The Cleavage and Inactivation of Plasminogen Activator Inhibitor Type 1 by Neutrophil Elastase: The Evaluation of Its Physiologic Relevance in Fibrinolysis

By Kai Wu, Tetsumei Urano, Hayato Ihara, Yumiko Takada, Michio Fujie, Michio Shikimori, Kenji Hashimoto, and Akikazu Takada

The effect of the proteolytic cleavage of plasminogen activator inhibitor type 1 (PAI-1) by human neutrophil elastase (HNE) on fibrinolysis was investigated. HNE cleaved active PAI-1 and produced low molecular weight forms of inactive PAI-1, as previously reported. Latent PAI-1 was resistant to HNE treatment. Vitronectin (VN) partially protected the cleavage. NH2-terminal sequence analysis indicated that the cleavage site was Val355-Ser356 (P4-P3). The effects of PAI-1 cleavage by HNE on clot lysis was studied in a purified system. Clot lysis time without PAI-1 was 20.0 ± 5.0 minutes and was prolonged to 86.7 ± 2.9 minutes by 68 nmol/L of PAI-1. It was shortened when HNE (from 0.6 nmol/L to 80 nmol/L) was added and returned to the value obtained without PAI-1 by 80 nmol/L of HNE (20.0 ± 5.8 minutes). However, in the absence of PAI-1, elastase did not enhance clot lysis at all. Euglobulin clot lysis time was also shortened after HNE treatment. The cleavage and inactivation of PAI-1 by HNE was shown to be a novel pathway to enhance fibrinolysis.

© 1995 by The American Society of Hematology.

MATERIALS AND METHODS

Purification of active recombinant prokaryotic PAI-1 (rpPAI-1). The cultivation of bacteria expressing PAI-1 and the purification of active nonglycosylated rpPAI-1 has been previously described.16 Escherichia coli strain SG20043 transformed with the pDL06 DNA16 was kindly provided by Dr. Tor Ny (Umeå University, Umeå, Sweden). Purified active rpPAI-1 was stored at −80°C before use. The concentration of active rpPAI-1 was determined by the titration with two-chain uPA,15 of which active site concentration was determined by the titration with 4-methylumbelliferyl-p-guanidinobenzoate (MUGB).17 This rpPAI-1 possessed almost full intrinsic activity and was used without any pretreatment by denaturant, which has been ordinarily employed to reanimate latent PAI-1.18 Others. Glu-plasminogen was prepared by an affinity chromatography of lysine-Sepharose19 from freshly frozen plasma. Fibrinogen was purchased from KabiVitrum (Stockholm, Sweden), and trace amounts of contaminated plasminogen and plasmin were removed by passing through lysine-Sepharose. Human α-thrombin and high molecular weight two-chain uPA were kind gifts from Green...
Cross (Osaka, Japan). Two-chain tPA was kindly provided by Sumitomo Pharmaceutical Co (Osaka, Japan). Human neutrophil elastase was purchased from Athens Research and Technology, Inc (Athens, GA). The two-chain uPA substrate S-2444 (L-Pyroglutamyl-glycyl-L-arginine-p-nitroanilide) was purchased from KabiVitrum (Stockholm, Sweden). Human vitronectin (VN) was purchased from Pro-mega (Madison, WI). A rabbit polyclonal antiserum raised against rpPAI-1 was kindly provided by Dr Tor Ny (Umeå University). Goat-antirabbit IgG horseradish peroxidase conjugate was purchased from Bio-Rad Laboratories (Richmond, CA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed according to Laemmli20 and protein bands were stained either by coomassie brilliant blue or by immunoblot analysis using a rabbit polyclonal antiserum against PAI-1 after blotting onto a nitrocellulose membrane. Nonstained and prestained low molecular weight standards and nitrocellulose membrane were purchased from Bio-Rad Laboratories.

Analyses of the proteolytic action of HNE on active and latent rpPAI-1. rpPAI-1 (0.36 μmol/L) was incubated with HNE (17 nmol/L) for various intervals at 37°C, and the reaction was stopped by the addition of the sample buffer for SDS-PAGE. The samples were then subjected to 10% SDS-PAGE. The latent form of rpPAI-1 was obtained by incubation of active rpPAI-1 at 37°C for 48 hours, and its specific activity was not detectable after the treatment. The latent form of rpPAI-1 was also treated by HNE, as mentioned above, and the proteolytic action of HNE was analyzed by SDS-PAGE.

Analyses of the cleavage and inactivation of rpPAI-1 by HNE. To study the effect of HNE on the inhibitory activity, 0.1 μmol/L of purified rpPAI-1 was incubated either in the presence or absence of 0.4 μmol/L of VN with various concentrations of HNE (0, 2.5, 5, 10, 21, 41, and 83 nmol/L) for 10 minutes or with 10 nmol/L HNE for various time intervals (0, 5, 10, 30, 60, and 120 minutes) at 37°C. The reaction mixtures were then diluted twice with 50 mmol/L Tris-HCl pH 7.4 containing 100 mmol/L NaCl and were subjected for the activity assay. The residual inhibitory activity toward uPA was expressed as a percentage of nontreated rpPAI-1 activity.

