine circulation is essentially identical to the clearance of trypsin complexes of native α_1PI, consistent with the results described above. To further confirm the shared hepatocyte uptake pathway for proteinase complexes of α_1PI and ATIII, clearance competition experiments were performed with large molar excesses of unlabeled ligands (Fig. 7). As demonstrated in Figure 7A, the clearance of α_1PI-trypsin can be inhibited by large molar excesses of unlabeled α_1PI-trypsin or ATIII-thrombin. Similarly, the clearance of ATIII-thrombin can be inhibited by large molar excesses of ATIII-thrombin or α_1PI-trypsin (Fig. 7B). In order to extend these observations to another proteinase, factor IX_a, which was not available in the relatively large quantities necessary to perform in vitro hepatocyte uptake studies, clearance competition studies were performed with radiolabeled ATIII-factor IX_a complexes and large molar excesses of unlabeled ATIII-thrombin and α_1PI-trypsin (Fig. 7C). These studies again demonstrate cross-competition of proteinase complexes of α_1PI and ATIII.

DISCUSSION

α_1-Proteinase inhibitor and antithrombin III are homologous serine proteinase inhibitors. Previous studies indicated that the removal from the circulation of α_1PI-trypsin and ATIII-thrombin complexes is accomplished by a common uptake mechanism located on hepatocytes [16,17]. This clearance pathway recognizes only the inhibitor-proteinase complexes; the native inhibitors alone are not cleared. Furthermore, the clearance of these inhibitor-proteinase complexes was not carbohydrate-mediated [17]. The formation of α_1PI-trypsin and ATIII-thrombin complexes is accompanied by conformational change [2,28]. These studies suggest that the hepatocyte uptake mechanism for these proteinase-inhibitor complexes is recognizing determinants present on the complexes which are not present on either the native inhibitor or the proteinase, as has been reported in previous studies from this laboratory [29–34].

In vitro hepatocyte uptake studies with ATIII-thrombin and ATIII-trypsin complexes confirmed the in vivo clearance studies described above. Complexes of ATIII with both proteinases were bound and endocytosed by the same receptor-mediated process [18]. In addition, only the proteinase complexes of ATIII were able to compete for the observed uptake of these complexes. Native ATIII and neoglycoproteins representing the known carbohydrate-mediated clearance pathway specificities did not alter the uptake of ATIII-proteinase complexes [18].

In order to extend the in vivo observations of a shared uptake mechanism for proteinase complexes of α_1PI and ATIII, in vitro hepatocyte uptake studies were performed with α_1PI-trypsin complexes. Primary cultured hepatocytes were chosen for these studies to allow time for membrane and metabolic lesions induced by the isolation procedure to be repaired, as previously described [18].

The studies described in this manuscript demonstrate that cultured hepatocytes specifically take up and degrade α_1PI-trypsin in a concentration- and time-dependent manner. Uptake of α_1PI-trypsin was inhibited by α_1PI-trypsin and ATIII-thrombin, but not by native α_1PI, consistent with in vivo studies [16,17,24].

SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide gels and autoradiography demonstrated degradation of the ^{125}I-α_1PI-trypsin by the hepatocytes. In addition this study provides direct evidence for the formation of a disulfide interchange product between the ligand and a cellular protein, as has been reported for ATIII-proteinase complexes [18]. The high molecular complex just enters the 7.5% acrylamide gel. Estimation of the molecular weight, however, is not possible because the electrophoresis must be performed under nonreducing conditions where such estimates are inaccurate. A similar observation has been reported for insulin, which undergoes disulfide interchange with the insulin receptor [19]. The identity of the cellular protein participating in the disulfide interchange with proteinase complexes of α_1PI and ATIII is unknown. This protein presumably is carried into the lysosomes with ligand, since it is found associated in disulfide linkage with peptides derived from the ^{125}I-α_1PI-trypsin complex.

ATIII has three disulfide bonds and no free sulfhydryl groups, and even mild reduction and alkylation abolish all ATIII inhibitory activity [35]. α_1PI, in contrast, has a single cysteine residue, which readily forms mixed disulfides [20]. In order to investigate the role of this highly reactive cysteine in the formation of this disulfide interchange product on uptake of α_1PI-trypsin by hepatocytes, α_1PI was reduced and

alkylated using iodoacetamide. The fully alkylated α_1PI retained 100% trypsin-inhibitory activity, consistent with previous studies [21]. Trypsin complexes of this alklylated α_1PI were taken up and degraded essentially identically to unmodified α_1PI-trypsin complexes, including formation of the disulfide interchange product. These results indicate that the sole cysteine residue in α_1PI is not involved in the formation of the disulfide interchange product, and implicate the proteinase in the formation of this species. These results were confirmed with in vivo clearance studies in our mouse model, which demonstrated no difference in the clearance of trypsin complexes of alkylated α_1PI as compared to unmodified α_1PI-trypsin complexes.

To further confirm the shared hepatocyte uptake mechanism for proteinase complexes of α_1PI and ATIII, clearance studies were performed using our mouse model. Large molar excesses of unlabeled α_1PI-trypsin or ATIII-thrombin inhibited the clearance of both ^{125}I-α_1PI-trypsin and ^{125}I-ATIII-thrombin. In order to extend these observations to another proteinase that was not available in the relatively large quantities required for the in vitro uptake studies, the clearance of ATIII-factor IX_a complexes was studied. Large molar excesses of unlabeled α_1PI-trypsin or ATIII-thrombin inhibited the clearance of ATIII-factor IX_a complexes.

In summary, these studies indicate that proteinase complexes of the homologous serine proteinase inhibitors, α_1PI and ATIII, are catabolized by the same uptake mechanism located on hepatocytes independent of the particular proteinase found in the complex. Furthermore, a disulfide interchange product between a cellular protein and proteinase complexes of α_1PI or ATIII is formed during this process. In the case of α_1PI-trypsin, at least, it is the proteinase that provides the sulfhydryl groups required for the formation of this complex. The precise role of this disulfide interchange product in the catabolism of proteinase complexes of α_1PI and ATIII, and the role of the proteinase in its formation, remains to be elucidated.

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