Measurement of clot lysis. For the clot lysis assay, 96-well microtiter plates were used. A total of 2 μmol/L fibrinogen, 1 μmol/L Glu-plasminogen, 0.2 nmol/L tPA, 68 nmol/L rpPAI-1, and various concentrations of HNE (0, 0.63, 1.25, 2.5, 5, 10, 20, 40, and 80 nmol/L) were added to individual wells, and the clot formation was initiated by 1 U/mL of human thrombin. Absorbance at 405 nm in each well was measured every 10 minutes for up to 6 hours, using an automatic microtiter plate reader (Plate Analyzer ERY-300 TOYO, Tokyo, Japan). The absorbance data were plotted against time, and the clot lysis time was obtained by an average of the maximum and the minimum absorbance values.4,22 The data were expressed by mean ± standard deviation.

Euglobulin clot lysis time (ECT). The euglobulin fraction of plasma was obtained as a precipitated fraction of human plasma after a 20-times dilution and acidification to pH 5.2 followed by a 1-hour incubation at 4°C.22,22 After centrifugation at 2,000g for 10 minutes at 4°C, the euglobulin fraction was dissolved by an original plasma volume of 0.1 mol/L TRIS-HCl buffer pH 7.4. After preincubation of the euglobulin fraction with various concentrations of HNE (0, 0.67, 133, 200, 267, and 333 nmol/L) for 30 minutes at 37°C, 150 μL of each sample was added to individual wells of a 96-well microtiter plate and clot formation was initiated by addition of 1 U/mL of human thrombin. The clot lysis time was measured as described above. The data were expressed by mean ± standard deviation.

Clot lysis time was obtained by an average of the maximum and minimum absorbance values.4,22 The data were expressed by mean ± standard deviation.

Edman degradation. Automatic amino acid sequence analyses were performed by using the Applied Biosystems protein sequencer (model 476A Applied Biosystems, Foster City, CA), according to established procedures.23 For N-terminal sequence analysis, rpPAI-1 (3.8 μmol/L) was incubated with 17 nmol/L HNE in a final volume of 100 μL for 60 minutes at 37°C. The reaction was stopped and the sample (57 pmol of rpPAI-1) was loaded onto a polybrene-treated glass-fiber filter. All data were acquired using a Macintosh IIfx workstation that was interfaced to the protein sequencer via an Applied Biosystems A/D Converter. Data analysis was performed using an Applied Biosystems Model 610A data analysis program.

RESULTS

The proteolytic action of HNE on active and latent rpPAI-1. As illustrated in Fig 1, rpPAI-1 was proteolytically cleaved by a catalytic concentration of HNE resulting in the formation of a lower molecular weight form (40 kD) in a time-dependent manner. Because the molecular weight of the lower band is similar to the proteolytically cleaved form of rpPAI-1 by PAs at its reactive site, the cleavage site in rpPAI-1 by HNE seems to be close to its reactive center. When latent rpPAI-1 was incubated with HNE, rpPAI-1 was not cleaved by HNE and remained as an intact form even after a 2-hour incubation (data not shown). Such different behaviors of HNE on different forms of rpPAI-1 suggest that the cleavage site of active rpPAI-1 by HNE exists in the reactive center loop, which is hidden in the latent form of rpPAI-1.24

Modification of the activity of rpPAI-1 after the cleavage by HNE. After incubation of 0.1 μmol/L active rpPAI-1 either in the presence or absence of 0.4 μmol/L of VN with HNE for various intervals, residual activity of rpPAI-1 was measured. Inhibitory activity decreased both in the presence and absence of VN with the time course (Fig 2). Approximately 100% of the activity in the absence of VN and 80% in the presence of VN were lost after a 30-minute incubation. Inactivation of rpPAI-1 was also in a dose-dependent manner of HNE at the concentration range from 0 to 83 nmol/L (Fig 3). Incubation of rpPAI-1 with 10 nmol/L of HNE resulted in a loss of approximately 56% of rpPAI-1 activity in the absence of VN and 38% in the presence of VN within 10 minutes at 37°C. VN seems not only to stabilize active PAI-1 but to tend to protect active PAI-1 from HNE digestion, although its protective potential is not strong.

Effect of HNE on fibrin clot lysis time in a purified system. To evaluate the relevance of rpPAI-1 cleavage by HNE on fibrin clot lysis initiated by tPA, fibrin clot was formed in the presence of rpPAI-1 and different concentrations of HNE, and the clot lysis time was measured. Clot lysis time was 20.0 ± 5.0 minutes in the absence of rpPAI-1 and was prolonged to 86.7 ± 2.9 minutes when rpPAI-1 was involved in the clot (mean ± SD, n = 3). Clot lysis time was shortened when increasing amounts of HNE were involved in the clot together with rpPAI-1 (Fig 4). In the presence of HNE at
the highest concentrations, clot lysis time showed essentially the same as those obtained in the absence of rpPAI-1. Because HNE is able to degrade many fibrinolytic factors and enhance fibrin clot lysis, we conducted a similar fibrin clot lysis assay in the absence of rpPAI-1. Although the same range of the concentration of HNE was used, the clot lysis time was not shortened. Therefore, proteolytic action of HNE on fibrinolytic factors other than PAI-1 is not responsible for the shortening of fibrin clot lysis observed in the presence of PAI-1 under the experimental condition used in the present study.

**Effect of HNE on ECLT.** To investigate the effect of PAI-1 cleavage by HNE on fibrinolysis under physiologic conditions, we employed ECLT, which is known to be well-controlled by PAI-1. HNE shortened ECLT by a concentration-dependent manner (Fig 5). Because ECLT was shown to correlate well to active PAI-1 concentration and to be regulated by PAI-1, the shortening was likely induced by a cleavage and inactivation of PAI-1 in the euglobulin fraction by HNE. To confirm this, we used western blot analysis and

---

**Fig 1.** The effect of HNE on active rpPAI-1. A total of 0.36 μmol/L of active rpPAI-1 was incubated with 17 nmol/L of HNE for 0 to 2 hours at 37°C. The data shown are representative of three experiments.

**Fig 2.** The time-dependent inactivation of rpPAI-1 by HNE. A total of 0.1 μmol/L of rpPAI-1 either in the presence or absence of 0.4 μmol/L of VN was incubated with 10 nmol/L of HNE at 37°C for various time intervals (0, 3, 5, 10, 30, 60, and 120 minutes). As control, rpPAI-1 was incubated without HNE at 37°C for the same intervals. The reaction mixture was then diluted two times for the assay of PAI-1 inhibitory activity. Inhibitory activity of PAI-1 was determined as described in Materials and Methods. Data are shown as mean ± SD (n = 3).

**Fig 3.** The dose-dependent inactivation of rpPAI-1 by HNE. A total of 0.1 μmol/L of rpPAI-1 either in the presence or absence of 0.4 μmol/L of VN was incubated with various concentrations of HNE (0, 2.5, 5, 10, 21, 41, and 83 nmol/L) at 37°C for 10 minutes. The reaction mixture was then diluted two times for the assay of PAI-1 inhibitory activity. Inhibitory activity of PAI-1 was determined as described in Materials and Methods. Data are shown as mean ± SD (n = 3).
investigated whether active rpPAI-1 could be cleaved by HNE in the euglobulin fraction of plasma. Supplemented rpPAI-1 was shown to be cleaved to smaller forms in the euglobulin fraction by HNE (Fig 6).

**Amino acid sequence around the cleavage site in rpPAI-1 by HNE.** Automated sequence analysis of an unfractionated HNE digest of rpPAI-1 gave two residues in about 1:0.8 ratio for the first 15 steps (Table 1). One sequence was consistent with the N-terminal sequence in rpPAI-1 (repetitive yield: 94.1%), and the other was the new sequence of Ser-Ala-Arg-Met-Ala (repetitive yield: 94.3%). This sequence originates from the C-terminal position of the intact PAI-1 molecule and corresponds to amino acid residues 356-360. The cleavage site of PAI-1 by HNE appears to be between the residues Val355-Ser356, which corresponds to three amino acids upstream (P4-P3) to its reactive site (P1-P1').

**DISCUSSION**

In the present study, we have shown that active rpPAI-1 was cleaved and inactivated by HNE and that fibrin clot lysis was augmented because of a higher expression of tPA activity induced most likely by a cleavage of PAI-1 by HNE. The major inhibitor of fibrinolysis, PAI-1, is of vital importance for the homeostatic balance in the fibrinolytic system. Hereditary or acquired PAI-1 deficiency was shown to be associated with an increased bleeding risk. The cleavage and inactivation of PAI-1 by HNE, therefore, are of special medical interest to express higher fibrinolytic activity. PAI-1 belongs to the SERPIN superfamily, and its reactive center is located at Arg358 and Met359 (P1 and P1').

**Table 1. Automated Sequencer Analysis of HNE Digest of rpPAI-1 (57 pmol)**

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Intact PA1 Sequence Determined</th>
<th>Cleaved Peptide Sequence Determined</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Val</td>
<td>Ser</td>
</tr>
<tr>
<td>2</td>
<td>His</td>
<td>Ala</td>
</tr>
<tr>
<td>3</td>
<td>His</td>
<td>Arg</td>
</tr>
<tr>
<td>4</td>
<td>Pro</td>
<td>Met</td>
</tr>
<tr>
<td>5</td>
<td>Pro</td>
<td>Ala</td>
</tr>
<tr>
<td>6</td>
<td>Ser</td>
<td>Pro</td>
</tr>
<tr>
<td>7</td>
<td>Tyr</td>
<td>Glu</td>
</tr>
<tr>
<td>8</td>
<td>Val</td>
<td>Glu</td>
</tr>
<tr>
<td>9</td>
<td>Ala</td>
<td>Ile</td>
</tr>
<tr>
<td>10</td>
<td>His</td>
<td>Ile</td>
</tr>
<tr>
<td>11</td>
<td>Leu</td>
<td>Met</td>
</tr>
<tr>
<td>12</td>
<td>Ala</td>
<td>Asp</td>
</tr>
<tr>
<td>13</td>
<td>Ser</td>
<td>Arg</td>
</tr>
<tr>
<td>14</td>
<td>Asp</td>
<td>Pro</td>
</tr>
<tr>
<td>15</td>
<td>Phe</td>
<td>Phe</td>
</tr>
</tbody>
</table>

For personal use only.
Its proteolytic cleavage by PAs after a modification of its tertiary structure by SDS treatment has been reported. The cleavage of PAI-1 by HNE was first shown by Levin et al. Considering the fact that the relative molecular mass of the cleaved form is similar to that cleaved at its reactive site by PAs and that rpPAI-1 lost its specific activity after cleavage by HNE, HNE most likely cleaved rpPAI-1 in its reactive center loop. Another fact of the failure to cleave the latent form of rpPAI-1 by HNE also supports this assumption as a part of the reactive site loop of the latent form of PAI-1 is buried in the molecule as shown by radiograph crystallography. HNE is a broad spectrum protease and preferentially cleaves as in the P1 position valine or alanine residues. Among large numbers of valine and alanine in the reactive center loop, Val355 (P4), Ala357 (P2), or Ala360 (P2') could be probable candidates. The amino acid sequence analyses of the cleaved rpPAI-1 by HNE showed that HNE cleaved rpPAI-1 at the Val355-Ser356 peptide bond, which corresponds to P4-P3 in intact PAI-1. The cleavage of PAI-1 by HNE, therefore, resulted in the release of C-terminal peptide, which contains its reactive site, and PAI-1 naturally lost its specific activity.

Indeed, as shown in the present report, isolated active rpPAI-1 is readily proteolytically cleaved by HNE, thereby loosing its inhibitory activity. The question of the clinical relevance of this phenomenon became substantial. Spontaneous lysis of either plasma or euglobulin clot is reported to be dependent on PAI-1 content in the clot. Not only in fluid phase, PAI-1 is shown to bind to fibrin and inactivate plasminogen activators on the surface of the fibrin resulting in the prolongation of clot lysis. To our surprise, when a clot was formed in the presence of tPA, rpPAI-1, and elastase, rpPAI-1 was inactivated very easily by HNE and higher tPA activity. Because plasminogen activation activity that results in the hemorrhagic features in APL. Besides APL, under the conditions when large amounts of HNE are released from leukocytes seem, at least in part, to be responsible for expressing higher fibrinolytic activity that results in the hemorrhagic features in APL. Besides APL, under the conditions when large amounts of HNE are released from neutrophils, such as endotoxin shock, septicemia, adult respiratory distress syndrome, hemodilysis, and cardiovascular surgery, such mechanisms to enhance fibrinolytic activity may also take place.

HNE-induced enhancement of fibrinolysis has been suggested for a long time. Direct proteolysis of fibrin and the conversion of the native form of Glu-plasminogen to a more susceptible form of plasminogen were suggested as main pathways for HNE to enhance fibrinolysis. In the present study, however, no significant shortening of fibrin clot lysis was observed in the absence of rpPAI-1, and the relevance of these mechanisms became questionable. Recently, the cleavage and inactivation of α2-antiplasmin, a main inhibitor for plasmin and a member of SERPIN superfamily, was suggested as another mechanism to enhance fibrinolysis. Taking our data shown in the present study into account, the cleavage and inactivation of relevant serine protease inhibitors involved in the fibrinolysis by HNE, could be an important mechanism to express higher fibrinolytic activity resulting in the enhancement of fibrinolysis.

REFERENCES
2. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J,
PROTEOLYTIC INACTIVATION OF PAI-1 BY ELASTASE


The cleavage and inactivation of plasminogen activator inhibitor type 1 by neutrophil elastase: the evaluation of its physiologic relevance in fibrinolysis

K Wu, T Urano, H Ihara, Y Takada, M Fujie, M Shikimori, K Hashimoto and A Takada